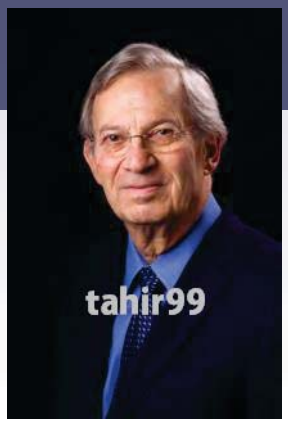
DeVita, Hellman, and Rosenberg's

Cancer

Principles & Practice of Oncology

10th Edition

Editors



Vincent T. DeVita, Jr., MD

Amy & Joseph Perella Professor of Medicine
Yale Comprehensive Cancer Center and Smilow
Cancer Hospital at Yale-New Haven
Professor of Epidemiology and Public Health
Yale University School of Public Health
New Haven, Connecticut

Theodore S. Lawrence, MD, PhD

Isadore Lampe Professor and Chair
Department of Radiation Oncology
University of Michigan
Ann Arbor, Michigan

Steven A. Rosenberg, MD, PhD

Chief, Surgery Branch, National Cancer Institute, National Institutes of Health
Professor of Surgery, Uniformed Services University of the Health Sciences
School of Medicine
Bethesda, Maryland
Professor of Surgery
George Washington University School of Medicine
Washington, District of Columbia

With 403 Contributing Authors

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In memoriam

Jonathan W. Pine, Jr.
1957–2013

We dedicate this edition to Jonathan Pine, our colleague and Senior Executive Editor at Wolters Kluwer Health, whose relaxed style belied his fierce dedication to turning out a quality book.

CONTRIBUTORS

Amy P. Abernethy, MD, PhD
Director, Center for Learning Health Care
Duke Clinical Research Institute
Director, Duke Cancer Care Research Program
Duke Cancer Institute
Associate Professor
Division of Medical Oncology
Department of Medicine
Duke University School of Medicine
Durham, North Carolina

Ghassan Abou-Alfa, MD
Associate Attending
Department of Medicine
Memorial Sloan-Kettering Cancer Center
Associate Professor
Department of Medicine
Weill Cornell Medical College
New York, New York

Ross A. Abrams, MD
Chairman and Hendrickson Professor of Radiation Oncology
Rush University Medical Center
Chicago, Illinois

Nadeem R. Abu-Rustum, MD
Professor
Weill Medical College of Cornell University
Chief, Gynecologic Oncology Service
Memorial Sloan-Kettering Cancer Center
New York, New York

Gregory P. Adams, PhD
Associate Professor Developmental Therapeutics Program
Director of Biological Research and Therapeutics
Fox Chase Cancer Center
Philadelphia, Pennsylvania

Anupriya Agarwal, PhD
Research Assistant Professor
The Knight Cancer Center
Oregon Health & Science University
Portland, Oregon

Bharat B. Aggarwal, PhD
Professor of Cancer Research
Professor of Cancer Medicine (Biochemistry)
Chief, Cytokine Research Laboratory
Department of Experimental Therapeutics
The University of Texas MD Anderson Cancer Center
Houston, Texas

Shahab Ahmed, MD
Data Analyst, Department of Gastrointestinal Medical Oncology
The University of Texas MD Anderson Cancer Center
Houston, Texas

Manmeet S. Ahluwalia, MD, FACP
Associate Professor, Department of Medicine
Cleveland Clinic Lerner College of Medicine of Case Western Reserve University
Associate Director, Clinical Trials, Operations
The Rose Ella Burkhardt Brain Tumor and Neuro-Oncology Center
Neurological Institute
Cleveland Clinic
Cleveland, Ohio

Kaled M. Alektiar, MD
Member Department of Radiation Oncology
Memorial Sloan-Kettering Cancer Center
New York, New York

James M. Allan, DPhil
Northern Institute for Cancer Research
Faculty of Medicine
Newcastle University
Newcastle-upon-Tyne, United Kingdom

Matthew L. Anderson, MD, PhD, FACOG
Assistant Professor
Division of Gynecology Oncology
Director of Clinical Research (Gynecology)
Department of Obstetrics & Gynecology
Research Member, Dan L. Duncan Cancer Center
Baylor College of Medicine
Houston, Texas

Kenneth C. Anderson, MD
Director, Jerome Lipper Multiple Myeloma Center and
Lebow Institute for Myeloma Therapeutics
American Cancer Society
Clinical Research Professor
Kraft Family Professor of Medicine
Dana-Farber Cancer Institute
Harvard Medical School
Boston, Massachusetts

Stephen Ansell, MD, PhD
Professor, Division of Hematology
Mayo Clinic
Rochester, Minnesota

Cristina R. Antonescu, MD
Attending Pathologist, Department of Pathology
Memorial Sloan-Kettering Cancer Center
Director, Bone and Soft Tissue Pathology
New York, New York

Shirin Arastu-Kapur, PhD
Associate Director
Biology at Onyx Pharmaceuticals
South San Francisco, California

Luiz Henrique de Lima Araujo, MD, MSc
Postdoctoral Researcher
James Cancer Center
The Ohio State University Medical Center
Columbus, Ohio

Alicia Y. Armstrong, MD, MHSCR
Chief, Gynecologic Services Program in Reproductive & Adult Endocrinology
Eunice Kennedy Shriver National Institute of Child Health and Human Development
Bethesda, Maryland

Alan Ashworth, FRS
Professor and Chief Executive
The Institute of Cancer Research
London, United Kingdom

Jon C. Aster, MD, PhD
Professor, Department of Pathology
Harvard Medical School
Head, Division of Hematopathology
Brigham and Women's Hospital
Boston, Massachusetts

David A. August, MD
Professor, Chief, Division of Surgical Oncology
Department of Surgery
Rutgers Robert Wood Johnson Medical School and the Rutgers Cancer Institute of New Jersey
New Brunswick, New Jersey

Itzhak Avital, MD, FACS
Professor of Surgery
Executive Medical Director
Bon Secours Cancer Institute
Richmond, Virginia

Jennifer E. Axilbund, MS, CGC
Cancer Risk Assessment Program
The Johns Hopkins Hospital
Baltimore, Maryland

Joachim M. Baehring, MD, DSc
Associate Professor
Departments of Neurology, Neurosurgery, and Medicine
Chief, Section of Neuro-Oncology
Yale Cancer Center
Yale University School of Medicine
New Haven, Connecticut

F. Amos Bailey, MD
Professor, Department of Medicine
Center for Palliative and Supportive Care
Division of Gerontology, Geriatrics, and Palliative Care
Department of Medicine
University of Alabama at Birmingham
Birmingham/Atlanta Geriatric, Research, Education, and Clinical Center
Birmingham Veterans Affairs Medical Center
Birmingham, Alabama

Sharyn D. Baker, PharmD, PhD
Associate Member
Pharmaceutical Sciences Department
St. Jude Children's Research Hospital
Memphis, Tennessee

Lodovico Balducci, MD
Program Leader, Senior Adult Oncology Program
Senior Member, H. Lee Moffitt Cancer Center & Research Institute
Professor of Oncologic Sciences & Medicine
University of South Florida College of Medicine
Tampa, Florida

Alberto Bardelli, MD
Laboratory of Molecular Genetics
Institute for Cancer Research and Treatment
University of Torino Medical School
Candiolo, Italy

Gene H. Barnett, MD, MBA
Professor and Director
Rose Ella Burkhardt Brain Tumor and Neuro-Oncology Center
Cleveland Clinic
Cleveland, Ohio

Tracy T. Batchelor, MD
Giovanni Armenise—Harvard Professor of Neurology
Harvard Medical School
Chief, Division of Neuro-Oncology
Massachusetts General Hospital Cancer Center
Co-Leader, Neuro-Oncology Program
Dana-Farber/Harvard Cancer Center
Boston, Massachusetts

Susan E. Bates, MD
Head, Molecular Therapeutics Section
Developmental Therapeutics Branch
Center for Cancer Research
National Cancer Institute
Bethesda, Maryland

Stephen B. Baylin, MD
Professor of Oncology
Professor of Medicine
Johns Hopkins University School of Medicine
Deputy Director of the Cancer Center
Baltimore, Maryland

Kevin P. Becker, MD, PhD
Assistant Professor
Department of Neurology
Section of Medical Neuro-Oncology
Yale Comprehensive Cancer
Yale University School of Medicine
New Haven, Connecticut

Andrew Berchuck, MD
Professor and Director
Gynecologic Oncology Program
Division of Gynecologic Oncology
Department of Obstetrics and Gynecology
Duke Cancer Institute
Duke University Medical Center
Durham, North Carolina

Jonathan S. Berek, MD, MMS
Laurie Kraus Lacob Professor
Director, Stanford Women's Cancer Center
Stanford Cancer Institute
Chair, Department of Obstetrics and Gynecology
Stanford University School of Medicine
Stanford, California

Ann M. Berger, MSN, MD
Pain and Palliative Care
Bethesda, Maryland

Ross S. Berkowitz, MD
William H. Baker Professor of Gynecology
Harvard Medical School
Director of Gynecology and Gynecologic Oncology
Brigham and Women's Hospital and Dana-Farber Cancer Institute
Co-Director of New England Trophoblastic Disease Center
Boston, Massachusetts

Leslie Bernstein, MS, PhD
Professor and Director
Division of Cancer Etiology
Department of Population Sciences
Beckman Research Institute
City of Hope Dean for Faculty Affairs
City of Hope National Medical Center and the Beckman Research Institute
Duarte, California

Bryan L. Betz, PhD
Assistant Professor
Department of Pathology
University of Michigan
Technical Director
Molecular Diagnostics Laboratory
University of Michigan Health System
Ann Arbor, Michigan

Smita Bhatia, MD, MPH
Professor and Chair
Department of Population Sciences
Associate Director, Population Research
City of Hope Comprehensive Cancer Center
Duarte, California

Ravi Bhatia, MD
Director, Division of Hematopoietic Stem Cell and Leukemia Research
Professor, Department of Hematology and Hematopoietic Cell Transplantation
City of Hope National Medical Center
Duarte, California

Michael J. Birrer, MD, PhD
Professor, Department of Medicine
Harvard Medical School
Director, Gynecologic Cancers Disease Center
Massachusetts General Hospital
Leader, Gynecologic Cancer Program
Dana-Farber Cancer Institute
Boston, Massachusetts

James S. Blachly, MD
Fellow, Division of Hematology
Department of Internal Medicine
The Ohio State University
Columbus, Ohio

Elizabeth M. Blanchard, MD
Director, Cancer Clinical Trials
Southcoast Centers for Cancer Care
Fairhaven, Massachusetts

Sharon L. Bober, PhD
Assistant Professor
Department of Psychiatry
Harvard Medical School Director
Sexual Health Program
Dana-Farber Cancer Institute
Boston, Massachusetts

Lawrence H. Boise, PhD
Professor
Winship Cancer Institute of Emory University
Departments of Hematology/Medical Oncology and Cell Biology
Emory School of Medicine
Atlanta, Georgia

Danielle C. Bonadies, MS, CGC
Director, Cancer Genetics Division
Gene Counsel, LLC
New Haven, Connecticut

Mitesh J. Borad, MD
Assistant Professor
Department of Medicine
Director, Phase I Drug Development
Mayo Clinic
Scottsdale, Arizona

Hossein Borghaei, MS, DO
Associate Professor
Chief, Thoracic Medical Oncology
Fox Chase Cancer Center
Philadelphia, Pennsylvania

Otis W. Brawley, MD, FACP
Chief Medical Officer
American Cancer Society, Inc.
Atlanta, Georgia

Rachel C. Brennan, MD
Assistant Member
Department of Oncology
Division of Solid Tumors
St. Jude's Children's Research Hospital
Memphis, Tennessee

Dean E. Brenner, MD
Kutsche Family Professor of Internal Medicine
Professor of Pharmacology
University of Michigan Comprehensive Cancer Center
Ann Arbor, Michigan

Jonathan R. Brody, PhD
Associate Professor
Director of Surgical Research
Department of Surgery
Jefferson Medical College of Thomas Jefferson University
Philadelphia, Pennsylvania

Paul D. Brown, MD
Professor
Co-Section Chief
Central Nervous System/Pediatrics
Director of Central Nervous System Stereotactic Radiosurgery
Department of Radiation Oncology
The University of Texas MD Anderson Cancer Center
Houston, Texas

Christopher B. Buck, PhD
Investigator
Head, Tumor Virus Molecular Biology Section
Laboratory of Cellular Oncology
Center for Cancer Research
National Cancer Institute
Bethesda, Maryland

Harold J. Burstein, MD, PhD
Associate Professor of Medicine
Harvard Medical School
Susan F. Smith Center for Women's Cancers
Dana-Farber Cancer Institute
Boston, Massachusetts

Tim E. Byers, MD, MPH
Associate Dean for Public Health Practice
Colorado School of Public Health
Associate Director for Cancer Prevention and Control
University of Colorado Cancer Center
Aurora, Colorado

John C. Byrd, MD
D. Warren Brown Chair of Leukemia Research
Director, Division of Hematology
Department of Internal Medicine
The Ohio State Comprehensive Cancer Center
Columbus, Ohio

A. Hilary Calvert
Professor
Gynecologic Oncology
University College Hospitals (UCLH)
London, United Kingdom

Robert B. Cameron, MD
Professor, Cardiothoracic Surgery and Surgical Oncology
David Geffen School of Medicine
University of California, Los Angeles
Chief, Thoracic Surgery
West Los Angeles Virginia Medical Center
Los Angeles, California

Stephen A. Cannistra, MD
Professor of Medicine
Harvard Medical School
Program Director, Gynecologic Medical Oncology
Division of Hematology and Oncology
Beth Israel Deaconess Medical Center
Boston, Massachusetts

Daniel J. Canter, MD
Vice Chairman
Department of Urology
Urologic Institute of Southeastern Pennsylvania
Einstein Healthcare Network
Associate Professor, Urologic Oncology
Fox Chase Cancer Center
Philadelphia, Pennsylvania

Antonino Carbone, MD
Professor and Chairman of the Department of Pathology, Centro di Riferimento Oncologico Aviano (CRO)
Instituto Nazionale dei Tumori, IRCCS
Aviano, Italy

David P. Carbone, MD, PhD
Barbara J. Bonner Chair in Lung Cancer Research
Professor of Medicine
Director, James Thoracic Center
James Cancer Center
The Ohio State University Medical Center
Columbus, Ohio

Michele Carbone, MD, PhD
Director
University of Hawaii Cancer Center
Professor, Department of Pathology
John A. Burns School of Medicine
Honolulu, Hawaii

Thomas E. Carey, PhD
Professor of Otolaryngology and Pharmacology
Associate Chair for Research
Department of Otolaryngology-Head and Neck Surgery
Co-Director-Head and Neck Oncology Program
Comprehensive Cancer Center
The University of Michigan School of Medicine
Ann Arbor, Michigan

Paolo G. Casali, MD
Head
Adult Mesenchymal Tumor Medical Oncology Unit
Fondazione Instituto di Ricovero Carattere Scientifico
Instituto Nazionale dei Tumori
Milano, Italy

Eric J. Cassell, MD, MACP
Emeritus Professor
Department of Public Health
Weill Medical College of Cornell University
Attending Physician
New York Presbyterian Hospital
New York, New York
Adjunct Professor
Department of Medicine
McGill University Faculty of Medicien
Montreal, Quebec, Canada

Jan Cerny, MD, PhD
Assistant Professor of Medicine
Division of Hematology
Department of Medicine
Director, Leukemia Program
University of Massachusetts Medical School
Associate Director, Cancer Research Office
UMass Memorial Cancer Center
University of Massachusetts
Worcester, Massachusetts

Ronald S. Chamberlain, MD, MPA, FACS
Chairman and Surgeon-in-Chief
Department of Surgery
Saint Barnabas Medical Center
Livingston, New Jersey

Richard Champlin, MD
Professor of Medicine and Chairman
Department of Stem Cell Transplantation and Cellular Therapy
The University of Texas MD Anderson Cancer Center
Houston, Texas

Gayun Chan-Smutko, MS, CGC
Senior Genetic Counselor
Center for Cancer Risk Assessment
Massachusetts General Hospital
Boston, Massachusetts

Susan M. Chang, MD
Professor in Residence
Department of Neurological Surgery
Director, Division of Neuro-Oncology
Lai Wan Kan Endowed Chair
University of California, San Francisco
San Francisco, California

Samuel T. Chao, MD
Brain Tumor and Neuro-Oncology Center
Radiation Oncology
Cleveland Clinic Main Campus
Cleveland, Ohio

Cindy H. Chau, PharmD, PhD
Scientist
Medical Oncology Branch
Center for Cancer Research
National Cancer Institute
National Institutes of Health
Bethesda, Maryland

Douglas B. Chepeha, MD, MSPH, FRCSC
Professor
Director of Microvascular Reconstructive Surgery
Department of Otolaryngology-Head and Neck Surgery
University of Michigan Health System
Ann Arbor, Michigan

Nathan I. Cherny, MBBS, FRACP, FRCP (Lon.)
Norman Levan Chair of Humanistic Medicine
Associate Professor of Medicine (BGU)
Director, Cancer Pain and Palliative Medicine Service
Department of Medical Oncology
Shaare Zedek Medical Center
Jerusalem, Israel

Arul M. Chinnaiyan, MD, PhD
Director, Michigan Center for Translational Pathology
S.P. Hicks Endowed Professor of Pathology
Investigator, Howard Hughes Medical Institute
American Cancer Society Research Professor
Professor of Urology
Ann Arbor, Michigan

Anu Chittenden, MS, CGC
Genetic Counselor
Center for Cancer Genetics & Prevention
Dana-Farber Cancer Institute
Boston, Massachusetts

Clifford S. Cho, MD
Associate Professor
Department of Surgery
University of Wisconsin School of Medicine and Public Health
Madison, Wisconsin

Edward Chow, MBBS, MSc, PhD, FRCPC
Professor, Department of Radiation Oncology
University of Toronto
Senior Scientist
Sunnybrook Research Institute
Chair, Rapid Response Radiotherapy Program and Bone Metastases Site Group
Sunnybrook Health Sciences Centre
Toronto, Ontario, Canada

Sean R. Christensen, MD, PhD
Assistant Professor Department of Dermatology
Section of Dermatologic Surgery and Cutaneous Oncology
Yale University School of Medicine
New Haven, Connecticut

Edward Chu, MD
Professor of Medicine and Pharmacology & Chemical Biology
Chief, Division of Hematology-Oncology
Deputy Director, University of Pittsburgh Cancer Institute
University of Pittsburgh School of Medicine
Pittsburgh, Pennsylvania

Nicki Chun, MS, LCGC
Clinical Assistant Professor of Pediatrics/Genetics
Stanford Cancer Genetics Clinic
Stanford, California

Jessica Clague, PhD, MPH
Assistant Research Professor
Division of Cancer Etiology
Department of Population Sciences
Beckman Research Institute
City of Hope National Medical Center
Duarte, California

Victoria Clark, MD
Departments of Neurosurgery and Genetics
Yale Program in Brain Tumor Research
Yale School of Medicine
New Haven, Connecticut

Robert E. Coleman, MBBS, MD, FRCP
Professor of Medical Oncology
Academic Unit of Clinical Oncology
Weston Park Hospital Cancer Research
Yorkshire Cancer Research
Sheffield Cancer Research Centre
Sheffield, South Yorkshire, United Kingdom

Louis S. Constine, MD, FASTRO
The Philip Rubin Professor of Radiation Oncology and Pediatrics
Vice Chair, Department of Radiation Oncology Director
Judy DiMarzo Cancer Survivorship Program
James P. Wilmot Cancer Center
University of Rochester Medical Center
Rochester, New York

M. Sitki Copur, MD, FACP
Medical Director of Oncology
Saint Francis Cancer Treatment Center
Grand Island, Nebraska
Professor, Department of Medicine
Division of Hematology Oncology
Adjunct Faculty
University of Nebraska Medical Center
Omaha, Nebraska

Aimee M. Crago, MD, PhD, FACS
Assistant Attending Surgeon
Sarcoma Disease Management Team
Gastric and Mixed Tumor Service
Department of Surgery
Memorial Sloan-Kettering Cancer Center
New York, New York

Jennifer Cuellar-Rodriguez, MD
Staff Clinician
Laboratory of Clinical Infectious Diseases
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Brian G. Czito, MD
Gary Hock and Lynn Proctor Associate Professor, Department of Radiation Oncology
Duke Cancer Institute
Duke University Medical Center
Durham, North Carolina

Bouthaina Dabaja, MD
Associate Professor and Section Chief, Hematology
Department of Radiation Oncology
The University of Texas MD Anderson Cancer Center
Houston, Texas

Douglas M. Dahl, MD, FACS
Associate Professor of Surgery
Harvard Medical School
Chief, Division of Urologic Oncology
Department of Urology
Massachusetts General Hospital
Boston, Massachusetts

Riccardo Dalla-Favera, MD
Professor of Pathology and Cell Biology
Director, Institute for Cancer Genetics
Columbia University
New York, New York

Molly S. Daniels, MS, CGC
Senior Genetic Counselor
Department of Clinical Cancer Genetics
The University of Texas MD Anderson Cancer Center
Houston, Texas

Caroline Davidge-Pitts, MB, BCh
Department of Endocrinology, Diabetes and Nutrition
Mayo Clinic
Rochester, Minnesota

Andrew M. Davidoff, MD
Professor Department of Surgery
University of Tennessee Health Science Center
Member and Chairman
Department of Surgery
St. Jude Children's Research Hospital
Memphis, Tennessee

Michael A. Davies, MD, PhD
Associate Professor
Department of Melanoma
Medical Oncology
Department of Systems Biology
The University of Texas MD Anderson Cancer Center
Houston, Texas

Marcos de Lima, MD
Professor of Medicine
University Hospitals
Case Medical Center
Case Western Reserve University
Cleveland, Ohio

Alan H. DeCherney, MD
Director, Program in Adult and Reproductive Endocrinology
National Institute of Child Health and Human Development
National Institutes of Health
Bethesda, Maryland

Roy H. Decker, MD, PhD
Associate Professor
Department of Therapeutic Radiology
Yale University School of Medicine
New Haven, Connecticut

Angelo Paolo Dei Tos, MD
Director, Department of Oncology
Director of Anatomic Pathology
Scientific Director
Veneto Region Cancer Registry
General Hospital of Treviso
Treviso, Italy

Marcello Deraco, MD
Peritoneal Surface Malignancies
Department of Surgery-Colorectal Unit
Fondazione IRCCS
Instituto Nazionale dei Tumori
Milano, Italy
Professor Postgraduation on Digestive Surgery
Tor Vergata University
Roma, Italy

Hari A. Deshpande, MD
Associate Professor of Medicine
Yale University School of Medicine
Section of Medical Oncology
Yale New Haven Hospital
New Haven, Connecticut

Frank C. Detterbeck, MD
Professor and Chief
Section of Thoracic Surgery
Yale University School of Medicine
New Haven, Connecticut

Khanh T. Do, MD
Senior Clinical Fellow
Division of Cancer Treatment and Diagnosis
National Cancer Institute
National Institutes of Health
Bethesda, Maryland

Jessica S. Donington, MD
Associate Professor
Department of Cardiothoracic Surgery
New York University School of Medicine
Director Thoracic Surgery
Bellevue Hospital
New York, New York

James H. Doroshow, MD
Director, Division of Cancer Treatment and Diagnosis
Deputy Director for Clinical and Translational Research
National Cancer Institute
National Institutes of Health
Bethesda, Maryland

Brian J. Druker, MD
Director, Oregon Health & Science University
Knight Cancer Institute
JELD-WEN Chair of Leukemia Research
Oregon Health & Science University
Investigator, Howard Hughes Medical Institute
Portland, Oregon

Steven G. DuBois, MD, MS
Associate Professor
Department of Pediatrics
University of California, San Francisco School of Medicine
University of California, San Francisco
Benioff Children's Hospital
San Francisco, California

Damian E. Dupuy, MD, FACR
Professor of Diagnostic Imaging
The Warren Alpert Medical School of Brown University
Director of Tumor Ablation
Rhode Island Hospital
Providence, Rhode Island

Craig C. Earle, MD, MSc, FRCP(C)
Director, Health Services Research Program
Cancer Care Ontario and the Ontario Institute for Cancer Research
Professor of Medicine
University of Toronto
Medical Oncologist
Sunnybrook Odette Cancer Centre
Toronto, Ontario, Canada

Richard L. Edelson, MD
Aaron B. & Marguerite Lerner Professor of Dermatology
Yale School of Medicine
New Haven, Connecticut

Jason A. Efstathiou, MD, DPhil
Assistant Professor
Department of Radiation Oncology
Massachusetts General Hospital
Harvard Medical School
Boston, Massachusetts

Christopher A. Eide, BA
Research Technician III
Howard Hughes Medical Institute
Division of Hematology and Medical Oncology
Oregon Health & Science University
Knight Cancer Institute
Portland, Oregon

Patricia J. Eifel, MD
Professor and Chief, Section of Gynecology
Department of Radiation Oncology
The University of Texas MD Anderson Cancer Center
Houston, Texas

Dominique M. Elias, MD, PhD
Professor, Department of Surgical Oncology
Villejuif, France

Tobias Else, MD
Assistant Professor
Department of Medicine
Division of Metabolism, Endocrinology & Diabetes
University of Michigan
Ann Arbor, Michigan

Cathy Eng, MD, FACP
Associate Professor
Associate Medical Director, Colorectal Center
Director, MD Anderson Cancer Center Clinical Trials Network
Gastrointestinal Medical Oncology Co-Chair
Southwest Oncology Group
Rectal Subcommittee
National Clinical Trials Network Institutional Grant
Lead Contact Principle Investigator
Department of Gastrointestinal Medical Oncology
The University of Texas MD Anderson Cancer Center
Houston, Texas

Charles Erlichman, MD
Professor, Department of Oncology
Deputy Director, Clinical Research
Peter and Frances Georgeson Professor of Gastroenterology Cancer Research
Mayo Clinic
Rochester, Minnesota

Elihu H. Estey, MD
Professor, Division of Hematology
University of Washington School of Medicine
Member and Director of Acute Myeloid Leukemia Clinical Research (Nontransplant)
Clinical Research Division
Fred Hutchinson Cancer Research Center
Seattle, Washington

Douglas B. Evans, MD
Professor and Chair
Department of Surgery
Medical College of Wisconsin
Milwaukee, Wisconsin

Jane M. Fall-Dickson, PhD, RN, AOCN
Associate Professor and Assistant Chair, Research
Department of Nursing
Georgetown University School of Nursing and Health Studies
Washington, District of Columbia

Adam S. Feldman, MD, MPH
Assistant Professor of Surgery
Harvard Medical School
Assistant in Urology, Department of Urology
Massachusetts General Hospital
Boston, Massachusetts

Steven A. Feldman, PhD
Staff Scientist
Director, Surgery Branch
Vector Production Facility
National Cancer Institute
Bethesda, Maryland

Mary Feng, MD
Associate Professor, Department of Radiation Oncology
University of Michigan Health System
Ann Arbor, Michigan

Felix Y. Feng, MD
Assistant Professor, Department of Radiation Oncology
Chief, Division of Translational Genomics
University of Michigan Health System
Ann Arbor, Michigan

William Douglas Figg, Sr., PharmD, MBA
Senior Investigator and Head of the Clinical Pharmacology Program
Clinical Director, Center for Cancer Research
Head of the Molecular Pharmacology Section
Medical Oncology Branch
Center for Cancer Research
National Cancer Institute
National Institutes of Health
Bethesda, Maryland

Paul T. Finger, MD
Clinical Professor of Ophthalmology
Director, Ocular Tumor Services
New York University School of Medicine
The New York Eye and Ear Infirmary
The New York Eye Cancer Center
New York, New York

Joel A. Finkelstein, MD, MSc, FRCS(C)
Division of Orthopaedic Surgery
Sunnybrook Health Sciences Centre
University of Toronto
Toronto, Ontario, Canada

Gini F. Fleming, MD
Professor, Department of Medicine
Director, Medical Gynecologic Oncology
Section of Hematology and Oncology
University of Chicago
Chicago, Illinois

Antonio Tito Fojo, MD, PhD
Medical Oncology Branch and Affiliates
Head, Experimental Therapeutics Section
Senior Investigator
Center for Cancer Research
National Cancer Institute
Bethesda, Maryland

Yuman Fong, MD
Chairman, Department of Surgery
Associate Director, International Relations
City of Hope Medical Center
Duarte, California

James M. Ford, MD
Associate Professor
Departments of Medicine, Pediatrics and Genetics
Divisions of Oncology and Medical Genetics
Director, Clinical Cancer Genomics Program
Stanford University School of Medicine
Stanford, California

Francine M. Foss, MD
Professor of Medicine, Hematology and Bone Marrow Transplantation
Yale University School of Medicine
New Haven, Connecticut

Arnold S. Freedman, MD
Associate Professor of Medicine
Harvard Medical School
Clinical Director, Lymphoma Program
Dana-Farber Cancer Institute
Associate Physician, Brigham and Women's Hospital
Boston, Massachusetts

Wayne L. Furman, MD
Member, Department of Oncology
St. Jude Children's Research Hospital
Professor, Department of Pediatrics
University of Tennessee Medical School
Memphis, Tennessee

Larissa V. Furtado, MD
Assistant Professor
Department of Pathology
Assistant Director
Division of Genomics and Molecular Pathology
University of Chicago
Chicago, Illinois

Sheryl G. A. Gabram-Mendola, MD, MBA, FACS
Surgeon-in-Chief
Grady Memorial Hospital
Emory University School of Medicine
Deputy Director
Georgia Cancer Center for Excellence
Director, AVON Comprehensive Breast Center at Grady
Director, High Risk Assessment Program
Winship Cancer Institute of Emory University
Georgia Cancer Coalition Distinguished Cancer Scholar
Atlanta, Georgia

Michele Gabree, MS
Certified Genetic Counselor
Center for Cancer Risk Assessment
Massachusetts General Hospital
Boston, Massachusetts

Patricia A. Ganz, MD
Distinguished University Professor
University of California, Los Angeles
Fielding School of Public Health
David Geffen School of Medicine at University of California, Los Angeles
Director, Cancer Prevention & Control Research
Jonsson Comprehensive Cancer Center
Los Angeles, California

Levi A. Garraway, MD, PhD
Associate Professor
Department of Medical Oncology
Dana-Farber Cancer Institute
Senior Associate Member
The Broad Institute of Massachusetts Institute of Technology and Harvard
Associate Physician
Brigham and Women's Hospital
Boston, Massachusetts

Jared J. Gartner, DO
Biologist, National Cancer Institute
Surgery Branch
National Institute of Health
Bethesda, Maryland

Juan C. Gea-Banacloche, MD
Head, Infectious Diseases Unit
Experimental Transplantation and Immunology Branch
National Cancer Institute
Chief, Infectious Diseases Consultation Service
National Institute of Allergy and Infectious Disease
Bethesda, Maryland

David M. Gershenson, MD
Professor and Chair
Department of Gynecologic Oncology
The University of Texas MD Anderson Cancer Center
Houston, Texas

Scott Nicholas Gettinger, MD
Associate Professor of Medicine
Thoracic Oncology Program
Developmental Therapeutics
Yale Cancer Center
New Haven, Connecticut

Alexander I. Geyer, MD
Assistant Clinical Member
Pulmonary Service
Department of Medicine
Memorial Sloan-Kettering Cancer Center
New York, New York

Juliet F. Gibson, BA
Research Fellow
Dermatology and Hematology
Yale University School of Medicine
New Haven, Connecticut

Hannah L. Gilmore, MD
Assistant Professor, Department of Pathology
Case Western Reserve University School of Medicine
Surgical Pathology Director
University Hospitals Case Medical Center
Cleveland, Ohio

Olivier Glehen, MD, PhD
Professor Lyon 1 University
Chief, Division of General Surgery
Hospices Civils de Lyon
Department of General and Oncologic Surgery
Centre Hospitalier Lyon Sud
Pierre-Bénite, France

Matthew P. Goetz, MD
Associate Professor of Pharmacology
Associate Professor of Oncology
Mayo Clinic
Rochester, Minnesota

Sarah B. Goldberg, MD, MPH
Assistant Professor of Internal Medicine
Medical Oncology
Yale Cancer Center
Yale University School of Medicine
New Haven, Connecticut

Donald P. Goldstein, MD
Professor of Obstetrics, Gynecology and Reproductive Biology
Harvard Medical School
Senior Obstetrician-Gynecologist
Brigham and Women's Hospital
Founder and Director Emeritus
New England Trophoblastic Disease Center
Dana-Farber and Brigham and Women's Cancer Center
Boston, Massachusetts

Leonard G. Gomella, MD, FACS
The Bernard W. Godwin Professor of Prostate Cancer
Chairman, Department of Urology
Associate Director
Jefferson Kimmel Cancer Center
Clinical Director, Jefferson Kimmel Cancer Center Network
Thomas Jefferson University
Philadelphia, Pennsylvania

Steven D. Gore, MD
Yale University School of Medicine
New Haven, Connecticut

Ramaswamy Govindan, MD
Professor of Medicine
Co-Director, Section of Medical Oncology
Division of Oncology
Washington University School of Medicine
St. Louis, Missouri

F. Anthony Greco, MD
Director, Sarah Cannon Cancer Center
Tennessee Oncology, PLLC and Sarah Cannon Research Institute
Nashville, Tennessee

Marcio L. Griebeler, MD
Fellow, Endocrinology, Metabolism and Nutrition
Mayo Clinic
Rochester, Minnesota

Ellen R. Gritz, PhD
Professor and Chair
Department of Behavioral Science
The University of Texas MD Anderson Cancer Center
Houston, Texas

Alessandro Gronchi, MD
Surgical Oncologist, Department of Surgery
Chief, Sarcoma Service
Fondazione IRCCS
Istituto Nazionale dei Tumori
Milan, Italy

Samuel D. Gross, MD
Professor and Chair, Department of Surgery
Jefferson Medical College of Thomas Jefferson University
Co-Director, Jefferson Pancreas, Biliary and Related Cancers Center
Philadelphia, Pennsylvania

José G. Guillem, MD, MPH
Department of Surgery
Memorial Sloan-Kettering Cancer Center
New York, New York

Caroline M. Gulati, MD
Fellow
Department of Medicine
Division of Pulmonary and Critical Care
New York Presbyterian Weill Cornell Medical Center
New York, New York

Jennifer Moliterno Günel, MD
Assistant Professor
Department of Neurosurgery
Yale University School of Medicine
New Haven, Connecticut

Murat Günel, MD
Nixdorff-German Professor
Chief, Yale Neurovascular Surgery Program
Co-Director, Yale Program on Neurogenetics
Director, Yale Program in Brain Tumor Research
Departments of Neurosurgery, Neurobiology and Genetics
Yale University School of Medicine
New Haven, Connecticut

Daphne A. Haas-Kogan, MD
Professor of Radiation Oncology and Neurological Surgery
Program Director and Vice Chair
Department of Radiation Oncology
Helen Diller Family Comprehensive Cancer Center
San Francisco, California

John D. Hainsworth, MD
Chief Scientific Officer
Sarah Cannon Research Institute
Nashville, Tennessee

Mehdi Hamadani, MD
Associate Professor of Medicine
Director-Myeloma & Lymphoma Service
Section of Hematology and Oncology
West Virginia University
Morgantown, West Virginia

Gary D. Hammer, MD, PhD
University of Michigan
Millie Schembechler Professor of Adrenal Cancer
Director Endocrine Oncology Program
Director, Center for Organogenesis
Ann Arbor, Michigan

Douglas Hanahan, PhD
Director
Swiss Institute for Experimental Cancer Research (ISREC)
Lausanne, Switzerland

Parameswaran N. Hari, MD
Professor Section Head, Hematological Malignancies and Transplantation
Division of Hematology Oncology
Department of Medicine
Medical College of Wisconsin
Milwaukee, Wisconsin

Jay R. Harris, MD
Professor and Chair
Department of Radiation Oncology
Dana-Farber Cancer Institute
Brigham and Women's Hospital
Harvard Medical School
Boston, Massachusetts

Lyndsay N. Harris, MD, FRCP(C)
Diana Hyland Chair in Breast Cancer
Director, Breast Cancer Program
Seidman Cancer Center
University Hospitals Case Medical Center
Professor of Medicine
Division of Hematology and Oncology
Case Western Reserve University
Cleveland, Ohio

Marc Haxer, MA, CCC-Sp
Departments of Speech Pathology and Otolaryngology/Head and Neck Surgery
University of Michigan Health System
Ann Arbor, Michigan

C. William Helm, MBBChir, FRCS
Northern Gynaecological Oncology Center
Queen Elizabeth Hospital, Gateshead
Tyne and Wear, United Kingdom

Lee J. Helman, MD
Scientific Director for Clinical Research
Center for Cancer Research National Cancer Institute
Bethesda, Maryland

James G. Herman, MD
Johns Hopkins University
Baltimore, Maryland

Paul J. Hesketh, MD
Professor, Department of Medicine
Tufts University School of Medicine
Boston, Massachusetts
Director Sophia Gordon Cancer Center
Lahey Hospital & Medical Center
Burlington, Massachusetts

Jay L. Hess, MD, PhD
Professor, Department of Pathology
Carl V. Weller Professor and Chair
Professor, Department of Internal Medicine
University of Michigan Health System
Ann Arbor, Michigan

Christopher J. Hoimes, DO
Assistant Professor
UH Case Medical Center
Department of Medicine-Hematology and Oncology
Cleveland, Ohio

Leora Horn, MD, MSc
Associate Professor, Department of Medicine
Vanderbilt University Medical Center
Nashville, Tennessee

Neil S. Horowitz, MD
Assistant Professor of Obstetrics, Gynecology, and Reproductive Medicine
Harvard Medical School
Director of Clinical Research
Division of Gynecologic Oncology
Brigham and Women's Hospital
Boston, Massachusetts

Ralph H. Hruban, MD
Professor, Department of Pathology
Director, The Sol Goldman Pancreatic Cancer Research Center
Johns Hopkins University School of Medicine
Baltimore, Maryland

Melissa M. Hudson, MD
Full Member
Director, Cancer Survivorship Division
Department of Oncology
St. Jude Children's Research Hospital
Memphis, Tennessee

Vanessa W. Hui, MD
Department of Surgery
Memorial Sloan-Kettering Cancer Center
New York, New York

Carolyn D. Hurst, BSc, MSc, PhD
Senior Postdoctoral Research Fellow
Section of Experimental Oncology
Leeds Institute of Cancer and Pathology
St. James's University Hospital
Leeds, United Kingdom

David H. Ilson, MD, PhD
Professor and Attending Physician
Department of Medicine
Memorial Sloan-Kettering Cancer Center and Weill Cornell Medical College
Memorial Hospital
New York, New York

Caron A. Jacobson, MD, MSc
Instructor, Department of Medicine
Harvard Medical School
Brigham and Women's Hospital
Division of Medical Oncology
Hematologic Malignancies
Dana-Farber Cancer Institute
Boston, Massachusetts

Kory W. Jasperson, MS, CGC
Genetic Counselor
Department of Internal Medicine
Huntsman Cancer Institute
University of Utah
Salt Lake City, Utah

Peter Johnson, MD, FRCP
Professor of Medical Oncology
Cancer Research United Kingdom Centre
University of Southampton Faculty of Medicine
Southampton, United Kingdom

Matthew Kalady, MD
Associate Professor of Surgery
Krause-Lieberman Chair in Colorectal Surgery
Department of Colorectal Surgery
Digestive Disease Institute
Cleveland Clinic
Cleveland, Ohio

Kala Y. Kamdar, MD, MS
Assistant Professor
Department of Pediatrics
Hematology/Oncology Section
Baylor College of Medicine
Associate Clinical Director
Texas Children's Cancer and Hematology Center
Houston, Texas

Robert J. Kaner, MD
Associate Attending Physician
New York Presbyterian Weill Cornell Center
Associate Professor of Clinical Medicine and Genetic Medicine
Weill Cornell Medical College
New York, New York

Joyson J. Karakunnel, MD, FACP
Potomac, Maryland

Brian D. Kavanagh, MD, MPH
Professor, Department of Radiation Oncology
University of Colorado School of Medicine
Anschutz Medical Campus
Aurora, Colorado

Partow Kebriaei, MD
Associate Professor
Division of Cancer Medicine
Department of Stem Cell Transplant and Cellular Therapy
The University of Texas MD Anderson Cancer Center
Houston, Texas

David P. Kelsen, MD
Edward S Gordon Chair in Medical Oncology
Attending Physician, Department of Medicine
Memorial Sloan-Kettering Cancer Center
Professor of Medicine Weill Cornell Medical College
New York, New York

Scott E. Kern, MD
Everett and Marjorie Kovler Professor of Pancreas Cancer Research
Department of Oncology
Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins
Baltimore, Maryland

Alok A. Khorana, MD, FACP
Professor of Medicine, Cleveland Clinic Lerner College of Medicine, Case Western Reserve University
Sondra and Stephen Hardis Chair in Oncology Research
Vice Chair for Clinical Services, Taussig Cancer Institute
Director, Gastrointestinal Malignancies Program
Cleveland Clinic
Cleveland, Ohio

Christopher J. Kirk, MD
Vice President of Research
Onyx Pharmaceuticals, Inc.
South San Francisco, California

Ann H. Klopp, MD, PhD
Assistant Professor
Section of Gynecology
Department of Radiation Oncology
The University of Texas MD Anderson Cancer Center
Houston, Texas

Margaret A. Knowles, PhD
Head, Section of Experimental Oncology
Leeds Institute of Cancer and Pathology
St. James's University Hospital
Leeds, United Kingdom

James N. Kochenderfer, MD
Investigator, Experimental Transplantation and Immunology Branch
National Cancer Institute
National Institutes of Health
Bethesda, Maryland

Manish Kohli, MD
Associate Professor of Oncology
Department of Oncology
College of Medicine
Mayo Clinic
Joint Appointment, Department of Urology
Mayo Clinic
Rochester, Minnesota

Rami S. Komrokji, MD
Clinical Director
Associate Member
Department of Malignant Hematology
H. Lee Moffitt Cancer Center & Research Institute
Tampa, Florida

Panagiotis A. Konstantinopoulos, MD, PhD
Assistant Professor of Medicine
Harvard Medical School
Gynecologic Medical Oncology Program
Dana-Farber Cancer Institute
Boston, Massachusetts

Vadim P. Koshenkov, MD
Assistant Professor of Surgery
Division of Surgical Oncology
Rutgers Cancer Institute of New Jersey
Robert Wood Johnson University Hospital
New Brunswick, New Jersey

Matthew J. Krasin, MD
Radiological Sciences
Radiation Oncology
St. Jude Children's Research Hospital
Memphis, Tennessee

Mark G. Kris, MD
Professor of Medicine
Weill Cornell Medical College
Attending Physician, Thoracic Oncology Service
Memorial Sloan-Kettering Cancer Center
New York, New York

Robert S. Krouse, MD, MS
Professor, Department of Surgery
University of Arizona College of Medicine
Staff General and Oncologic Surgeon
Surgical Care Line
Southern Arizona Veterans Affairs Health Care System
Tucson, Arizona

Lee M. Krug, MD
Associate Attending Physician
Department of Medicine
Memorial Sloan-Kettering Cancer Center
Associate Professor of Medicine
Weill Medical College of Cornell University
New York, New York

Shivaani Kummar, MD, FACP
Head, Early Clinical Trials Development
Office of the Director
Division of Cancer Treatment and Diagnosis
National Cancer Institute
Bethesda, Maryland

Pamela L. Kunz, MD
Assistant Professor of Medicine/Oncology
Stanford University School of Medicine
Division of Oncology
Stanford Cancer Institute
Stanford, California

John Kuruvilla, MD, FRCPC
Hematologist, Princess Margaret Cancer Centre
Assistant Professor Medicine
University of Toronto
Toronto, Ontario, Canada

Wendy Landier, PhD, RN, CPNP
Assistant Professor, Department of Population Sciences
Clinical Director, Center for Cancer Survivorship
City of Hope
Duarte, California

Brian R. Lane, MD, PhD
Associate Professor, Department of Surgery
Michigan State University College of Human Medicine
Betz Family Endowed Chair for Cancer Research
Spectrum Health Cancer Program
Grand Rapids, Michigan

Theodore S. Lawrence, MD, PhD
Isadore Lampe Professor and Chair
Department of Radiation Oncology
University of Michigan Health System
Ann Arbor, Michigan

Hillard M. Lazarus, MD, FACP
Professor of Medicine
Case Western Reserve University
Director, Novel Cell Therapy
The George & Edith Richman Professor and Distinguished Scientist in Cancer Research
University Hospitals
Case Medical Center
Cleveland, Ohio

Thomas W. LeBlanc, MD, MA
Assistant Professor, Division of Hematologic Malignancies and Cellular Therapy
Department of Medicine
Duke University School of Medicine
Durham, North Carolina

Richard J. Lee, MD, PhD
Assistant Professor, Department of Medicine
Harvard Medical School
Assistant Physician, Division of Hematology and Oncology
Massachusetts General Hospital
Boston, Massachusetts

Percy P. Lee, MD
Associate Professor of Radiation Oncology
Chief, Thoracic Radiation Oncology
Chief, Gastrointestinal Radiation Oncology
Director, Stereotactic Body Radiation Oncology Program
David Geffen School of Medicine at University of California, Los Angeles
University of California Los Angeles Jonsson Comprehensive Cancer Center
Los Angeles, California

Agnes Y. Y. Lee, MC, MSc, FRCPC
Associate Professor, Department of Medicine
Director, Thrombosis Program
University of British Columbia and British Columbia Cancer Agency
Vancouver, British Columbia, Canada

David J. Leffell, MD
David Paige Smith Professor of Dermatology & Surgery
Chief, Section of Dermatologic Surgery and Cutaneous Oncology
Department of Dermatology
Yale University School of Medicine
New Haven, Connecticut

Antonio M. Lerario, MD
Adrenal Disorders Unit
Department of Endocrinology and Metabolism
Hospital das Clinicas da Faculdade de Medicina da Universidade de Sao Paulo
Sao Paulo, Brazil

Rebecca A. Levine, MD
Assistant Professor, Department of Surgery
Montefiore Medical Center
Albert Einstein College of Medicine
Bronx, New York

Nancy L. Lewis, MD
Associate Professor
Department of Medical Oncology
Clinical Director
Experimental Therapeutics
Thomas Jefferson University
Kimmel Cancer Center
Philadelphia, Pennsylvania

Steven K. Libutti, MD, FACS
Director, Montefiore-Einstein Center for Cancer Care
Vice Chairman, Department of Surgery
Montefiore-Einstein Center for Cancer Care
Bronx, New York

Rogerio C. Lilenbaum, MD
Chief Medical Officer of Smilow Cancer Hospital
Thoracic Oncology Program
Yale University School of Medicine
New Haven, Connecticut

W. Marston Linehan, MD
Chief, Urologic Oncology Branch
Center for Cancer Research
National Cancer Institute
Bethesda, Maryland

Scott M. Lippman, MD
Director, Senior Associate Dean, & Associate Vice Chancellor
Cancer Research and Care
Chugai Pharmaceutical Chair
Professor of Medicine
University of California, San Diego
Moores Cancer Center
La Jolla, California

Roy Lirov, MD
Resident in General Surgery
Department of Surgery
University of Michigan Hospital and Health Systems
University of Michigan Health System
Ann Arbor, Michigan

Alan F. List, MD
President and CEO
Senior Member
H. Lee Moffitt Cancer Center
Tampa, Florida

Richard F. Little, MD, MPH
Senior Investigator
Head, Blood and AIDS Related Cancer Therapeutics
Clinical Investigations Branch
Cancer Therapy Evaluation Program
National Cancer Institute
Bethesda, Maryland

Mats Ljungman, PhD
Professor, Departments of Radiation Oncology and Environmental Health Sciences
Translational Oncology Program
University of Michigan Medical School
Ann Arbor, Michigan

Patrick J. Loehrer, Sr, MD
H. H. Gregg Professor of Oncology
Director, Indiana University Melvin and Bren Simon Cancer Center
Associate Dean for Cancer Research
Indiana University School of Medicine
Indianapolis, Indiana

Christopher J. Logothetis, MD
Principle Investigator
Genitourinary Medical Oncology
The University of Texas MD Anderson Cancer Center
Houston, Texas

Carlos López-Otin, PhD
Professor, Department of Biochemistry and Molecular Biology
Universidad de Oviedo
Principality of Asturias, Spain

Charles L. Loprinzi, MD
Regis Professor of Breast Cancer Research
Department of Oncology
Mayo Clinic
Rochester, Minnesota

Yani Lu, PhD
Assistant Research Professor
Division of Cancer Etiology
Department of Population Science
Beckman Research Institute of the City of Hope
Duarte, California

Samuel J. Lubner, MD
Assistant Professor
Department of Medicine
Hematology/Oncology Section
Associate Program Director
Hematology/Oncology Fellowship
University of Wisconsin
Madison, Wisconsin

Matthew A. Lunning, DO
Assistant Professor
Division of Hematology/Oncology
Department of Medicine
University of Nebraska Medical Center
Omaha, Nebraska

Teresa H. Lyden, MA, CCC-SLP
Speech Language Pathologist
Department of Otolaryngology-Head and Neck Surgery
University of Michigan Medical Center
Ann Arbor, Michigan

Xiaomei Ma, PhD
Associate Professor, Department of Chronic Disease Epidemiology
Yale University School of Public Health
New Haven, Connecticut

Krishnaraj Mahendraraj, MD
Resident, Department of Surgery
St. Barnabas Medical Center
Livingston, New Jersey

Ajay V. Maker, MD, FACS
Assistant Professor, Department of Surgery
Division of Surgical Oncology
Department of Microbiology and Immunology
University of Illinois at Chicago
Director of Surgical Oncology
Advocate Illinois Masonic Medical Center
Chicago, Illinois

David Malkin, MD
Staff Oncologist, Division of Hematology/Oncology
Senior Scientist, Genetics & Genome Biology Program
The Hospital for Sick Children
University of Toronto
Toronto, Ontario, Canada

Eddie W. Manning, III, MD
Senior Resident
Division of Cardiothoracic Surgery
The DeWitt Daughtry Family Department of Surgery
Miller School of Medicine
University of Miami
Miami, Florida

Judith F. Margolin, MD
Associate Professor
Department of Pediatrics
Baylor College of Medicine
Attending Oncologist
Texas Children's Cancer Center
Houston, Texas

David Marin, MD, DM, FRCP
Department of Stem Cell Transplantation and Cellular Therapy
The University of Texas MD Anderson Cancer Center
Houston, Texas

Jens U. Marquardt, MD
Resident Physician
Department of Medicine
University of Mainz
Mainz, Germany
Laboratory of Experimental Carcinogenesis
Center for Cancer Research
National Cancer Institute
National Institutes of Health
Bethesda, Maryland

Ellen T. Matloff, MS, CGC
President & CEO
Gene Counsel, LLC
New Haven, Connecticut

Peter Mauch, MD
Professor of Radiation Oncology
Department of Radiation Oncology
Harvard Medical School
Boston, Massachusetts

Susan T. Mayne, PhD
C.-E.A. Winslow Professor of Epidemiology
Chair, Department of Chronic Disease Epidemiology
Yale University School of Public Health
Associate Director for Population Sciences
Yale Cancer Center
New Haven, Connecticut

Howard L. McLeod, PharmD
Medical Director, DeBartolo Family Personalized Medicine Institute
Senior Member, Division of Population Sciences
H. Lee Moffitt Cancer Center
Tampa, Florida

Minesh P. Mehta, MBChB, FASTRO
Professor, Department of Radiation Oncology
Director, Maryland Proton Treatment Center
University of Maryland
Baltimore, Maryland

Laleh G. Melstrom, MD, MSCI
Assistant Professor, Department of Surgery
Rutgers Cancer Institute of New Jersey
Robert Wood Johnson School of Medicine
Robert Wood Johnson Hospital and the Rutgers Cancer Institute of New Jersey
New Brunswick, New Jersey

William M. Mendenhall, MD
Professor Department of Radiation Oncology
University of Florida
Shands Hospital at the University of Florida
Gainesville, Florida
University of Florida Proton Therapy Institute
Jacksonville, Florida

M. Dror Michaelson, MD, PhD
Associate Professor of Medicine
Harvard Medical School
Clinical Director, Urologic Oncology
Massachusetts General Hospital Cancer Center
Boston, Massachusetts

Karin B. Michels, ScD, PhD
Associate Professor
Obstetrician/Gynecologist
Epidemiology Center
Department of Obstetrics, Gynecology and Reproductive Biology
Brigham and Women's Hospital
Harvard Medical School
Boston, Massachusetts

Bruce D. Minsky, MD
Professor and Frank T. McGraw Memorial Chair
Deputy Head, Division of Radiation Oncology
The University of Texas MD Anderson Cancer Center
Houston, Texas

Sandra A. Mitchell, PhD, CRNP
Research Scientist and Program Director
Outcomes Research Branch
Applied Research Program
Division of Cancer Control and Population Sciences
National Cancer Institute
Rockville, Maryland

Jeffrey F. Moley, MD
Chief, Section of Endocrine and Oncologic Surgery
Professor of Surgery
Washington University School of Medicine
St. Louis, Missouri

Meredith A. Morgan, PhD
Research Assistant Professor
Department of Radiation Oncology
University of Michigan
Ann Arbor, Michigan

Daniel Morgensztern, MD
Associate Professor of Medicine
Director of Thoracic Oncology
Clinical Research Division of Oncology
Alvin J. Siteman Cancer Center
Washington University School of Medicine
St. Louis, Missouri

Monica Morrow, MD
Chief, Breast Service
Anne Burnett Windfohr Chair of Clinical Oncology
Department of Surgery
Memorial Sloan-Kettering Cancer Center
Professor of Surgery
Weill Cornell Medical College
New York, New York

Nikhil C. Munshi, MD
Associate Director, Jerome Lipper Multiple Myeloma Center
Department of Medical Oncology
Dana-Farber Cancer Institute
Associate Professor of Medicine
Harvard Medical School
Boston, Massachusetts

Rebecca Nagy, MS, LGC
Associate Professor
Clinical Internal Medicine
The Ohio State University
Department of Internal Medicine
Licensed Genetic Counselor
Division of Human Genetics
The Ohio State University Wexner Medical Center
Columbus, Ohio

Patrick Nana-Sinkam, MD
Associate Professor, Department of Internal Medicine
Division of Pulmonary, Allergy, Critical Care and Sleep Medicine
The Ohio State University
Columbus, Ohio

Fariba Navid, MD
Associate Member, Department of Oncology
Division of Solid Malignancies
St. Jude Children's Research Hospital
Memphis, Tennessee

Christian J. Nelson, PhD
Assistant Attending Psychologist
Department of Psychiatry and Behavioral Sciences
Memorial Sloan-Kettering Cancer Center
New York, New York

Anna C. Newlin, MS, CGC
Certified Genetic Counselor
Center for Medical Genetics
NorthShore University HealthSystem
Evanston, Illinois

Andrea Ng, MD, MPH
Associate Professor, Department of Radiation Oncology
Brigham and Women's Hospital
Dana-Farber Cancer Institute
Harvard Medical School
Boston, Massachusetts

Dao M. Nguyen, MD, MSc, FRCSC, FACS
The B. and Donald Carlin Endowed Chair of Thoracic Surgical Oncology
Professor and Chief
Section of Thoracic Surgery Section
Division of Cardiothoracic Surgery
The DeWitt Daughtry Family Department of Surgery
Miller School of Medicine
University of Miami
Miami, Florida

Torsten O. Nielsen, MD, PhD, FRCPC
Professor, Department of Pathology and Laboratory Medicine
University of British Columbia
Vancouver, British Colombia, Canada

Jeffrey A. Norton, MD
Professor, Department of Surgery
Chief, Section of Surgical Oncology and Division of General Surgery
Department of Surgery
Stanford University Hospital
Stanford, California

Susan M. O'Brien, MD
Professor, Department of Leukemia
The University of Texas MD Anderson Cancer Center
Houston, Texas

Richard J. O'Connor, PhD
Associate Member, Department of Health Behavior
Division of Cancer Prevention and Population Sciences
Roswell Park Cancer Institute
Buffalo, New York

Richard J. O'Donnell, MD
Professor of Clinical Orthopaedic Surgery
Chief, Orthopaedic Oncology Service
Director, Sarcoma Program
University of California, San Francisco Medical Center
University of California, San Francisco
Benioff Children's Hospitals
University of California, San Francisco
Bakar Cancer Hospital University of California, San Francisco
Helen Diller Family Comprehensive Cancer Center
San Francisco, California

Kunle Odunsi, MD, PhD
The M. Steven Piver Professor and Chair
Department of Gynecologic Oncology Director
Center for Immunotherapy
Professor of Obstetrics and Gynecology
Department of Gynecology-Obstetrics
School of Medicine and Biomedical Sciences
University at Buffalo-State University of New York
Buffalo, New York

Peter J. O'Dwyer, MD
Professor of Medicine
Abramson Cancer Center
University of Pennsylvania
Philadelphia, Pennsylvania

Kevin C. Oeffinger, MD
Director, Adult Long-Term Follow-Up Program
Co-Leader, Survivorship, Outcomes and Risk Program
Full Member, Departments of Medicine and Pediatrics
Memorial Sloan-Kettering Cancer Center
New York, New York

Brian O'Sullivan, MD, FRCPC
Bartley-Smith/Wharton Chair in Radiation Oncology
Professor, Department of Radiation Oncology
University of Toronto
Toronto, Ontario, Canada

Gregory A. Otterson, MD
Professor, Department of Internal Medicine
Division of Medical Oncology
Director, Hematology/Oncology Fellowship Program
The Ohio State University Comprehensive Cancer Center
Columbus, Ohio

Eric Padron, MD
Assistant Member
Malignant Hematology Section
Head, Genomics and Personalized Medicine
H. Lee Moffitt Cancer Center and Research Institute
Tampa, Florida

Lance C. Pagliaro, MD
Professor, Department of Genitourinary Medical Oncology
The University of Texas MD Anderson Cancer Center
Houston, Texas

Tara N. Palmore, MD
Hospital Epidemiologist
National Institutes of Health Clinical Center
Staff Clinician
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Alberto S. Pappo, MD
Member
Director, Solid Tumor Section
St. Jude Children's Research Hospital
Memphis, Tennessee

Howard L. Parnes, MD
Chief
Prostate and Urologic Cancer Research Group
Division of Cancer Prevention
National Cancer Institute
Rockville, Maryland

Mark Parta, MD
Clinical Research Directorate/Clinical Monitoring Research Program
Leodos Biomedical Research, Inc.
Frederick National Laboratory for Cancer Research
Frederick, Maryland

Laura Pasqualucci, MD
Associate Professor of Pathology and Cell Biology
Department of Pathology and Cell Biology
Institute for Cancer Genetics
Herbert Irving Comprehensive Cancer Center
Columbia University
New York, New York

Harvey I. Pass, MD
Stephen E. Banner Professor of Thoracic Oncology
Vice-Chairman, Research Department of Cardiothoracic Surgery
Director, General Thoracic Surgery
New York University Langone Medical Center
New York, New York

Tushar Patel, MBChB
Professor of Medicine
Mayo Clinic
Jacksonville, Florida

George Patounakis, MD, PhD
Clinical Fellow
Department of Reproductive Endocrinology and Infertility
National Institute of Child Health and Human Development
National Institutes of Health
Bethesda, Maryland

David M. Peereboom, MD, FACP
Professor of Medicine
Cleveland Clinic Lerner College of Medicine
Director, Clinical Research
The Rose Ella Burkhardt Brain Tumor & Neuro-Oncology Center
Solid Tumor Oncology
Cleveland Clinic
Cleveland, Ohio

Tanja Pejovic, MD, PhD
Associate Professor
Director, Gynecologic Cancer Research
Department of Obstetrics & Gynecology
Knight Cancer Institute
Oregon Health & Science University
Portland, Oregon

David G. Pfister, MD
Chief, Head and Neck Oncology Service
Memorial Sloan-Kettering Cancer Center
New York, New York

Giao Q. Phan, MD, FACS
Associate Professor
Division of Surgical Oncology
Massey Cancer Center
Virginia Commonwealth University
Richmond, Virginia

M. Catherine Pietanza, MD
Thoracic Oncology
Memorial Sloan-Kettering Cancer Center
New York, New York

Robert Pilarski, MS, LGC, MSW
Associate Professor
Clinical Internal Medicine
James Comprehensive Cancer Center
Department of Internal Medicine
The Ohio State University Wexner Medical Center
Columbus, Ohio

Peter W. T. Pisters, MD, FACS
Professor, Department of Surgical Oncology
Division of Surgery
The University of Texas MD Anderson Cancer Center
Houston, Texas

Mark N. Polizzotto, MB, BS, BMedSc
Assistant Clinical Investigator
HIV and AIDS Malignancy Branch
Center for Cancer Research National Cancer Institute
National Institutes of Health
Bethesda, Maryland

Yves Pommier, MD, PhD
Chief, Laboratory of Molecular Pharmacology
Head, DNA Topoisomerase/Integrase Group
Center for Cancer Research
National Cancer Institute
Bethesda, Maryland

David G. Poplack, MD
Elise C. Young Professor of Pediatric Oncology
Head, Hematology-Oncology Section
Department of Pediatrics
Baylor College of Medicine
Director, Texas Children's Cancer Center
Texas Children's Hospital
Houston, Texas

Edwin M. Posadas, MD, FACP, KM
Medical Director, Urologic Oncology Program
Assistant Professor, Department of Medicine
Samuel Ochsin Comprehensive Cancer Institute
Cedars-Sinai Medical Center
Los Angeles, California

Mitchell C. Posner, MD, FACS
Thomas D. Jones Professor of Surgery and Vice-Chairman
Chief, Section of General Surgery and Surgical Oncology
Professor, Radiation and Cellular Oncology
Medical Director, Clinical Cancer Programs
The University of Chicago Medicine
Chicago, Illinois

Sahdeo Prasad, PhD
Cytokine Research Laboratory
Department of Experimental Therapeutics
The University of Texas MD Anderson Cancer Center
Houston, Texas

Mark E. P. Prince, MD, FRCS(C)
Professor, Department of Otolaryngology-Head and Neck Surgery
Chief, Division of Head and Neck Surgery
University of Michigan Health System
Ann Arbor, Michigan

Ibrahim M. Qazi, PharmD
University of Maryland
Baltimore, Maryland

Karen R. Rabin, MD, PhD
Assistant Professor, Department of Pediatrics
Division of Pediatric Hematology and Oncology
Baylor College of Medicine
Houston, Texas

Glen D. Raffel, MD, PhD
Assistant Professor, Department of Medicine
Division of Hematology-Oncology
University of Massachusetts Medical School
Worcester, Massachusetts

Lee Ratner, MD, PhD
Professor Departments of Medicine and Molecular Microbiology
Co-Director, Medical & Molecular Oncology
Washington University School of Medicine
Barnes-Jewish Hospital
St. Louis, Missouri

Abram Recht, MD, FASTRO
Professor, Department of Radiation Oncology
Harvard Medical School
Deputy Chief, Department of Radiation Oncology
Beth Israel Deaconess Medical Center
Boston, Massachusetts

Michelle B. Riba, MD, MS
Professor and Associate Chair for Integrated Medical and Psychiatric Services
Department of Psychiatry
Director, PsychOncology Program
University of Michigan Comprehensive Cancer Center
Ann Arbor, Michigan

Antoni Ribas, MD, PhD
Professor of Medicine
Professor of Surgery
Professor of Molecular and Medical Pharmacology
Director, Tumor Immunology Program
Jonsson Comprehensive Cancer Center
David Geffen School of Medicine
University of California Los Angeles
Chair, Melanoma Committee
Southwest Oncology Group
Los Angeles, California

Stanley R. Riddell, MD
Member, Fred Hutchinson Cancer Research Center
Professor, Department of Medicine
University of Washington
Seattle, Washington

Andreas Rimner, MD
Assistant Professor, Department of Radiation Oncology
Assistant Attending, Department of Radiation Oncology
Memorial Sloan-Kettering Cancer Center
New York, New York

Brian I. Rini, MD, FACP
Professor of Medicine
Lerner College of Medicine
Department of Solid Tumor Oncology
Cleveland Clinic Taussig Cancer Institute
Glickman Urological Institute
Cleveland, Ohio

Paul F. Robbins, PhD
National Institutes of Health
Bethesda, Maryland

Matthew K. Robinson, PhD
Assistant Professor, Developmental Therapeutics Program
Fox Chase Cancer Center
Philadelphia, Pennsylvania

Steven A. Rosenberg, MD, PhD
Chief, Surgery Branch, National Cancer Institute, National Institutes of Health
Professor of Surgery, Uniformed Services University of the Health Sciences
School of Medicine
Bethesda, Maryland
Professor of Surgery
George Washington University School of Medicine
Washington, District of Columbia

Kenneth E. Rosenzweig, MD
Professor, Department of Radiation Oncology
Icahn School of Medicine at Mount Sinai
Chair, Department of Radiation Oncology
Mount Sinai Health System
New York, New York

Charles M. Rudin, MD, PhD
Chief, Thoracic Oncology Service
Memorial Sloan-Kettering Cancer Center
New York, New York

Anil K. Rustgi, MD
T. Grier Miller Professor of Medicine & Genetics
Chief of Gastroenterology
University of Pennsylvania Perelman School of Medicine
Philadelphia, Pennsylvania

Arjun Sahgal, MD
Associate Professor of Radiation Oncology and Surgery
Department of Radiation Oncology
Toronto, Ontario, Canada

M. Wasif Saif, MD, MBBS
Director, Gastrointestinal Oncology Program
Leader, Experimental Therapeutics Program
Tufts Medical Center
Tufts University School of Medicine
Boston, Massachusetts

Leonard B. Saltz, MD
Professor, Department of Medicine
Chief, Gastrointestinal Oncology Service
Memorial Sloan-Kettering Cancer Center
New York, New York

Yardena Samuels, PhD
Knell Family Professorial Chair
Department of Molecular Cell Biology
Weizmann Institute of Science
Rehovot, Israel

Charles L. Sawyers, MD
Investigator, Howard Hughes Medical Institute
Chair, Human Oncology and Pathogenesis Program
Memorial Sloan-Kettering Cancer Center
New York, New York

Peter T. Scardino, MD
Chairman, Department of Surgery
The David H. Koch Chair
Memorial Sloan-Kettering Cancer Center
New York, New York

Sanne B. Schagen, PhD
Division of Psychosocial Research and Epidemiology
The Netherlands Cancer Institute
Amsterdam, The Netherlands

Howard I. Scher, MD
Chief, Genitourinary Oncology Service
Member and Attending Physician
Department of Medicine
Memorial Sloan-Kettering Cancer Center
Professor of Medicine
Weill Cornell Medical College
New York, New York

Laura S. Schmidt, PhD
Principal Scientist
Leidos Biomedical Research, Inc.
Frederick National Laboratory for Cancer Research
Frederick, Maryland
Urologic Oncology Branch
National Cancer Institute
National Institutes of Health
Bethesda, Maryland

Meredith L. Seidel, MS, CGC
Genetic Counselor
Center for Cancer Risk Assessment
Massachusetts General Hospital
Boston, Massachusetts

Leigha Senter-Jamieson, MS, CGC
Director of Clinical Supervison
Associate Professor
Division of Human Genetics
Department of Internal Medicine
The Ohio State University
Columbuis, Ohio

Bijal Shah, MD
Associate Member
H. Lee Moffitt Cancer Center
Tampa, Florida

Syed Ammer Shah, MD
Postgraduate Year Four
Surgical Resident
Saint Barnabas Medical Center
Caldwell, New Jersey

Kristen M. Shannon, MS, CGC
Director, Cancer Center Genetics Program
Licensed Genetic Counselor
Massachusetts General Cancer Center
Massachusetts General Hospital
Boston, Massachusetts

Peter G. Shields, MD
Deputy Director, Comprehensive Cancer Center
Professor, College of Medicine
James Cancer Hospital
The Ohio State University
Columbus, Ohio

Ramesh A. Shivdasani, MD, PhD
Associate Professor, Department of Medical Oncology
Dana-Farber Cancer Institute
Department of Medicine
Harvard Medical School
Boston, Massachusetts

Richard M. Simon, DSc
Chief, Biometric Research Branch
National Cancer Institute
Rockville, Maryland

Samuel Singer, MD
Member, Department of Surgery
Chief, Gastric and Mixed Tumor Service
Vincent Astor Chair of Clinical Research
Memorial Sloan-Kettering Cancer Center
New York, New York

Craig L. Slingluff, Jr., MD
Joseph Helms Farrow Professor of Surgery
Division of Surgical Oncology
Vice-Chair for Research Director
Human Immune Therapy Center
University of Virginia
Charlottesville, Virginia

Eliezer Soto, MD, DABPM, FIPP
Anesthesia Pain Care Consultants
Tamarac, Florida

Alex Sparreboom, PhD
Associate Member, Department of Pharmaceutical Sciences
St. Jude Children's Research Hospital
Memphis, Tennessee

David Spiegel, MD
Willson Professor and Associate Chair
Department of Psychiatry & Behavioral Sciences
Director, Center on Stress and Health
Medical Director, Center for Integrative Medicine
Stanford University School of Medicine
Stanford, California

Thomas E. Stinchcombe, MD
Associate Professor, Division of Hematology/Oncology
University of North Carolina
Chapel Hill, North Carolina

Alexander Stojadinovic, MD
Bon Secours Cancer Institute
Richmond, Virginia

Diane E. Stover, MD
Professor of Clinical Medicine
Cornell University Medical College
Chief, Pulmonary Service
Department of Medicine
Memorial Sloan-Kettering Cancer Center
New York, New York

Michael D. Stubblefield, MD
Associate Attending Physiatrist
Rehabilitation Medicine Service
Department of Neurology
Memorial Sloan-Kettering Cancer Center
Associate Professor of Rehabilitation Medicine
Division of Rehabilitation Medicine
Weill Cornell Medical College
New York City, New York

Paul H. Sugarbaker, MD, FACS, FRCS
Medical Director, Center for Gastrointestinal Malignancies
Chief, Program in Peritoneal Surface Oncology
MedStar Washington Hospital Center
Washington, District of Columbia

John H. Suh, MD
Professor of Medicine
Chariman, Department of Radiation Oncology
Cleveland Clinic Taussig Cancer Institute
Burkhardt Brain Tumor and Neuro-Oncology Center
Cleveland Clinic
Cleveland, Ohio

Irene M. Tamí-Maury, DMD, DrPH, MSc
The University of Texas MD Anderson Cancer Center
Houston, Texas

Lynn Tanoue, MD
Professor of Internal Medicine
Medical Director
Yale Cancer Center
Thoracic Oncology Program
Yale University School of Medicine
New Haven, Connecticut

William D. Tap, MD
Associate Member, Department of Medicine
Section Chief, Sarcoma Oncology
Melanoma and Sarcoma Service
Division of Solid Tumors
Memorial Sloan-Kettering Cancer Center
New York, New York

Michael D. Taylor, MD, PhD, FRCSC
Associate Professor
Departments of Surgery and of Laboratory Medicine and Pathobiology
University of Toronto
Hospital for Sick Children Research Institute
Toronto, Ontario, Canada

Randall K. Ten Haken, PhD, FAAPM, FInstP, FASTRO, FACR
Professor, Associate Chair, and Physics Division Director
Department of Radiation Oncology
University of Michigan Medical School
Ann Arbor, Michigan

Kenneth D. Tew, PhD, DSc
Chairman and John C. West Chair in Cancer Research
Cell and Molecular Pharmacology
Medical University of South Carolina
Charleston, South Carolina

Geoffrey B. Thompson, MD, FACS, FACE
Professor, Department of Surgery
Section Head, Endocrine Surgery
College of Medicine, Mayo Clinic
Consultant, Division of Gastroenterologic and General Surgery
Mayo Clinic
Rochester, Minnesota

Snorri S. Thorgeirsson, MD, PhD
Head, Center of Excellence in Integrative Cancer Biology and Genomics
Chief, Laboratory of Experimental Carcinogenesis Center for Cancer Research
National Cancer Institute
National Institutes of Health
Bethesda, Maryland

Benjamin A. Toll, PhD
Associate Professor of Psychiatry
Yale University School of Medicine
Yale Comprehensive Cancer Center
Program Director, Smoking Cessation Service
Smilow Cancer Hospital at Yale-New Haven
New Haven, Connecticut

Christopher W. Towe, MD
Chief Resident
Department of Cardiothoracic Surgery
New York University School of Medicine
New York, New York

Edouard J. Trabulsi, MD, FACS
Associate Professor, Department of Urology
Kimmel Cancer Center
Jefferson Medical College
Director, Division of Urologic Oncology
Thomas Jefferson University
Philadelphia, Pennsylvania

Lois B. Travis, MD, ScD
Director, Rubin Center for Cancer Survivorship
Professor and Chief, Division of Cancer Survivorship
Department of Radiation Oncology
University of Rochester Medical Center
Rochester, New York

William D. Travis, MD
Professor of Pathology
Weill Medical College of Cornell University
Memorial Sloan-Kettering Cancer Center
New York, New York

Brian B. Tuch, PhD
Associate Director, Translational Genomics
Onyx Pharmaceuticals
South San Francisco, California

Catherine E. Ulbricht, PharmD
Founder, Natural Standard
Vice President, Integrated Therapeutics & Clinical Solutions
Therapeutic Research Center
Senior Attending Pharmacist
Massachusetts General Hospital
Somerville, Massachusetts

Thomas S. Uldrick, MD, MS
Staff Clinician, HIV & AIDS Malignancy Branch
Center for Cancer Research
National Cancer Institute
Bethesda, Maryland

Robert G. Uzzo, MD
Professor and Chairman, Department of Surgery
Fox Chase Cancer Center
Temple University Health System
Philadelphia, Pennsylvania

Veronica Sanchez Varela, PhD
Assistant Professor
Department of Behavioral Sciences & Department of Internal Medicine
Section of Bone Marrow Transplant and Cell Therapy
Rush University Medical Center
Chicago, Illinois

Vic J. Verwaal, MD, PhD
The Netherlands Cancer Institute
Amsterdam, The Netherlands

Shaveta Vinayak, MD, MS
Instructor, Department of Medicine
Division of Oncology
Stanford University School of Medicine
Stanford, California

Michael A. Vogelbaum, MD, PhD, FAANS, FACS
The Robert W. and Kathryn B. Lamborn Chair for Neuro-Oncology
Professor of Surgery (Neurosurgery)
Cleveland Clinic
Lerner College of Medicine of Case Western Reserve University
Associate Director, Rose Ella Burkhardt Brain Tumor and Neuro-Oncology Center
Cleveland Clinic
Cleveland, Ohio

Christine M. Walko, PharmD, BCOP
Clinical Pharmacogenetic Scientist
DeBartolo Family Personalized Medicine Institute
Applied Clinical Scientist, Division of Population Science
H. Lee Moffitt Cancer Center and Research Institute
Tampa, Florida

Edus H. Warren, MD, PhD
Member, Clinical Research Division
Fred Hutchinson Cancer Research Center
Professor, Department of Medicine
University of Washington
Seattle, Washington

Graham W. Warren, MD, PhD
Associate Professor
Vice Chair for Research in Radiation Oncology
Department of Radiation Oncology
Department of Cell and Molecular Pharmacology
Hollings Cancer Center
Medical University of South Carolina
Charleston, South Carolina

Jeffrey S. Wefel, PhD, ABPP/CN
Associate Professor, Department of Neuro-Oncology
Chief, Section of Neuropsychology
The University of Texas MD Anderson Cancer Center
Houston, Texas

Robert A. Weinberg, PhD
Member, Whitehead Institute for Biomedical Research
Department of Biology
Massachusetts Institute of Technology
Director, Ludwig Center for Molecular Oncology
Whitehead Institute for Biomedical Research
Cambridge, Massachusetts

Louis M. Weiner, MD
Director, Lombardi Comprehensive Cancer Center
Professor and Chair, Department of Oncology
Francis L. and Charlotte G. Gragnani Chair
Georgetown University Medical Center
Washington, District of Columbia

Shelly M. Weiss, MS, CGC
Regional Medical Specialist
Myriad Genetics
Chicago, Illinois

Scott M. Weissman, MS, CGC
Certified Genetic Counselor
GeneDx
Gaithersburg, Maryland

Samuel A. Wells, Jr., MD
Adjunct Investigator, Medical Oncology Branch
Center for Cancer Research
National Cancer Institute
Bethesda, Maryland

Batsheva Werman, MD
Senior Physician
Oncology Department
Shaare Zedek Medical Center
Jerusalem, Israel

John W. Werning, MD, DMD
Associate Professor
Department of Otolaryngology
University of Florida, College of Medicine
Gainesville, Florida

William G. Wierda, MD, PhD
Professor, Department of Leukemia
The University of Texas MD Anderson Cancer Center
Houston, Texas

Elizabeth L. Wiley, MD
Professor and Director, Division of Surgical Pathology
Department of Pathology
University of Illinois Hospital and Health Sciences System
University of Illinois College of Medicine
Chicago, Illinois

Christopher G. Willett, MD
Professor and Chairman, Department of Radiation Oncology
Duke University
Durham, North Carolina

Walter C. Willett, MD, DrPH
Professor and Chair, Department of Nutrition
Harvard School of Public Health
Boston, Massachusetts

Terence M. Williams, MD, PhD
Assistant Professor, Department of Radiation Oncology
The Ohio State University
Arthur G. James and Richard Solove Research Institute
Columbus, Ohio

Lynn D. Wilson, MD, MPH
Professor, Vice Chairman, and Clinical Director
Department of Therapeutic Radiology
Yale University School of Medicine
New Haven, Connecticut

Jordan M. Winter, MD
Assistant Professor, Department of Surgery
Thomas Jefferson University
Philadelphia, Pennsylvania

Abraham J. Wu, MD
Assistant Attending Physician
Department of Radiation Oncology
Memorial Sloan-Kettering Cancer Center
New York, New York

Joachim Yahalom, MD
Vice Chairman for Academic Programs
Department of Radiation Oncology
Attending Physician and Member
Memorial Sloan-Kettering Cancer Center
Professor of Radiation Oncology
Weill Medical College of Cornell University
New York, New York

James C. Yao, MD
Professor Deputy Chairman, Department of Gastrointestinal Medical Oncology
The University of Texas MD Anderson Cancer Center
Houston, Texas

Robert Yarchoan, MD
Principal Investigator and Branch Chief
HIV and AIDS Malignancy Branch
Center for Cancer Research
National Cancer Institute
Bethesda, Maryland

Charles J. Yeo, MD
Samuel D. Gross Professor and Chair
Department of Surgery
Jefferson Medical College
Philadelphia, Pennsylvania

Anas Younes, MD
Professor, Department of Medicine
Chief, Lymphoma Service
Memorial Sloan-Kettering Cancer Center
New York, New York

Herbert Yu, MD, PhD
Professor and Director
Cancer Epidemiology Program
Associate Director for Population Sciences and Cancer Control
University of Hawaii Cancer Center
Adjunct Professor, Department of Chronic Disease Epidemiology
Yale School of Public Health
Honolulu, Hawaii

Stuart H. Yuspa, MD
Chief, Laboratory of Cancer Biology and Genetics
Center for Cancer Research
National Cancer Institute
Bethesda, Maryland

Michael J. Zelefsky, MD
Chief, Brachytherapy Service
Department of Radiation Oncology
Memorial Sloan-Kettering Cancer Center
New York, New York

Eric S. Zhou, PhD
Clinical Fellow, Dana-Farber Cancer Institute
Research Fellow, Harvard Medical School
Boston, Massachusetts

Anthony L. Zietman, MD
Jenot and William Shipley Professor of Radiation Oncology
Department of Radiation Oncology
Massachusetts General Hospital
Harvard Medical School
Boston, Massachusetts

Kenneth Zuckerman, MD
Senior Member
H. Lee Moffitt Cancer Center
Tampa, Florida

PREFACE

The first edition of *Cancer: Principles & Practice of Oncology* was published in 1982. Now, 32 years later, we present the 10th edition, a milestone of sorts. Our intention with the first edition was to publish a book that was comprehensive and balanced, covering not just one field, as had been the practice of cancer texts before 1982, but providing in-depth, expert coverage of all the specialties. In fact, a feature of the disease-oriented chapters then and now has been co-authorship by each of the major specialties.

Even in the early 1980s, it was apparent the field of cancer was changing rapidly and the regular production of new editions would be necessary to keep information fresh. Ten editions in 32 years has to be some kind of record for textbooks and accounts for the fact that *Cancer: Principles & Practice of Oncology* is the most popular cancer textbook in the world.

But times have changed. With the increase in the rate of new information and the digital information revolution, the text has changed too. Updates cannot wait for a new edition. Doctors need new information as it appears. For this reason, the 10th edition will be updated quarterly by a team of experts selected by the editors. The new information will be inserted and highlighted in the appropriate chapters, with references, and updates will be posted to the online version. This makes *Cancer: Principles & Practice of Oncology* the most up-to-date, easily searchable cancer text in the world, and the only comprehensive cancer text that is continuously updated. A perusal of how the contents have evolved from the first edition to the tenth shows the breathtaking pace of change in our understanding of the biology of cancer and the application of this information to the practice of medicine in the past 32 years. All these changes have been chronicled in the 10 editions of the book and the text has been a major vehicle for the translation of new information into practice. With the new flexible format of the 10th edition, we expect this will continue.

Vincent T. DeVita, Jr., MD
Theodore S. Lawrence, MD, PhD
Steven A. Rosenberg, MD, PhD

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To
Mary Kay,
Wendy, and
Alice

And to the invaluable Zia Raven

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PART I

Principles of Oncology

1 The Cancer Genome

Yardena Samuels, Alberto Bardelli, Jared J. Gartner, and Carlos López-Otin

INTRODUCTION

There is a broad consensus that cancer is, in essence, a genetic disease, and that accumulation of molecular alterations in the genome of somatic cells is the basis of cancer progression (Fig. 1.1).[1] In the past 10 years, the availability of the human genome sequence and progress in DNA sequencing technologies has dramatically improved knowledge of this disease. These new insights are transforming the field of oncology at multiple levels:

1. The genomic maps are redesigning the tumor taxonomy by moving it from a histologic- to a genetic-based level.
2. The success of cancer drugs designed to target the molecular alterations underlying tumorigenesis has proven that somatic genetic alterations are legitimate targets for therapy.
3. Tumor genotyping is helping clinicians individualize treatments by matching patients with the best treatment for their tumors.
4. Tumor-specific DNA alterations represent highly sensitive biomarkers for disease detection and monitoring.
5. Finally, the ongoing analyses of multiple cancer genomes will identify additional targets, whose pharmacologic exploitation will undoubtedly result in new therapeutic approaches.

This chapter will review the progress that has been made in understanding the genetic basis of sporadic cancers. An emphasis will be placed on an introduction to novel integrated genomic approaches that allow a comprehensive and systematic evaluation of genetic alterations that occur during the progression of cancer. Using these powerful tools, cancer research, diagnosis, and treatment are poised for a transformation in the next years.

CANCER GENES AND THEIR MUTATIONS

Cancer genes are broadly grouped into oncogenes and tumor suppressor genes. Using a classical analogy, oncogenes can be compared to a car accelerator, so that a mutation in an oncogene would be the equivalent of having the accelerator continuously pressed.[2] Tumor suppressor genes, in contrast, act as brakes,[2] so that when they are not mutated, they function to inhibit tumorigenesis. Oncogene and tumor suppressor genes may be classified by the nature of their somatic mutations in tumors. Mutations in oncogenes typically occur at specific hotspots, often affecting the same codon or clustered at neighboring codons in different tumors.[1] Furthermore, mutations in oncogenes are almost always missense, and the mutations usually affect only one allele, making them heterozygous. In contrast, tumor suppressor genes are usually mutated throughout the gene; a large number of the mutations may truncate the encoded protein and generally affect both alleles, causing loss of heterozygosity (LOH). Major types of somatic mutations present in malignant tumors include nucleotide substitutions, small insertions and deletions (*indels*), chromosomal rearrangements, and copy number alterations.

IDENTIFICATION OF CANCER GENES

The completion of the Human Genome Project marked a new era in biomedical sciences.[3] Knowledge of the sequence and organization of the human genome now allows for the systematic analysis of the genetic alterations underlying the origin and evolution of tumors. Before elucidation of the human genome, several cancer genes, such as *KRAS*, *TP53*, and *APC*, were successfully discovered using approaches based on an oncovirus analysis, linkage studies, LOH, and cytogenetics.[4,5] The first curated version of the Human Genome Project was released in 2004,[3] and provided a sequence-based map of the normal human genome. This information, together with the construction of the HapMap, which contains single nucleotide polymorphisms (SNP), and the underlying genomic structure of natural human genomic variation,[6,7] allowed an extraordinary throughput in cataloging somatic mutations in cancer. These projects now offer an unprecedented opportunity: the identification of all the genetic changes associated with a human cancer. For the first time, this ambitious goal is within reach of the scientific community. Already, a number of studies have demonstrated the usefulness of strategies aimed at the systematic identification of somatic mutations associated with cancer progression. Notably, the Human Genome Project, the HapMap project, as well as the candidate and family gene approaches (described in the following paragraphs), utilized capillary-based DNA sequencing (first-generation sequencing, also known as Sanger sequencing).[8] Figure 1.2 clearly illustrates the developments in the search of cancer genes, its increased pace, as well as the most relevant findings in this field.

Cancer Gene Discovery by Sequencing Candidate Gene Families

The availability of the human genome sequence provides new opportunities to comprehensively search for somatic mutations in cancer on a larger scale than previously possible. Progress in the field has been closely linked to improvements in the throughput of DNA analysis and in the continuous reduction in sequencing costs. What follows are some of the achievements in this research area, as well as how they affected knowledge of the cancer genome.

A seminal work in the field was the systematic mutational profiling of the genes involved in the RAS-RAF pathway in multiple tumors. This candidate gene approach led to the discovery that *BRAF* is frequently mutated in melanomas and is mutated at a lower frequency in other tumor types.[9] Follow-up studies quickly revealed that mutations in *BRAF* are mutually exclusive with alterations in *KRAS*,[9,10] genetically emphasizing that these genes function in the same pathway, a concept that had been previously demonstrated in lower organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster*.[11,12]

In 2003, the identification of cancer genes shifted from a candidate gene approach to the mutational analyses of gene families. The first gene families to be completely sequenced were those that

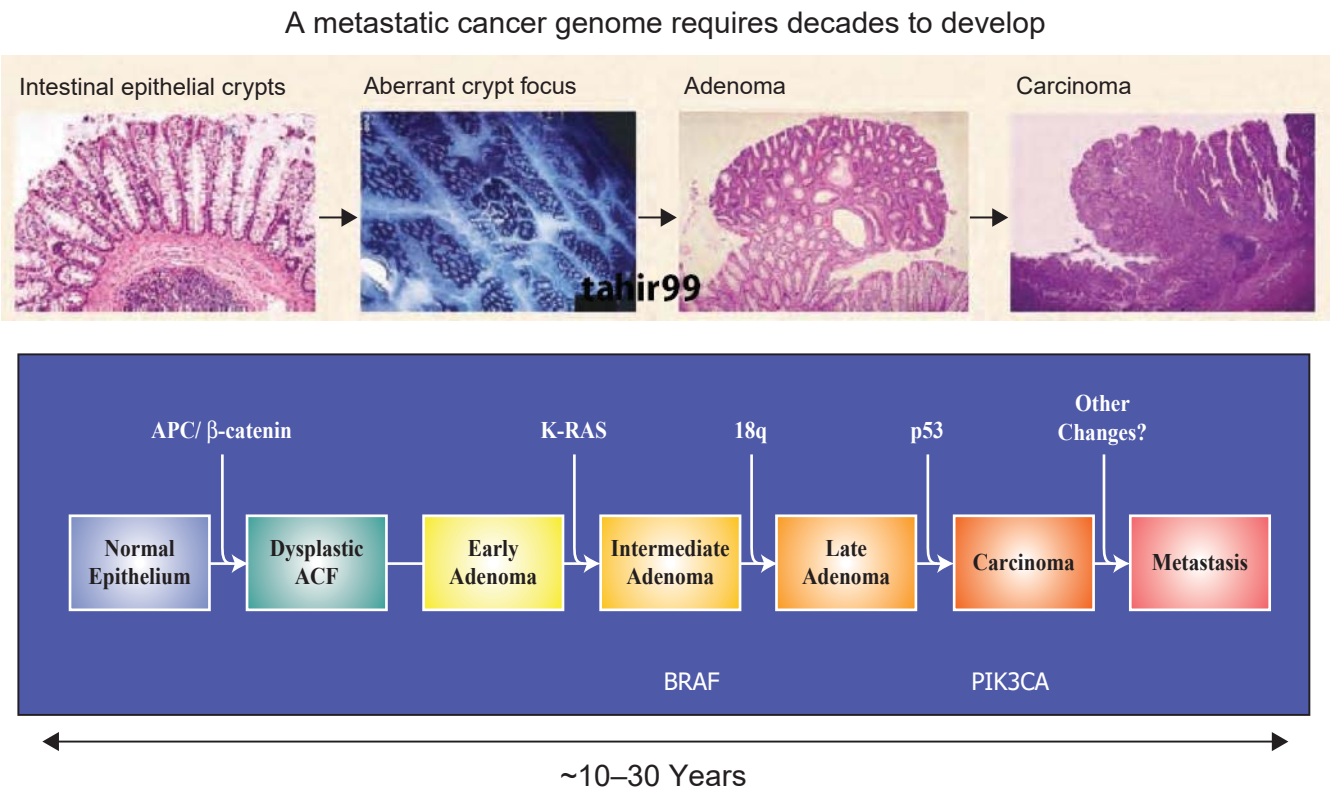

Figure 1.1 Schematic representation of the genomic and histopathologic steps associated with tumor progression: from the occurrence of the initiating mutation in the founder cell to metastasis formation. It has been convincingly shown that the genomic landscape of solid tumors such as that of pancreatic and colorectal tumors requires the accumulation of many genetic events, a process that requires decades to complete. This timeline offers an incredible window of opportunity for the early detection, which is often associated with an excellent prognosis, of this disease.

involved protein[13,14] and lipid phosphorylation.[15] The rationale for initially focusing on these gene families was threefold:

- The corresponding proteins were already known at that time to play a pivotal role in the signaling and proliferation of normal and cancerous cells.
- Multiple members of the protein kinases family had already been linked to tumorigenesis.
- Kinases are clearly amenable to pharmacologic inhibition, making them attractive drug targets.

The mutational analysis of all the tyrosine-kinase domains in colorectal cancers revealed that 30% of cases had a mutation in at least one tyrosine-kinase gene, and overall mutations were identified in eight different kinases, most of which had not previously been linked to cancer.[13] An additional mutational analysis of the coding exons of 518 protein kinase genes in 210 diverse human cancers, including breast, lung, gastric, ovarian, renal, and acute lymphoblastic leukemia, identified approximately 120 mutated genes that probably contribute to oncogenesis.[14] Because kinase activity is attenuated by enzymes that remove phosphate groups called phosphatases, the rational next step in these studies was to perform a mutation analysis of the protein tyrosine phosphatases. A mutational investigation of this family in colorectal cancer identified that 25% of cases had mutations in six different phosphatase genes (*PTPRF*, *PTPRG*, *PTPRT*, *PTPN3*, *PTPN13*, or *PTPN14*).[16] A combined analysis of the protein tyrosine kinases and the protein tyrosine phosphatases showed that 50% of colorectal cancers had mutations in a tyrosine-kinase gene, a protein tyrosine phosphatase gene, or both, further emphasizing the pivotal role of protein phosphorylation in neoplastic progression. Many of the identified genes had previously been linked to human cancer, thus validating the unbiased comprehensive mutation profiling. These landmark studies led to additional gene family surveys.

The phosphatidylinositol 3-kinase (*PI3K*) gene family, which also plays a role in proliferation, adhesion, survival, and motility, was also comprehensively investigated.[17] Sequencing of the exons encoding the kinase domain of all 16 members belonging to this family pinpointed *PIK3CA* as the only gene to harbor somatic mutations. When the entire coding region was analyzed, *PIK3CA* was found to be somatically mutated in 32% of colorectal cancers. At that time, the *PIK3CA* gene was certainly not a newcomer in the cancer arena, because it had previously been shown to be involved in cell transformation and metastasis.[17] Strikingly, its staggeringly high mutation frequency was discovered only through systematic sequencing of the corresponding gene family.[15] Subsequent analysis of *PIK3CA* in other tumor types identified somatic mutations in this gene in additional cancer types, including 36% of hepatocellular carcinomas, 36% of endometrial carcinomas, 25% of breast carcinomas, 15% of anaplastic oligodendrogliomas, 5% of medulloblastomas and anaplastic astrocytomas, and 27% of glioblastomas.[18–22] It is known that *PIK3CA* is one of the two (the other being *KRAS*) most commonly mutated oncogenes in human cancers. Further investigation of the *PI3K* pathway in colorectal cancer showed that 40% of tumors had genetic alterations in one of the *PI3K* pathway genes, emphasizing the central role of this pathway in colorectal cancer pathogenesis.[23]

Although most cancer genome studies of large gene families have focused on the kinome, recent analyses have revealed that members of other families highly represented in the human genome are also a target of mutational events in cancer. This is the case of proteases, a complex group of enzymes consisting of at least 569 components that constitute the so-called human degradome.[24] Proteases exhibit an elaborate interplay with kinases and

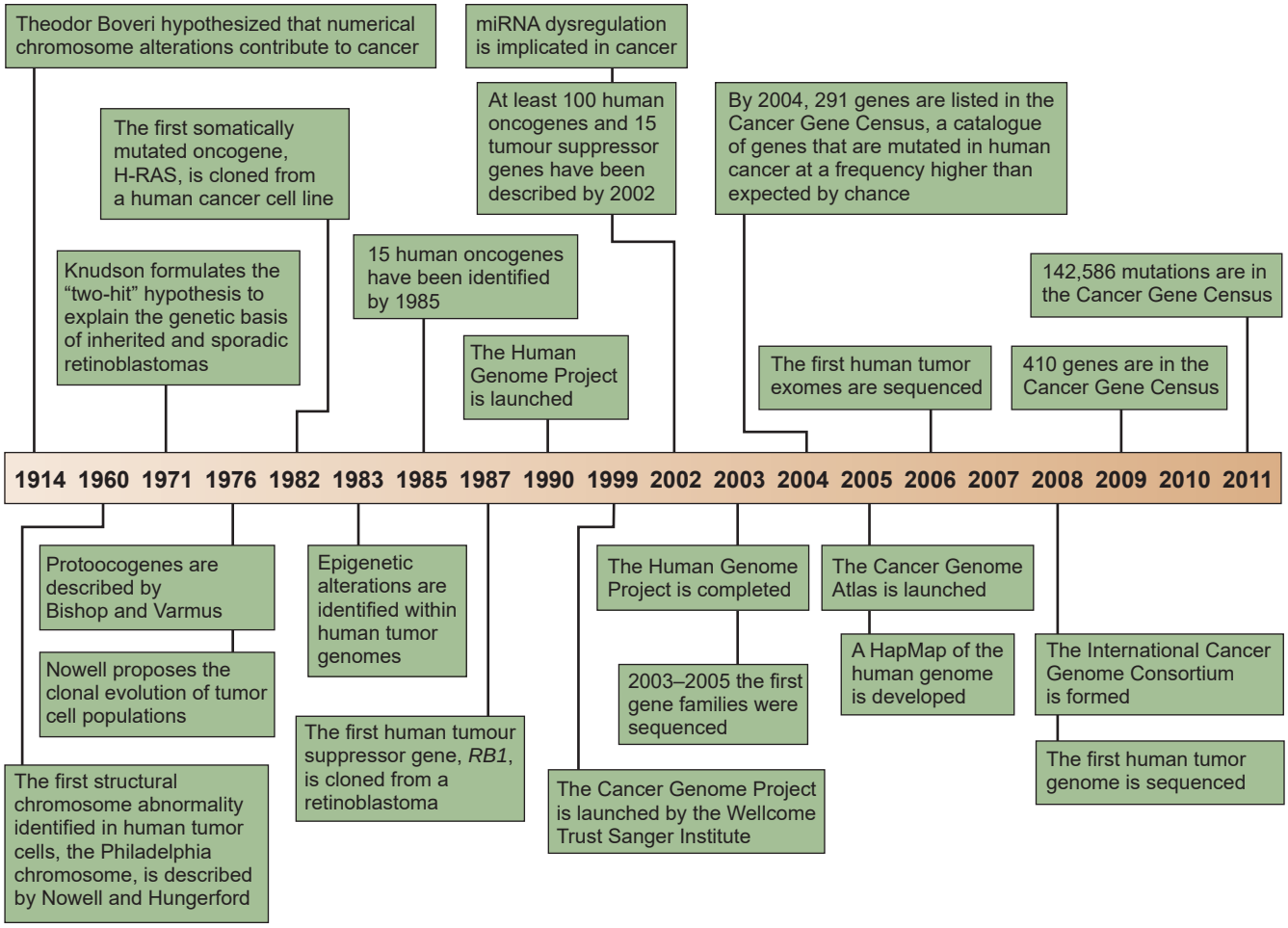

Figure 1.2 Timeline of seminal hypotheses, research discoveries, and research initiatives that have led to an improved understanding of the genetic etiology of human tumorigenesis within the past century. The consensus cancer gene data were obtained from the Wellcome Trust Sanger Institute Cancer Genome Project Web site (http://www.sanger.ac.uk/genetics/CGP). (Redrawn from Bell DW. Our changing view of the genomic landscape of cancer. *J Pathol* 2010;220:231–243.)

have traditionally been associated with cancer progression because of their ability to degrade extracellular matrices, thus facilitating tumor invasion and metastasis.[25,26] However, recent studies have shown that these enzymes hydrolyze a wide variety of substrates and influence many different steps of cancer, including early stages of tumor evolution.[27] These functional studies have also revealed that beyond their initial recognition as prometastatic enzymes, they play dual roles in cancer, as assessed by the identification of a growing number of tumor-suppressive proteases.[28]

These findings emphasized the possibility that mutational activation or inactivation of protease genes occurs in cancer. A systematic analysis of genetic alterations in breast and colorectal cancers revealed that proteases from different catalytic classes were somatically mutated in cancer.[29] These results prompted the mutational analysis of entire protease families such as matrix metalloproteinases (MMP), a disintegrin and metalloproteinase (ADAM), and ADAMs with thrombospondin domains (ADAMTS) in different tumors. These studies led to the identification of protease genes frequently mutated in cancer, such as *MMP8*, which is mutated and functionally inactivated in 6.3% of human melanomas.[30,31]

The mutational status of caspases has also been extensively analyzed in different tumors because these proteases play a fundamental role in the execution of apoptosis, one of the hallmarks of cancer.[32] These studies demonstrated that *CASP8* is deleted in neuroblastomas and inactivated by somatic mutations in a variety of human malignancies, including head and neck, colorectal, lung, and gastric carcinomas.[33–35] Other large protease families whose components are often mutated in cancer are the deubiquitinating enzymes (DUB), which catalyze the removal of ubiquitin and ubiquitin-like modifiers of their target proteins.[36] Some DUBs were initially identified as oncogenic proteins, but further work has shown that other deubiquitinases, such as CYLD, A20, and BAP1, are tumor suppressors inactivated in cancer. *CYLD* is mutated in patients with familial cylindromatosis, a disease characterized by the formation of multiple tumors of skin appendages.[37] A20 is a DUB family member encoded by the *TNFAIP3* gene, which is mutated in a large number of Hodgkin lymphomas and primary mediastinal B-cell lymphomas.[38–41] Finally, the *BAP1* gene, encoding an ubiquitin C-terminal hydrolase, is frequently mutated in metastasizing uveal melanomas[42] and in other human malignancies, such as mesothelioma and renal cell carcinoma.[43]

Mutational Analysis of Exomes Using Sanger Sequencing

Although the gene family approach for the identification of cancer genes has proven extremely valuable, it still is a candidate approach and thus biased in its nature. The next step forward in the mutational profiling of cancer has been the sequencing of exomes, which is the entire coding portion of the human genome (18,000 protein-encoding genes). The exomes of many different tumors—including breast, colorectal, pancreatic, and ovarian clear cell carcinomas; glioblastoma multiforme; and medulloblastoma—have been analyzed

using Sanger sequencing. For the first time, these large-scale analyses allowed researchers to describe and understand the genetic complexity of human cancers.[29,44–48] The declared goals of these exome studies were to provide methods for exomewide mutational analyses in human tumors, to characterize their spectrum and quantity of somatic mutations, and, finally, to discover new genes involved in tumorigenesis as well as novel pathways that have a role in these tumors. In these studies, sequencing data were complemented with gene expression and copy number analyses, thus providing a comprehensive view of the genetic complexity of human tumors.[45–48] A number of conclusions can be drawn from these analyses, including the following:

- Cancer genomes have an average of 30 to 100 somatic alterations per tumor in coding regions, which was a higher number than previously thought. Although the alterations included point mutations, small insertions, deletions, or amplifications, the great majority of the mutations observed were single-base substitutions.[45,46]
- Even within a single cancer type, there is a significant intertumor heterogeneity. This means that multiple mutational patterns (encompassing different mutant genes) are present in tumors that cannot be distinguished based on histologic analysis. The concept that individual tumors have a unique genetic milieu is highly relevant for personalized medicine, a concept that will be further discussed.
- The spectrum and nucleotide contexts of mutations differ between different tumor types. For example, over 50% of mutations in colorectal cancer were C:G to T:A transitions, and 10% were C:G to G:C transversions. In contrast, in breast cancers, only 35% of the mutations were C:G to T:A transitions, and 29% were C:G to G:C transversions. Knowledge of mutation spectra is vital because it allows insight into the mechanisms underlying mutagenesis and repair in the various cancers investigated.
- A considerably larger number of genes that had not been previously reported to be involved in cancer were found to play a role in the disease.
- Solid tumors arising in children, such as medulloblastomas, harbor on average 5 to 10 times less gene alterations compared to a typical adult solid tumor. These pediatric tumors also harbor fewer amplifications and homozygous deletions within coding genes compared to adult solid tumors.

Importantly, to deal with the large amount of data generated in these genomic projects, it was necessary to develop new statistical and bioinformatic tools. Furthermore, an examination of the overall distribution of the identified mutations allowed for the development of a novel view of cancer genome landscapes and a novel definition of cancer genes. These new concepts in the understanding of cancer genetics are further discussed in the following paragraphs. The compiled conclusions derived from these analyses have led to a paradigm shift in the understanding of cancer genetics.

A clear indication of the power of the unbiased nature of the whole exome surveys was revealed by the discovery of recurrent mutations in the active site of *IDH1*, a gene with no known link to gliomas, in 12% of tumors analyzed.[46] Because malignant gliomas are the most common and lethal tumors of the central nervous system, and because glioblastoma multiforme (GBM; World Health Organization grade IV astrocytoma) is the most biologically aggressive subtype, the unveiling of *IDH1* as a novel GBM gene is extremely significant. Importantly, mutations of *IDH1* predominantly occurred in younger patients and were associated with a better prognosis.[49] Follow-up studies showed that mutations of *IDH1* occur early in glioma progression; the R132 somatic mutation is harbored by the majority (greater than 70%) of grades II and III astrocytomas and oligodendrogliomas, as well as in secondary GBMs that develop from these lower grade lesions.[49–55] In contrast, less than 10% of primary GBMs harbor these alterations. Furthermore, analysis of the associated *IDH2* revealed recurrent somatic mutations in the R172 residue, which is the exact analog of the frequently mutated R132 residue of *IDH1*. These mutations occur mostly in a mutually exclusive manner with *IDH1* mutations,[49,51] suggesting that they have equivalent phenotypic effects. Subsequently, *IDH1* mutations have been reported in additional cancer types, including hematologic neoplasias.[56–58]

Next-Generation Sequencing and Cancer Genome Analysis

In 1977, the introduction of the Sanger method for DNA sequencing with chain-terminating inhibitors transformed biomedical research.[8] Over the past 30 years, this first-generation technology has been universally used for elucidating the nucleotide sequence of DNA molecules. However, the launching of new large-scale projects, including those implicating whole-genome sequencing of cancer samples, has made necessary the development of new methods that are widely known as next-generation sequencing technologies.[59–61] These approaches have significantly lowered the cost and the time required to determine the sequence of the 3×10^9 nucleotides present in the human genome. Moreover, they have a series of advantages over Sanger sequencing, which are of special interest for the analysis of cancer genomes.[62] First, next-generation sequencing approaches are more sensitive than Sanger methods and can detect somatic mutations even when they are present in only a subset of tumor cells.[63] Moreover, these new sequencing strategies are quantitative and can be used to simultaneously determine both nucleotide sequence and copy number variations.[64] They can also be coupled to other procedures such as those involving paired-end reads, allowing for the identification of multiple structural alterations, such as insertions, deletions, and rearrangements, that commonly occur in cancer genomes.[63] Nonetheless, next-generation sequencing still presents some limitations that are mainly derived from the relatively high error rate in the short reads generated during the sequencing process. In addition, these short reads make the task of de novo assembly of the generated sequences and the mapping of the reads to a reference genome extremely complex. To overcome some of these current limitations, deep coverage of each analyzed genome is required and a careful validation of the identified variants must be performed, typically using Sanger sequencing. As a consequence, there is a substantial increase in both the cost of the process and in the time of analysis. Therefore, it can be concluded that whole-genome sequencing of cancer samples is already a feasible task, but not yet a routine process. Further technical improvements will be required before the task of decoding the entire genome of any malignant tumor of any cancer patient can be applied to clinical practice.

The number of next-generation sequencing platforms has substantially grown over the past few years and currently includes technologies from Roche/454, Illumina/Solexa, Life/APG's SOLiD3, Helicos BioSciences/HeliScope, and Pacific Biosciences/PacBio RS.[61] Noteworthy also are the recent introduction of the Polonator G.007 instrument, an open source platform with freely available software and protocols; the Ion Torrent's semiconductor sequencer; as well as those involving self-assembling DNA nanoballs or nanopore technologies.[65–67] These new machines are driving the field toward the era of third-generation sequencing, which brings enormous clinical interest because it can substantially increase the speed and accuracy of analyses at reduced costs and can facilitate the possibility of single-molecule sequencing of human genomes. A comparison of next-generation sequencing platforms is shown in Table 1.1. These various platforms differ in the method utilized for template preparation and in the nucleotide sequencing and imaging strategy, which finally result in their different performance. Ultimately, the most suitable approach depends on the specific genome sequencing projects.[61]

Current methods of template preparation first involve randomly shearing genomic DNA into smaller fragments, from which

TABLE 1.1

Comparative Analysis of Next-Generation Sequencing Platforms

Platform	Library/Template Preparation	Sequencing Method	Average Read-Length (Bases)	Run Time (Days)	Gb Per Run	Instrument Cost (U.S.$)	Comments
Roche 454 GS FLX	Fragment, mate-pair Emulsion PCR	Pyrosequencing	400	0.35	0.45	500,000	Fast run times High reagent cost
Illumina HiSeq 2000	Fragment, mate-pair Solid phase	Reversible terminator	100–125	8 (mate-pair run)	150–200	540,000	Most widely used platform Low multiplexing capability
Life/APG's SOLiD 5500xl	Fragment, mate-pair Emulsion PCR	Cleavable probe, sequencing by ligation	35–75	7 (mate-pair run)	180–300	595,000	Inherent error correction Long run times
Helicos BioSciences HeliScope	Fragment, mate-pair Single molecule	Reversible terminator	32	8 (fragment run)	37	999,000	Nonbias template representation Expensive, high error rates
Pacific Biosciences PacBio RS	Fragment Single molecule	Real-time sequencing	1,000	1	0.075	NA	Greatest potential for long reads Highest error rates
Polonator G.007	Mate pair Emulsion PCR	Noncleavable probe, sequencing by ligation	26	5 (mate-pair run)	12	170,000	Least expensive platform Shortest read lengths

NA, not available.
Data represent an update of information provided in Metzker ML. Sequencing technologies—the next generation. *Nat Rev Genet* 2010;11:31–46.

a library of either fragment templates or mate-pair templates are generated. Then, clonally amplified templates from single DNA molecules are prepared by either emulsion polymerase chain reaction (PCR) or solid-phase amplification.[68,69] Alternatively, it is possible to prepare single-molecule templates through methods that require less starting material and that do not involve PCR amplification reactions, which can be the source of artifactual mutations.[70] Once prepared, templates are attached to a solid surface in spatially separated sites, allowing thousands to billions of nucleotide sequencing reactions to be performed simultaneously.

The sequencing methods currently used by the different next-generation sequencing platforms are diverse and have been classified into four groups: cyclic reversible termination, single-nucleotide addition, real-time sequencing, and sequencing by ligation (Fig. 1.3).[61,71] These sequencing strategies are coupled with different imaging methods, including those based on measuring bioluminescent signals or involving four-color imaging of single molecular events. Finally, the extraordinary amount of data released from these nucleotide sequencing platforms is stored, assembled, and analyzed using powerful bioinformatic tools that have been developed in parallel with next-generation sequencing technologies.[72]

Next-generation sequencing approaches represent the newest entry into the cancer genome decoding arena and have already been applied to cancer analyses. The first research group to apply these methodologies to whole cancer genomes was that of Ley et al.,[73] who reported in 2008 the sequencing of the entire genome of a patient with acute myeloid leukemia (AML) and its comparison with the normal tissue from the same patient, using the Illumina/Solexa platform. As further described, this work allowed for the identification of point mutations and structural alterations of putative oncogenic relevance in AML and represented proof of principle of the relevance of next-generation sequencing for cancer research.

Whole-Genome Analysis Utilizing Second-Generation Sequencing

The sequence of the first whole cancer genome was reported in 2008, where AML and normal skin from the same patient were described.[73] Numerous additional whole genomes, together with the corresponding normal genomes of patients with a variety of malignant tumors, have been reported since then.[56,63,74–86]

The first available whole genome of a cytogenetically normal AML subtype M1 (AML-M1) revealed eight genes with novel mutations along with another 500 to 1,000 additional mutations found in noncoding regions of the genome. Most of the identified genes had not been previously associated with cancer. However, validation of the detected mutations did not identify novel recurring mutations in AML.[73] Concomitantly, with the expansion in the use of next-generation sequencers, many other whole genomes from a number of cancer types started to be evaluated in a similar manner (Fig. 1.4).[87]

In contrast to the first AML whole genome, the second did observe a recurrent mutation in *IDH1*, encoding isocitrate dehydrogenase.[56] Follow-up studies extended this finding and reported that mutations in *IDH1* and the related gene *IDH2* occur at a 20% to 30% frequency in AML patients and are associated with a poor prognosis in some subgroups of patients.[79,80,88] A good example illustrating the high pace at which second-generation technologies and their accompanying analytical tools are found is demonstrated by the following finding derived from a reanalysis of the first AML whole genome. Thus, when improvements in sequencing

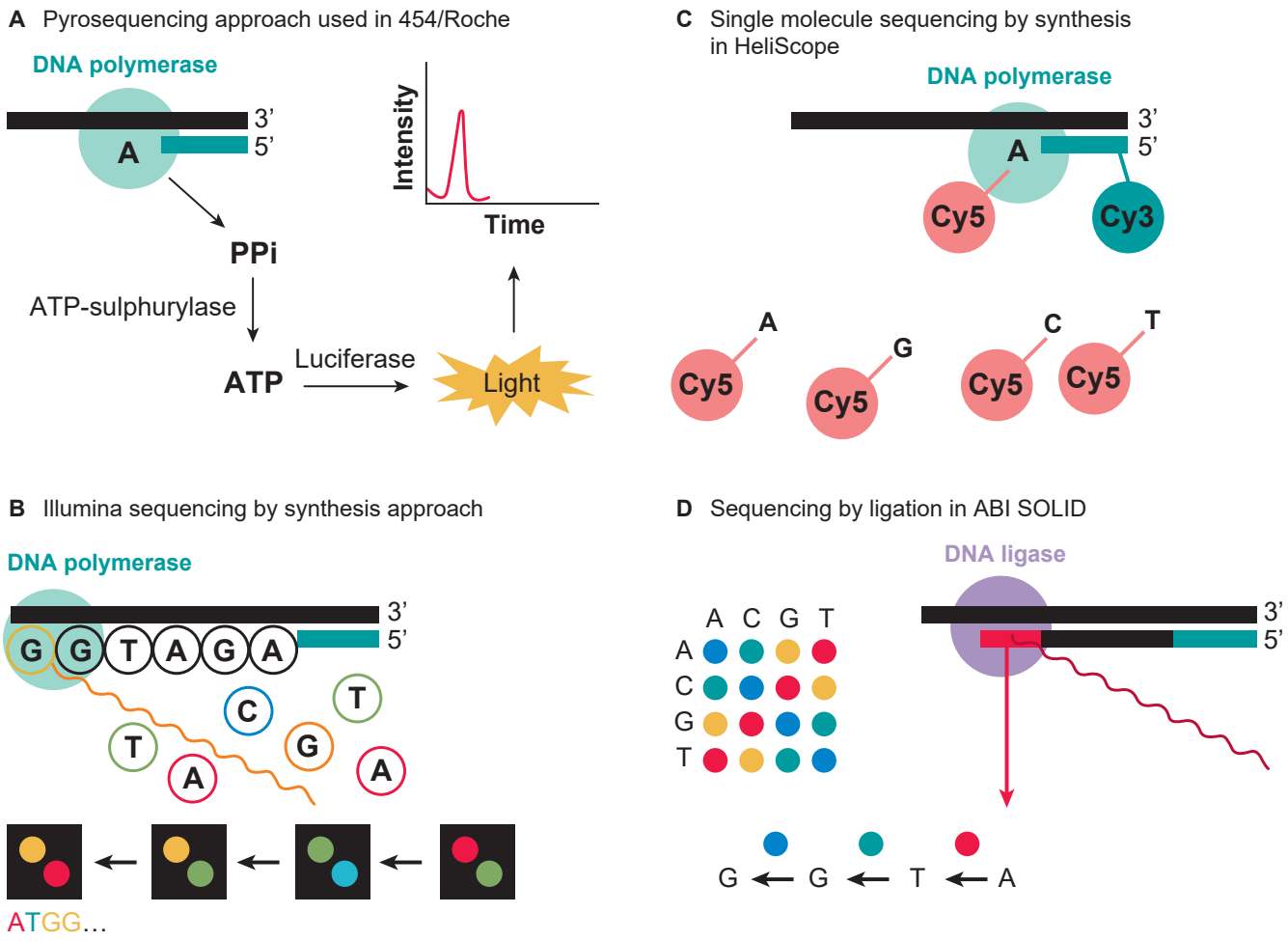

Figure 1.3 Advances in sequencing chemistry implemented in next-generation sequencers. **(A)** The pyrosequencing approach implemented in 454/Roche sequencing technology detects incorporated nucleotides by chemiluminescence resulting from PPi release. **(B)** The Illumina method utilizes sequencing by synthesis in the presence of fluorescently labeled nucleotide analogs that serve as reversible reaction terminators. **(C)** The single-molecule sequencing by synthesis approach detects template extension using Cy3 and Cy5 labels attached to the sequencing primer and the incoming nucleotides, respectively. **(D)** The SOLiD method sequences templates by sequential ligation of labeled degenerate probes. Two-base encoding implemented in the SOLiD instrument allows for probing each nucleotide position twice. (From Morozova O, Hirst M, Marra MA. Applications of new sequencing technologies for transcriptome analysis. *Annu Rev Genomics Hum Genet* 2009;10:135–151.)

techniques were available, the first AML whole genome (described previously), which identified no recurring mutations and had a 91.2% diploid coverage, was reevaluated by deeper sequence coverage, yielding 99.6% diploid coverage of the genome. This improvement, together with more advanced mutation calling algorithms, allowed for the discovery of several nonsynonymous mutations that had not been identified in the initial sequencing. This included a frameshift mutation in the DNA methyltransferase gene *DNMT3A*. Validation of *DNMT3A* in 280 additional de novo AML patients to define recurring mutations led to the significant discovery that a total of 22.1% of AML cases had mutations in *DNMT3A* that were predicted to affect translation. The median overall survival among patients with *DNMT3A* mutations was significantly shorter than that among patients without such mutations (12.3 months versus 41.1 months; $p < 0.001$).

Shortly after this study, complete sequences of a series of cancer genomes, together with matched normal genomes of the same patients, were reported.[56,78,83,84] These works opened the way to more ambitious initiatives, including those involving large international consortia, aimed at decoding the genome of malignant tumors from thousands of cancer patients. Thus, over the last 2 years, many whole genomes of different human malignancies have been made available.[74–76]

In addition to direct applications of next-generation sequencing technologies for the mutational analysis of cancer genomes, these methods have an additional range of applications in cancer research. Thus, genome sequencing efforts have begun to elucidate the genomic changes that accompany metastasis evolution through a comparative analysis of primary and metastatic lesions from breast and pancreatic cancer patients.[77,81,82,85] Likewise, massively parallel sequencing has been used to analyze the evolution of a tongue adenocarcinoma in response to selection by targeted kinase inhibitors.[89] Detailed information of several of these whole genome projects is found in the following paragraph.

The first solid cancer to undergo whole-genome sequencing was a malignant melanoma that was compared to a lymphoblastoid cell line from the same individual.[83] Impressively, a total of 33,345 somatic base substitutions were identified, with 187 nonsynonymous substitutions in protein-coding sequences, at least one order of magnitude higher than any other cancer type. Most somatic base substitutions were C:G > T:A transitions, and of the 510 dinucleotide substitutions, 360 were CC.TT/GG.AA changes, which is consistent with ultraviolet light exposure mutation signatures previously reported in melanoma.[14] Such results from the most comprehensive catalog of somatic mutations not only provide

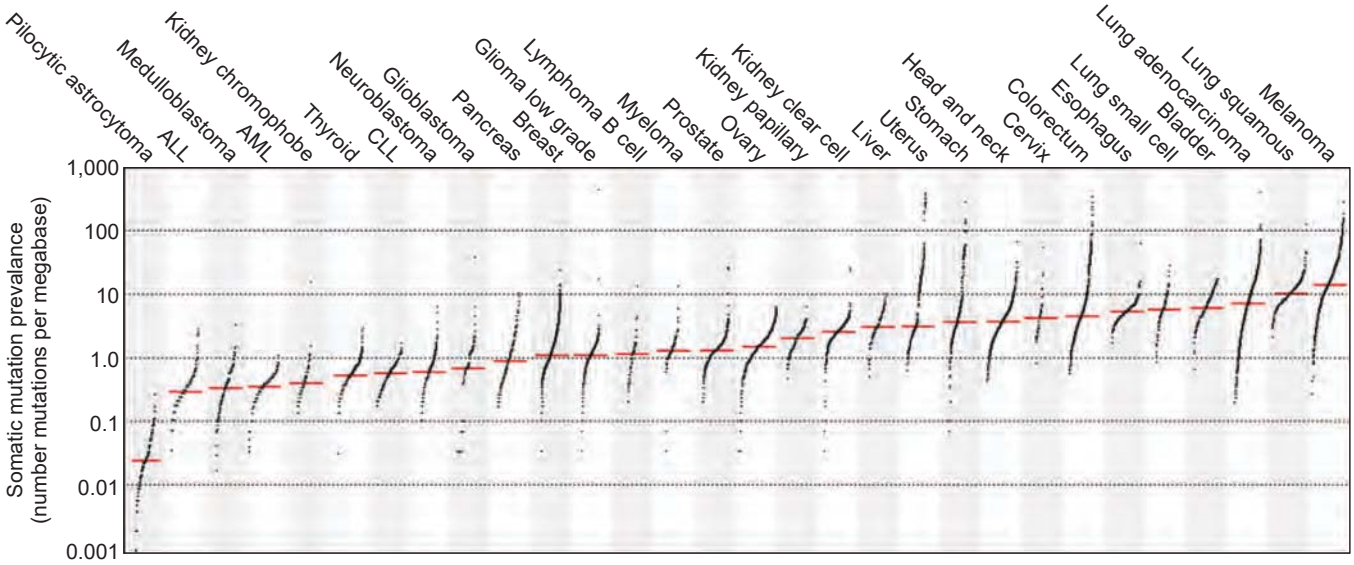

Figure 1.4 The prevalence of somatic mutations across human cancer types. Every *dot* represents a sample, whereas the *red horizontal lines* are the median numbers of mutations in the respective cancer types. The vertical axis (log scaled) shows the number of mutations per megabase, whereas the different cancer types are ordered on the horizontal axis based on their median numbers of somatic mutations. ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia. (Used with permission from Alexandrov LB, Nik-Zainal S, Wedge DC, et al. Signatures of mutational processes in human cancer. *Nature* 2013;500:415–421.)

insight into the DNA damage signature in this cancer type, but can also be useful in determining the relative order of some acquired mutations. Indeed, this study shows that a significant correlation exists between the presence of a higher proportion of C.A/G.T transitions in early (82%) compared to late mutations (53%). Another important aspect that the comprehensive nature of this melanoma study provided was that cancer mutations are spread out unevenly throughout the genome, with a lower prevalence in regions of transcribed genes, suggesting that DNA repair occurs mainly in these areas.

An interesting and pioneering example of the power of whole-genome sequencing in deciphering the mutation evolution in carcinogenesis was seen in a study in which a basallike breast cancer tumor, a brain metastasis, a tumor xenograft derived from the primary tumor, and the peripheral blood from the same patient were compared (Fig. 1.5).[85] This analysis showed a wide range of

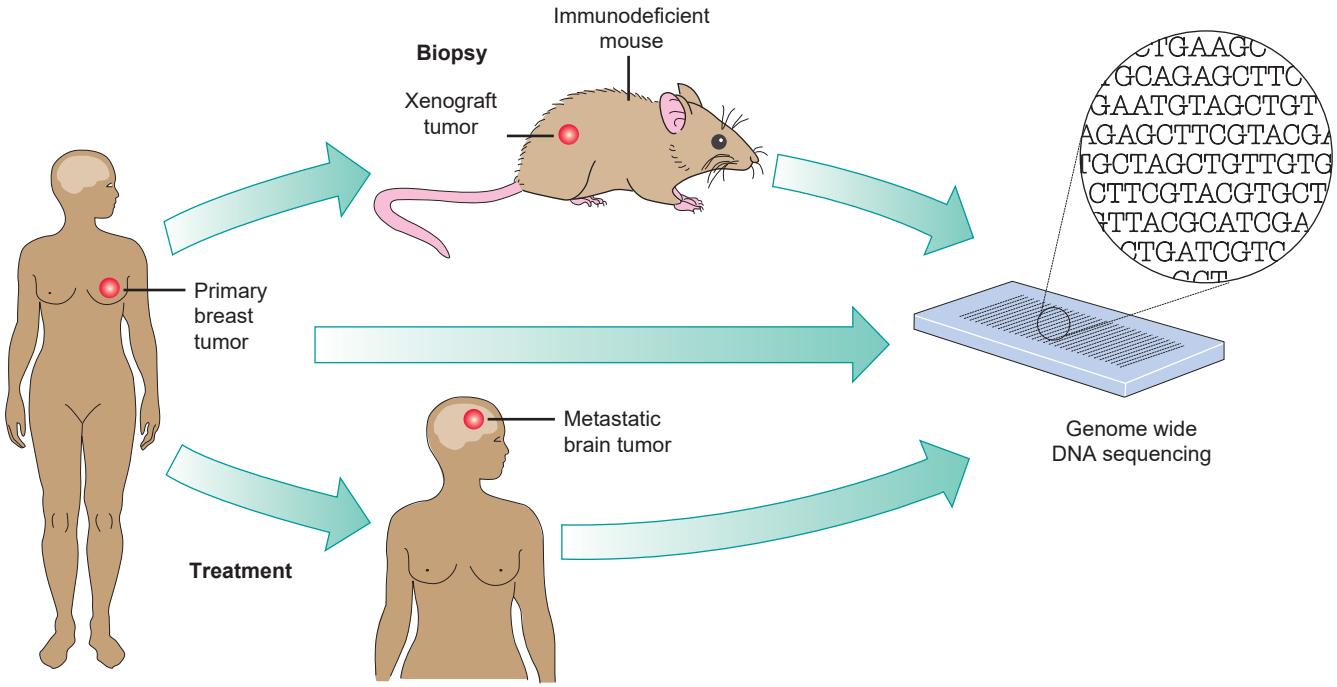

Figure 1.5 Covering all the bases in metastatic assessment. Ding et al.[85] performed a genomewide analysis on three tumor samples: a patient's primary breast tumor; her metastatic brain tumor, which formed despite therapy; and a xenograft tumor in a mouse, originating from the patient's breast tumor. They find that the primary tumor differs from the metastatic and xenograft tumors mainly in the prevalence of genomic mutations. (With permission from Gray J. Cancer: genomics of metastasis. *Nature* 2010;464:989–990.)

mutant allele frequencies in the primary tumor, which was narrowed in the metastasis and xenograft samples. This suggested that the primary tumor was significantly more heterogeneous in its cell populations compared to its matched metastasis and xenograft samples because these underwent selection processes whether during metastasis or transplantation. The clear overlap in mutation incidence between the metastatic and xenograft cases suggests that xenografts undergo similar selection as metastatic lesions and, therefore, are a reliable source for genomic analyses. The main conclusion of this whole-genome study was that, although metastatic tumors harbor an increased number of genetic alterations, the majority of the alterations found in the primary tumor are preserved. Interestingly, single-cell genome sequencing of a breast primary tumour and its liver metastasis indicated that a single clonal expansion formed the primary tumor and seeded the metastasis.[90] Further studies have confirmed and extended these findings to metastatic tumors from different types, including renal and pancreatic carcinomas.[91]

The importance of performing whole-genome sequencing has also been emphasized by the recent identification of somatic mutations in regulatory regions, which can also elicit tumorigenesis. In a study reviewing the noncoding mutations in 19 melanoma whole-genome samples, two recurrent mutations in 17 of the 19 cases studied within the *telomerase reverse transcriptase* (*TERT*) promoter region were revealed.[92] When these two mutations were investigated in an extension of 51 additional tumors and their matched normal tissues, it was observed that 33 tumors harbored one of the mutations and that the mutations occurred in a mutually exclusive manner. These two mutations generate an identical 11 bp nucleotide stretch that contains the consensus binding site for E-twenty-six (ETS) transcription factors. When cloned into a luciferase reporter assay system, it was shown that these mutations conferred a two- to fourfold increase in transcriptional activity of this promoter in five melanoma cell lines. Although this alteration is much more frequent in melanoma, it is also present in other cancer types because 16% of the cancers listed in the Cancer Cell Line Encyclopedia harbor one of the two *TERT* mutations. In combination, these *TERT* mutations are seen in a greater frequency than *BRAF*- and *NRAS*-activating mutations. They occur in a mutually exclusive manner and in regions that do not show a large background mutation rate, all suggesting that these mutations are important driver events contributing to oncogenesis. Further supporting this was another recent study that identified these same two mutations in the germ line of familial melanoma patients.[93]

As the *TERT* promoter mutation discovery shows, regions of the genome that do not code for proteins are just as vital in our understanding of the biology behind tumor development and progression. Another class of non–protein-coding regions in the genome are the noncoding RNAs. One class of noncoding RNAs are microRNAs (miRNA). Discovered 20 years ago, miRNAs are known to be expressed in a tissue or developmentally specific manner and their expression can influence cellular growth and differentiation along with cancer-related pathways such as apoptosis or stress response. miRNAs do this through either overexpression, leading to the targeting and downregulation of tumor suppressor genes, or inversely through their own downregulation, leading to increased expression of their target oncogene. miRNAs have been extensively studied in cancer and their functional effects have been noted in a wide variety of cancers like glioma[94] and breast cancer,[95] to name just a few.

Another class of noncoding RNAs (ncRNA) are the long noncoding RNAs (lncRNA). These RNAs are typically greater than 200 bp and can range up to 100 kb in size. They are transcribed by RNA polymerase II and can undergo splicing and polyadenylation. Although much less extensively studied when compared to miRNAs for their role in cancer, lncRNAs are beginning to come under much more scrutiny. A recent study of the steroid receptor RNA activator (SRA) revealed two transcripts, a lncRNA (SRA) and a translated transcript (steroid receptor RNA activator protein [SRAP]), that coexist within breast cancer cells. However, their expression varies within breast cancer cell lines with different phenotypes. It was shown that in a more invasive breast cancer line, higher relative levels of the noncoding transcript were seen.[96] Because this ncRNA acts as part of a ribonucleoprotein complex that is recruited to the promoter region of regulatory genes, it has been hypothesized that this shift in balance between both noncoding and coding transcripts may be associated with growth advantages. When this balance was shifted in vitro, it led to a large increase in transcripts associated with invasion and migration. The results of this study highlight the importance of the investigation into the roles of ncRNA in tumor development or progression and confirm again that the study of coding variants is not sufficient in determining the full genomic spectrum of cancer.

It must be also noted that the recent analysis of whole genomes of many different human tumors has provided additional insights into cancer evolution. Thus, it has been demonstrated that multiple mutational processes are operative during cancer development and progression, each of which has the capacity to leave its particular mutational signature on the genome. A remarkable and innovative study in this regard was aimed at the generation of the entire catalog of somatic mutations in 21 breast carcinomas and the identification of the mutational signatures of the underlying processes. This analysis revealed the occurrence of multiple, distinct single- and double-nucleotide substitution signatures. Moreover, it was reported that breast carcinomas harboring *BRCA1* or *BRCA2* mutations showed a characteristic combination of substitution mutation signatures and a particular profile of genomic deletions. An additional contribution of this analysis was the identification of a distinctive phenomenon of localized hypermutation, which has been termed *kataegis*, and which has also subsequently been observed in other malignancies distinct from breast carcinomas.[87]

Whole-genome sequencing of human carcinomas has also allowed for the ability to characterize other massive genomic alterations, termed *chromothripsis* and *chromoplexy*, occurring across different cancer subtypes.[97] Chromothripsis implies a massive genomic rearrangement acquired in a one-step catastrophic event during cancer development and has been detected in about 2% to 3% of all tumors, but is present at high frequency in some particular cases, such as bone cancers.[98] Chromoplexy has been originally described in prostate cancer and involves many DNA translocations and deletions that arise in a highly interdependent manner and result in the coordinate disruption of multiple cancer genes.[99] These newly described phenomena represent powerful strategies of rapid genome evolution, which may play essential roles during carcinogenesis.

Whole-Exome Analysis Utilizing Second-Generation Sequencing

Another application of second-generation sequencing involves utilizing nucleic acid "baits" to capture regions of interest in the total pool of nucleic acids. These could either be DNA, as described previously,[100,101] or RNA.[102] Indeed, most areas of interest in the genome can be targeted, including exons and ncRNAs. Despite inefficiencies in the exome-targeting process—including the uneven capture efficiency across exons, which results in not all exons being sequenced, and the occurrence of some off-target hybridization events—the higher coverage of the exome makes it highly suitable for mutation discovery in cancer samples.

Over the last few years, thousands of cancer samples have been subjected to whole-exome sequencing. These studies, combined with data from whole-genome sequencing, have provided an unprecedented level of information about the mutational landscape of the most frequent human malignancies.[74–76] In addition, whole-exome sequencing has been used to identify the somatic mutations characteristic of both rare tumors and those that are prevalent in certain geographical regions.[76]

Overall, these studies have provided very valuable information about mutation rates and spectra across cancer types and subtypes.[87,103,104] Remarkably, the variation in mutational frequency between different tumors is extraordinary, with hematologic and pediatric cancers showing the lowest mutation rates (0.001 per Mb of DNA), and melanoma and lung cancers presenting the highest mutational burden (more than 400 per Mb). Whole-exome sequencing has also contributed to the identification of novel cancer genes that had not been previously described to be causally implicated in the carcinogenesis process. These genes belong to different functional categories, including signal transduction, RNA maturation, metabolic regulation, epigenetics, chromatin remodeling, and protein homeostasis.[74] Finally, a combination of data from whole-exome and whole-genome sequencing has allowed for the identification of the signatures of mutational processes operating in different cancer types.[87] Thus, an analysis of a dataset of about 5 million mutations from over 7,000 cancers from 30 different types has allowed for the extraction of more than 20 distinct mutational signatures. Some of them, such as those derived from the activity of APOBEC cytidine deaminases, are present in most cancer types, whereas others are characteristic of specific tumors. Known signatures associated with age, smoking, ultraviolet (UV) light exposure, and DNA repair defects have been also identified in this work, but many of the detected mutational signatures are of cryptic origin. These findings demonstrate the impressive diversity of mutational processes underlying cancer development and may have enormous implications for the future understanding of cancer biology, prevention, and treatment.

SOMATIC ALTERATION CLASSES DETECTED BY CANCER GENOME ANALYSIS

Whole-genome sequencing of cancer genomes has an enormous potential to detect all major types of somatic mutations present in malignant tumors. This large repertoire of genomic abnormalities includes single nucleotide changes, small insertions and deletions, large chromosomal reorganizations, and copy number variations (Fig. 1.6).

Nucleotide substitutions are the most frequent somatic mutations detected in malignant tumors, although there is a substantial variability in the mutational frequency among different cancers.[60] On average, human malignancies have one nucleotide change per million bases, but melanomas reach mutational rates 10-fold higher, and tumors with mutator phenotype caused by DNA mismatch repair deficiencies may accumulate tens of mutations per million nucleotides. By contrast, tumors of hematopoietic origin have less than one base substitution per million. Several bioinformatic tools and pipelines have been developed to efficiently detect somatic nucleotide substitutions through comparison of the genomic information obtained from paired normal and tumor samples from the same patient. Likewise, there are a number of publicly available computational methods to predict the functional relevance of the identified mutations in cancer specimens.[60] Most of these bioinformatic tools exclusively deal with nucleotide changes in protein coding regions and evaluate the putative structural or functional effect of an amino acid substitution in a determined protein, thus obviating changes in other genomic regions, which can also be of crucial interest in cancer. In any case, current computational methods used in this regard are far from being optimal, and experimental validation is finally required to assess the functional relevance of nucleotide substitutions found in cancer genomes.

For years, the main focus of cancer genome analyses has been on identifying coding mutations that cause a change in the amino acid sequence of a gene. The rationale behind this is quite sound because any mutation that creates a novel protein or truncates an essential protein has the potential to drastically change the cellular environment. Examples of this have been shown earlier in the chapter with *BRAF* and *KRAS* along with many others. With the advancements in next-generation sequencing, larger studies are able to be conducted. These studies give the power to detect mutations occurring in the cancer genome at a lower frequency. Interesting to note is that these studies are leading to the discovery that recurrent synonymous mutations occur in cancer. Previously believed to be merely neutral mutations that maintain no functional role in tumorigenesis, these mutations were largely ignored, but a recent study shows[105] that simply dismissing these mutations as silent may be premature.

In a review of only 29 melanoma exomes and genomes, 16 recurring synonymous mutations were discovered. When these mutations were screened in additional samples, a synonymous mutation in the gene *BCL2L12* was discovered in 12 out of 285 total samples. The observed frequency of this recurrent mutation is greater than expected by chance, suggesting that it has undergone some type of selective pressure during tumor development.[105] Noting that *BCL2L12* had previously been linked to tumorigenesis, the mutation was further evaluated for its functional effect, with the finding that it led to an abrogation of the effect of a miRNA, leading to the deregulated expression of *BCL2L12*. BCL2L12 is a negative regulator of the gene p53, which functions by binding and inhibiting apoptosis in glioma.[106] Accordingly, the dysregulation observed in *BCL2L12* led to a reduction in p53 target gene expression.

Small insertions and deletions (*indels*) represent a second category of somatic mutations that can be discovered by whole-genome sequencing of cancer specimens. These mutations are about 10-fold less frequent than nucleotide substitutions, but may also have an obvious impact in cancer progression. Accordingly, specific bioinformatic tools have been created to detect these *indels* in the context of the large amount of information generated by whole-genome sequencing projects.[107]

The systematic identification of large chromosomal rearrangements in cancer genomes represents one of the most successful applications of next-generation sequencing methodologies. Previous strategies in this regard had mainly been based on the utilization of cytogenetic methods for the identification of recurrent translocations in hematopoietic tumors. More recently, a combination of bioinformatics and functional methods has allowed for the finding of recurrent translocations in solid epithelial tumors such as *TMPRSS2–ERG* in prostate cancer and *EML4–ALK* in non–small-cell lung cancer.[108,109] Now, by using a next-generation sequencing analysis of genomes and transcriptomes, it is possible to systematically search for both intrachromosomal and interchromosomal rearrangements occurring in cancer specimens. These studies have already proven their usefulness for cancer research through the discovery of recurrent translocations involving genes of the *RAF* kinase pathway in prostate and gastric cancers and in melanomas.[110] Likewise, massively parallel paired-end genome and transcriptome sequencing has already been used to detect new gene fusions in cancer and to catalog all major structural rearrangements present in some tumors and cancer cell lines.[63,111–113] The ongoing cancer genome projects involving thousands of tumor samples will likely lead to the detection of many other chromosomal rearrangements of relevance in specific subsets of cancers. It is also remarkable that whole-genome sequencing may also facilitate the identification of other types of genomic alterations, including rearrangements of repetitive elements, such as active retrotransposons, or insertions of foreign gene sequences, such as viral genomes, which can contribute to cancer development. Indeed, a next-generation sequencing analysis of the transcriptome of Merkel cell carcinoma samples has revealed the clonal integration within the tumor genome of a previously unknown polyomavirus likely implicated in the pathogenesis of this rare but aggressive skin cancer.[114]

Finally, next-generation sequencing approaches have also demonstrated their feasibility to analyze the pattern of copy number

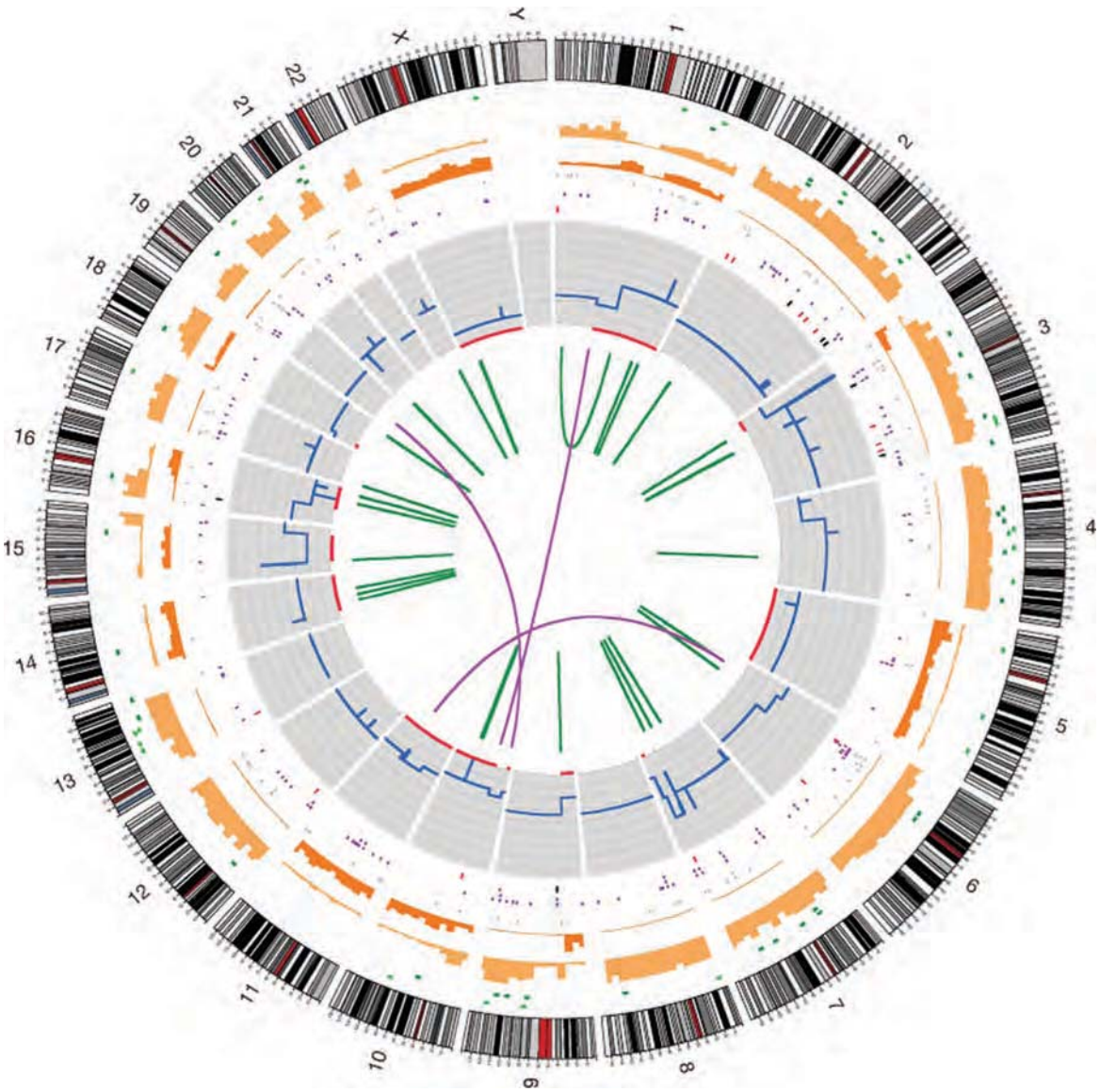

Figure 1.6 The catalog of somatic mutations in COLO-829. Chromosome ideograms are shown around the *outer ring* and are oriented pter–qter in a clockwise direction with centromeres indicated in *red*. Other tracks contain somatic alterations (*from outside to inside):* validated insertions *(light green rectangles);* validated deletions *(dark green rectangles);* heterozygous *(light orange bars),* and homozygous *(dark orange bars)* substitutions shown by density per 10 megabases; coding substitutions *(colored squares: silent in gray, missense in purple, nonsense in red, and splice site in black);* copy number *(blue lines);* regions of loss of heterozygosity (LOH) *(red lines);* validated intrachromosomal rearrangements *(green lines*); validated interchromosomal rearrangements *(purple lines).* (From Pleasance ED, Cheetham RK, Stephens PJ, et al. A comprehensive catalogue of somatic mutations from a human cancer genome. *Nature* 2010;463:191–196.)

alterations in cancer, because they allow researchers to count the number of reads in both tumor and normal samples at any given genomic region and then to evaluate the tumor-to-normal copy number ratio at this particular region. These new methods offer some advantages when compared with those based on microarrays, including much better resolution, precise definition of the involved breakpoints, and absence of saturation, which facilitates the accurate estimation of high copy number levels occurring in some genomic loci of malignant tumors.[60]

PATHWAY-ORIENTED MODELS OF CANCER GENOME ANALYSIS

Genomewide mutational analyses suggest that the mutational landscape of cancer is made up of a handful of genes that are mutated in a high fraction of tumors, otherwise known as *mountains*, and most mutated genes are altered at relatively low frequencies, otherwise known as *hills* (Fig. 1.7).[29] The mountains probably give a high selective advantage to the mutated cell, and the hills might provide a lower advantage, making it hard to distinguish them from passenger mutations. Because the hills differ between cancer types, it seems that the cancer genome is more complex and heterogeneous than anticipated. Although highly heterogeneous, bioinformatic studies suggest that the mountains and hills can be grouped into sets of pathways and biologic processes. Some of these pathways are affected by mutations in a few pathway members and others by numerous members. For example, pathway analyses have allowed for the stratification of mutated genes in pancreatic adenocarcinomas to 12 core pathways that have at least one member mutated in 67% to 100% of the tumors analyzed (Fig. 1.8).[45] These core pathways deviated to some that harbored one single highly mutated gene,

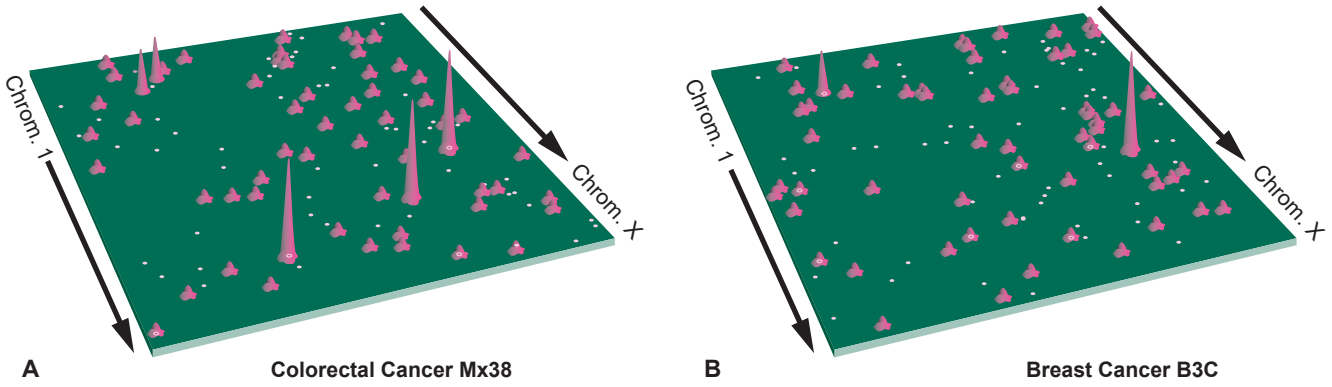

Figure 1.7 Cancer genome landscapes. Nonsilent somatic mutations are plotted in a two-dimensional space representing chromosomal positions of RefSeq genes. The telomere of the short arm of chromosome 1 is represented in the rear left corner of the *green plane* and ascending chromosomal positions continue in the direction of the arrow. Chromosomal positions that follow the front edge of the plane are continued at the back edge of the plane of the adjacent row, and chromosomes are appended end to end. Peaks indicate the 60 highest ranking CAN genes for each tumor type, with peak heights reflecting CaMP scores. The *dots* represent genes that were somatically mutated in the individual colorectal (Mx38) **(A)** or breast tumor (B3C) **(B)**. The *dots* corresponding to mutated genes that coincided with hills or mountains are black with white rims; the remaining *dots* are white with red rims. The mountain on the right of both landscapes represents *TP53* (chromosome 17), and the other mountain shared by both breast and colorectal cancers is *PIK3CA* (upper left, chromosome 3). (Redrawn from Wood LD, Parsons DW, Jones S, et al. The genomic landscapes of human breast and colorectal cancers. *Science* 2007;318:1108–1113. Reprinted with permission from the American Association for the Advancement of Science).

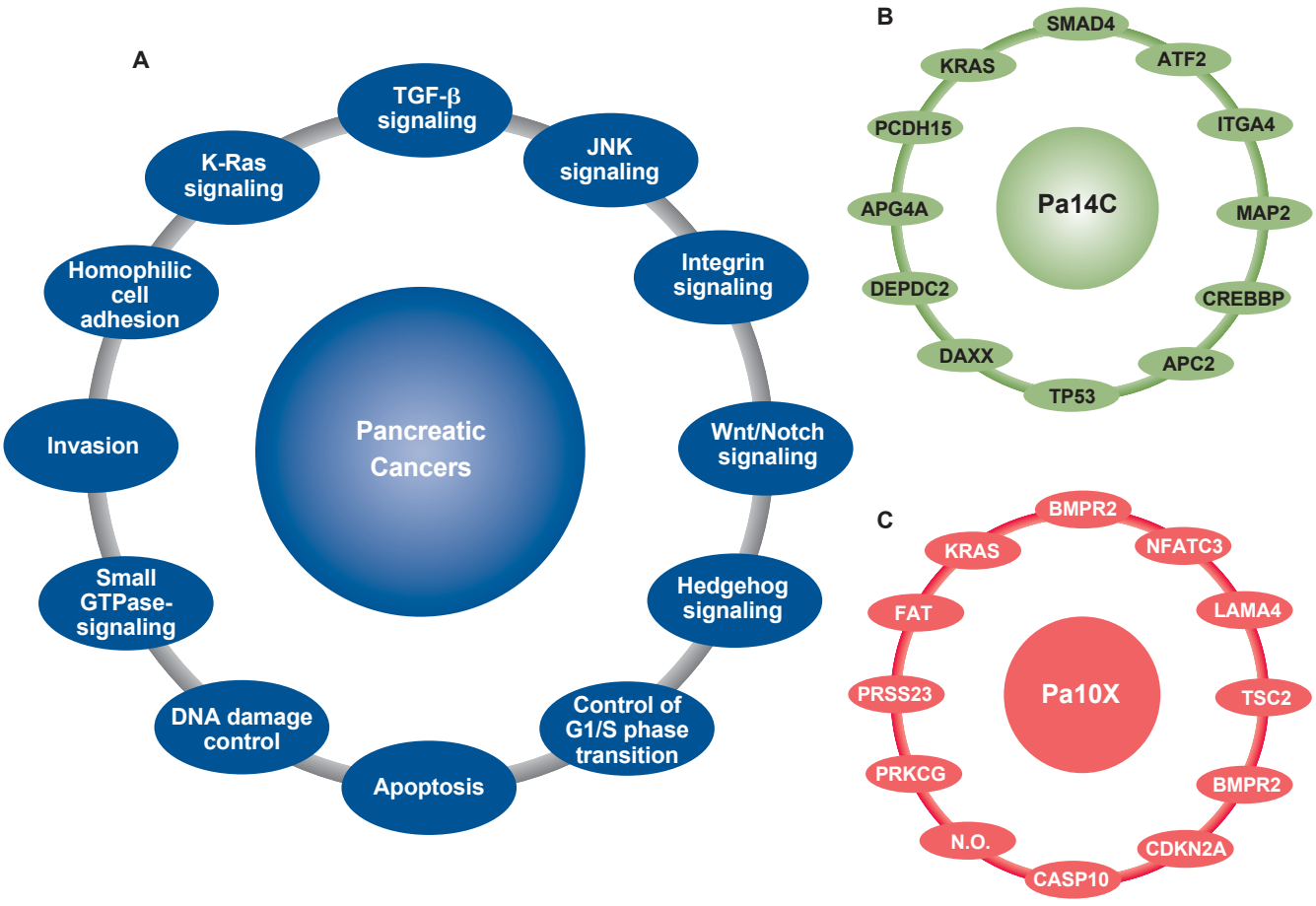

Figure 1.8 Signaling pathways and processes. **(A)** The 12 pathways and processes whose component genes were genetically altered in most pancreatic cancers. **(B,C)** Two pancreatic cancers (Pa14C and Pa10X) and the specific genes that are mutated in them. The positions around the circles in **(B)** and **(C)** correspond to the pathways and processes in **(A).** Several pathway components overlapped, as illustrated by the BMPR2 mutation that presumably disrupted both the SMAD4 and Hedgehog signaling pathways in Pa10X. Additionally, not all 12 processes and pathways were altered in every pancreatic cancer, as exemplified by the fact that no mutations known to affect DNA damage control were observed in Pa10X. NO, not observed. (Redrawn from Jones S, Zhang X, Parsons DW, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science* 2008;321:1801–1806. Reprinted with permission from the American Association for the Advancement of Science).

such as in *KRAS* in the G1/S cell cycle transition pathway and pathways where a few mutated genes were found, such as the transforming growth factor (TGF-β) signaling pathway. Finally, there were pathways in which many different genes were mutated, such as invasion regulation molecules, cell adhesion molecules, and integrin signaling. Importantly, independent of how many genes in the same pathway are affected, if they are found to occur in a mutually exclusive fashion in a single tumor, they most likely give the same selective pressure for clonal expansion.

The idea of genetically analyzing pathways rather than individual genes has been applied previously, revealing the concept of mutual exclusivity. Mutual exclusivity has been shown elegantly in the case of *KRAS* and *BRAF*, where a *KRAS*-mutated cancer generally does not also harbor a *BRAF* mutation, because *KRAS* is upstream of *BRAF* in the same pathway.[9] A similar concept was applied for *PIK3CA* and *PTEN*, where both mutations do not usually occur in the same tumor.[23]

With the ever expanding amounts of genetic information being gathered, the ability to search for common pathways being affected in cancer is increasing. One new pathway that is beginning to emerge is the glutamate-signaling pathway. Glutamate dysregulation has been implicated in a number of cancers. In a study of pancreatic duct adenocarcinoma (PDAC), it was seen that glutamate levels were significantly higher in the tissue of individuals with chronic pancreatitis (CP) and PDAC when compared to normal pancreas tissue.[115] It was also observed that the increased glutamate levels led to proinvasion and antiapoptotic signaling through the activation of AMPA receptors.

Also in this regard, and through the use of whole-exome sequencing, it has been recently shown that the glutamate receptor gene *GRIN2A* is highly mutated in melanoma. The finding that many of these mutations are nonsense has suggested that *GRIN2A* is a novel tumor suppressor. Additional genes in the glutamate pathway have also found mutated in melanomas.[116] Pathway analyses and statistical testing on the whole-exome data have also revealed the glutamate signaling pathway to be dysregulated. These results have been further corroborated in another study reporting mutations in the metabotropic glutamate receptor GRM3[117,118] in melanoma. A functional analysis of mutations found in *GRM3* in melanoma tumor samples has shown an increased activation of MEK1/2 kinase, increased migration, and anchorage-independent growth.[117]

Passenger and Driver Mutations

By the time a cancer is diagnosed, it is comprised of billions of cells carrying DNA abnormalities, some of which have a functional role in malignant proliferation; however, many genetic lesions acquired along the way have no functional role in tumorigenesis.[14] The emerging landscapes of cancer genomes include thousands of genes that were not previously linked to tumorigenesis but are found to be somatically mutated. Many of these changes are likely to be *passengers*, or neutral, in that they have no functional effects on the growth of the tumor.[14] Only a small fraction of the genetic alterations are expected to drive cancer evolution by giving cells a selective advantage over their neighbors. Passenger mutations occur incidentally in a cell that later or in parallel develops a *driver* mutation, but are not ultimately pathogenic.[119] Although neutral, cataloging passengers mutations is important because they incorporate the signatures of the previous exposures the cancer cell underwent as well as DNA repair defects the cancer cell has. In many cases, the passenger and driver mutations occur at similar frequencies and the identification of drivers versus the passenger is of utmost relevance and remains a pressing challenge in cancer genetics.[120–122] This goal will eventually be achieved through a combination of genetic and functional approaches, some of which are listed as follows.

The most reliable indicator that a gene was selected for and therefore is highly likely to be pathogenic is the identification of recurrent mutations, whether at the same exact amino acid position or in neighboring amino acid positions in different patients. More than that, if somatic alterations in the same gene occur very frequently (mountains in the tumor genome landscape), these can be confidently classified as drivers. For example, cancer alleles that are identified in multiple patients and different tumors types, such as those found in *KRAS*, *TP53*, *PTEN*, and *PIK3CA*, are clearly selected for during tumorigenesis.

However, most genes discovered thus far are mutated in a relatively small fraction of tumors (hills), and it has been clearly shown that genes that are mutated in less than 1% of patients can still act as *drivers*.[123] The systematic sequencing of newly identified putative cancer genes in the vast number of specimens from cancer patients will help in this regard. However, even if examining large numbers of samples can provide helpful information to classify drivers versus passengers, this approach alone is limited by the marked variation in mutation frequency among individual tumors and individual genes. The statistical test utilized in this case calculates the probability that the number of mutations in a given gene reflects a mutation frequency that is greater than expected from the nonfunctional background mutation rate,[29,124] which is different between different cancer types. These analyses incorporate the number of somatic alterations observed, the number of tumors studied, and the number of nucleotides that were successfully sequenced and analyzed.

Another approach often used to distinguish driver from passenger mutations exploits the statistical analysis of synonymous versus nonsynonymous changes.[125] In contrast to nonsynonymous mutations, synonymous mutations do not alter the protein sequence. Therefore, they do not usually apply a growth advantage and would not be expected to be selected during tumorigenesis. This strategy works by comparing the observed-to-expected ratio of synonymous with that of nonsynonymous mutation. An increased proportion of nonsynonymous mutations from the expected 2:1 ratio implies selection pressure during tumorigenesis.

Other approaches are based on the concept that driver mutations may have characteristics similar to those causing Mendelian disease when inherited in the germ line and may be identifiable by constraints on tolerated amino acid residues at the mutated positions. In contrast, passenger mutations may have characteristics more similar to those of nonsynonymous SNPs with high minor allele frequencies. Based on these premises, supervised machine learning methods have been used to predict which missense mutations are drivers.[126] Additional approaches to decipher drivers from passengers include the identification of mutations that affect locations that have previously been shown to be cancer causing in protein members of the same gene family. Enrichment for mutations in evolutionarily conserved residues are analyzed by algorithms, such as SIFT (sorting intolerant from tolerant (SIFT),[127] which estimates the effects of the different mutations identified.

Probably the most conclusive methods to identify driver mutations will be rigorous functional studies using biochemical assays as well as model organisms or cultured cells, using knockout and knockin of individual cancer alleles.[128] Unfortunately, these methods are not well suited to the analysis of the hundreds of gene candidates that arise from every large-scale cancer genome project. In conclusion, it is fair to say that sequencing cancer genomes is only the beginning of a journey that will ultimately be completed when the thousands of the newly discovered alleles are annotated as being the drivers of this disease. A summary of the various next-generation applications and approaches for their analysis is summarized in Figure 1.9 and Table 1.2.

NETWORKS OF CANCER GENOME PROJECTS

The repertoire of oncogenic mutations is extremely heterogeneous, suggesting that it would be difficult for independent cancer genome initiatives to address the generation of comprehensive

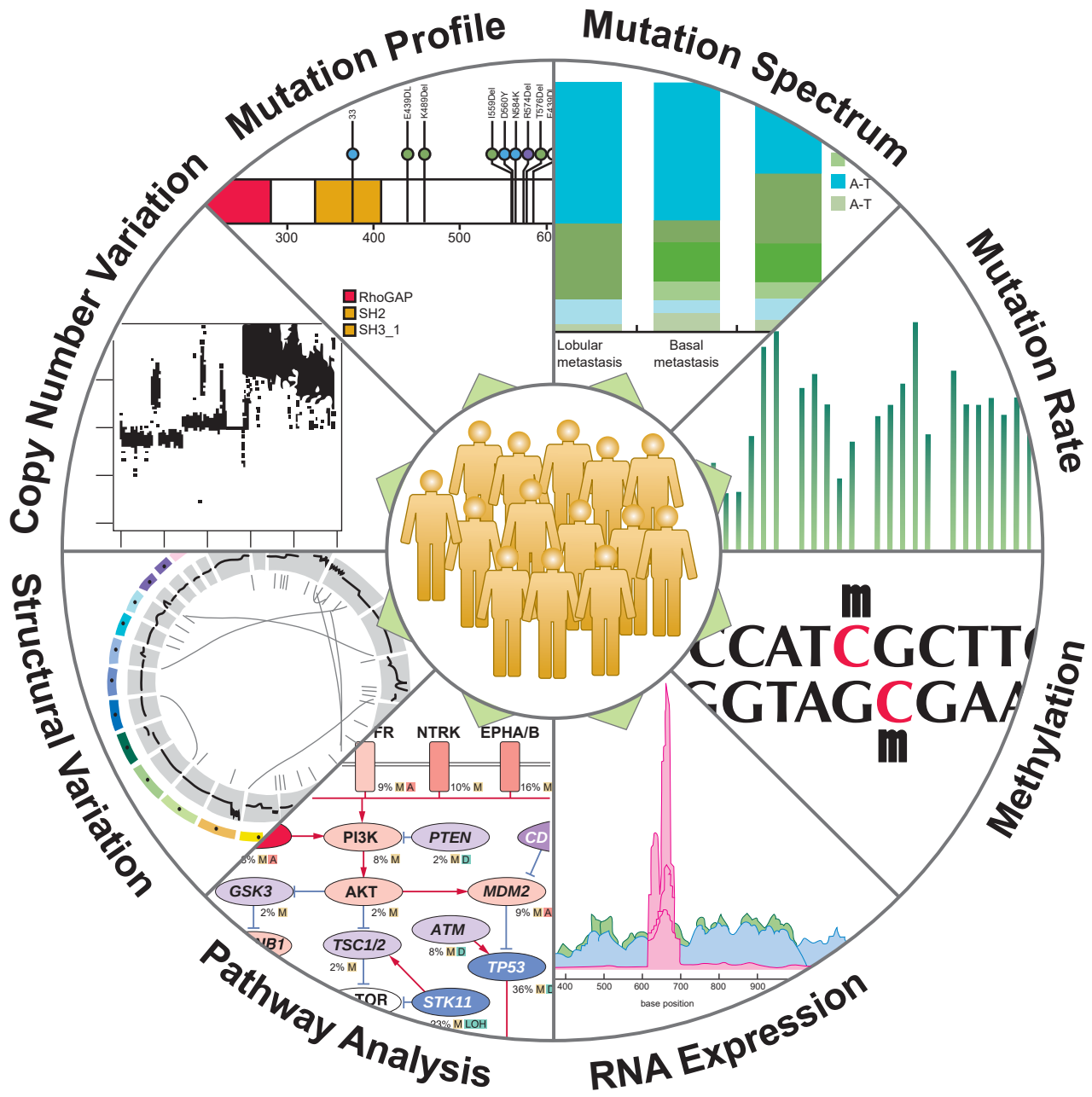

Figure 1.9 Landscape of cancer genomics analyses. NGS data will be generated for hundreds of tumors from all major cancer types in the near future. The integrated analysis of DNA, RNA, and methylation sequencing data will help elucidate all relevant genetic changes in cancers. (Used with permission from Ding L, Wendl MC, Koboldt DC, et al. Analysis of next-generation genomic data in cancer: accomplishments and challenges. *Hum Mol Genet* 2010;19:R188–R196.)

catalogs of mutations in the wide spectrum of human malignancies. Accordingly, there have been different efforts to coordinate the cancer genome sequencing projects being carried out around the world, including The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC). Moreover, there are other initiatives that are more focused on specific tumors, such as that led by scientists at St. Jude Children's Research Hospital in Memphis, and Washington University, which aims at sequencing multiple pediatric cancer genomes.[129]

TCGA began in 2006 in the United States as a comprehensive program in cancer genomics supported by the U.S. National Institutes of Health (NIH). The initial project focused on three tumors: GBM, serous cystadenocarcinoma of the ovary, and lung squamous carcinoma. These studies have already generated novel and interesting information regarding genes mutated in these malignancies.[134] On the basis of these positive results, the NIH announced an expansion of the TCGA program with the aim to produce genomic data sets for at least 20 to 25 cancers during the next few years.

The ICGC was formed in 2008 to coordinate the generation of comprehensive catalogs of genomic abnormalities in tumors from 50 different cancer types or subtypes that are of clinical and societal importance across the world.[130] The project aims to perform systematic studies of over 25,000 cancer genomes at the genomic level and integrate this information with epigenomic and transcriptomic studies of the same cases as well as with clinical features of patients. At present, there are a total of 69 committed projects involving at least 16 different countries coordinated by

TABLE 1.2

Computational Tools and Databases Useful for Cancer Genome Analyses

Category	Tool/Database	URL
Alignment	Maq[a]	http://maq.sourceforge.net
	Burrows-Wheeler Aligner (BWA)[b]	http://bio-bwa.sourceforge.net
Mutation calling	SNVMix[c]	http://www.bcgsc.ca/platform/bioinfo/software/SNVMix
	SAMtools[d]	http://samtools.sourceforge.net
	VarScan[e]	http://varscan.sourceforge.net
	MuTect[f]	http://www.broadinstitute.org/cancer/cga/mutect
Indel calling	Pindel[g]	http://gmt.genome.wustl.edu/pindel/current/
Copy number analysis	CBS[h]	http://www.bioconductor.org
	SegSeq[i]	http://www.broadinstitute.org/cgi-bin/cancer/publications/pub_paper.cgi?mode=view&paper_id=182
Functional effect	SIFT[j]	http://sift.jcvi.org/
	PolyPhen-2[k]	http://genetics.bwh.harvard.edu/pph2
Visualization	CIRCOS[l]	http://mkweb.bcgsc.ca/circos
	Integrative Genomics Viewer (IGV)[m]	http://www.broadinstitute.org/igv
Repository	Catalogue of Somatic Mutations in Cancer (COSMIC)[n]	http://www.sanger.ac.uk/genetics/CGP/cosmic
	Cancer Genome Project (CGP)[o]	http://www.sanger.ac.uk/genetics/CGP
	dbSNP[p]	http://www.ncbi.nlm.nih.gov/SNP
	Gene Ranker[q]	http://cbio.mskcc.org/tcga-generanker/

[a] Li H, Durbin R. Fast and accurate short read alignment with Burrows–Wheeler transform. *Bioinformatics* 2009;25:1754–1760.
[b] Li H, Durbin R. Fast and accurate long-read alignment with Burrows–Wheeler transform. *Bioinformatics* 2010;26:589–595.
[c] Goya R, Sun MG, Morin RD, et al. SNVMix: predicting single nucleotide variants from next-generation sequencing of tumors. *Bioinformatics* 2010;26:730–736.
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[e] Koboldt DC, Chen K, Wylie T, et al. VarScan: variant detection in massively parallel sequencing of individual and pooled samples. *Bioinformatics* 2009;25:2283–2285.
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[q] The Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 2008;455:1061–1068.
Based on Meyerson M, Stacey G, Getz G. Advances in understanding cancer genomes through second generation sequencing. *Nature Rev Genet* 2010;11:685–696, Table 2.

the ICGC. All of these projects deal with at least 500 samples per cancer type from cancers affecting a variety of human organs and tissues, including blood, the brain, the breast, the esophagus, the kidneys, the liver, the oral cavity, the ovaries, the pancreas, the prostate, the skin, and the stomach.[130]

All of these coordinated projects have already provided new insights into the catalog of genes mutated in cancer and have unveiled specific signatures of the mutagenic mechanisms, including carcinogen exposures or DNA-repair defects, implicated in the development of different malignant tumors.[83,84,87,131] Furthermore, these cancer genome studies have also contributed to define clinically relevant subtypes of tumors for prognosis and therapeutic management, and in some cases have identified new targets and strategies for cancer treatment.[74–76] The rapid technological advances in DNA sequencing will likely drop the costs of sequencing cancer genomes to a small fraction of the current price and will allow researchers to overcome some of the current limitations of these global sequencing efforts. Hopefully, worldwide coordination of cancer genome projects, including Pan-Cancer initiative, with those involving large-scale, functional analyses of genes in both cellular and animal models will likely provide us with the most comprehensive collection of information generated to date about the causes and molecular mechanisms of cancer.

THE GENOMIC LANDSCAPE OF CANCERS

Examining the overall distribution of the identified mutations redefined the cancer genome landscapes whereby the *mountains* are the handful of commonly mutated genes and the *hills* represent the vast majority of genes that are infrequently mutated.

One of the most striking features of the tumor genomic landscape is that it involves different sets of cancer genes that are mutated in a tissue-specific fashion.[132,133] To continue with the analogy, the scenery is very different if we observe a colorectal, a lung, or a breast tumor. This indicates that mutations in specific genes cause tumors at specific sites, or are associated with specific stages of development, cell differentiation, or tumorigenesis, despite many of those genes being expressed in various fetal and adult tissues. Moreover, different types of tumors follow specific genetic pathways in terms of the combination of genetic alterations that it must acquire. For example, no cancer outside the bowel has been shown to follow the classic genetic pathway of colorectal tumorigenesis. Additionally, *KRAS* mutations are almost always present in pancreatic cancers but are very rare or absent in breast cancers. Similarly, *BRAF* mutations are present in 60% of melanomas, but are very infrequent in lung cancers.[1] Another intriguing feature is that alterations in ubiquitous housekeeping genes, such as those involved in DNA repair or energy production, occur only in particular types of tumors.

In addition to tissue specificity, the genomic landscape of tumors can also be associated with gender and hormonal status. For example, *HER2* amplification and *PIK3C2A* mutations, two genetic alterations associated with breast cancer development, are correlated with the estrogen-receptor hormonal status.[134] The molecular basis for the occurrence of cancer mutations in tissue- and gender-specific profiles is still largely unknown. Organ-specific expression profiles and cell-specific neoplastic transformation requirements are often mentioned as possible causes for this phenomenon. Identifying tissue and gender cancer mutations patterns is relevant because it may allow for the definition of individualized therapeutic avenues.

INTEGRATIVE ANALYSIS OF CANCER GENOMICS

The implementation of novel high-throughput technologies is generating an extraordinary amount of information on cancer samples in many different ways other than those derived from whole-exome or whole-genome sequencing. Accordingly, there is a growing need to integrate genomic, epigenomic, transcriptomic, and proteomic landscapes from tumor samples, and then linking this integrated information with clinical outcomes of cancer patients. There are some examples of human malignancies in which this integrative approach has been already performed, such as for AML, glioblastoma, medulloblastoma, and renal cell, colorectal, ovarian, endometrial, prostate, and breast carcinomas.[135–142] In these cases, the integration of whole-exome and whole-genome sequencing with studies involving genomic DNA copy number arrays, DNA methylation, transcriptomic arrays, miRNA sequencing, and proteomic profiling has contributed to improving the molecular classification of complex and heterogeneous tumors. These integrative molecular analyses have also provided new insights into the mechanisms disrupted in each particular cancer type or subtype and have facilitated the association of genomic information with distinct clinical parameters of cancer patients and the discovery of novel therapeutic targets.[143] Also in this regard, there has been significant progress in the definition of the mechanisms by which the cancer genome and epigenome influence each other and cooperate to facilitate malignant transformation.[144,145] Thus, many tumor-suppressor genes are inactivated by either mutation or epigenetic silencing, and in some cases such as colorectal carcinomas, both mechanisms work coordinately to create a permissive environment for oncogenic transformation.[146] Moreover, mutations in epigenetic regulators such as DNA methyl transferases, chromatin remodelers, histones, and histone modifiers, are very frequent events in many tumors, including hepatocellular carcinomas, renal carcinomas leukemias, lymphomas, glioblastomas, and medulloblastomas. These genetic alterations of epigenetic modulators cause widespread transcriptomic changes, thereby amplifying the initial effect of the mutational event at the cancer genome level.[145]

The recent availability of different platforms for integrative cancer genome analyses will be very helpful in enabling the classification, biologic characterization, and personalized clinical management of human cancers (Table 1.3).[144,147]

THE CANCER GENOME AND THE NEW TAXONOMY OF TUMORS

Deciphering the cancer genome has already impacted clinical practice at multiple levels. On the one hand, it allowed for the identification of new cancer genes such as *IDH1*, a gene involved in glioma, which was discovered recently (see previous), and on the other hand, it is redesigning the taxonomy of tumors.

Until the genomic revolution, tumors had been classified based on two criteria: their localization (site of occurrence) and their appearance (histology). These criteria are also currently used as primary determinants of prognosis and to establish the best treatments. For many decades, it has been known that patients with histologically similar tumors have different clinical outcomes. Furthermore, tumors that cannot be distinguished based on an histologic analysis can respond very differently to identical therapies.[148]

It is becoming increasingly clear that the frequency and distribution of mutations affecting cancer genes can be used to redefine the histology-based taxonomy of a given tumor type. Lung and colorectal tumors represent paradigmatic examples. Genomic analyses led to the identification of activating mutations in the receptor tyrosine kinase *EGFR* in lung adenocarcinomas.[149] The occurrence of *EGFR* mutations molecularly defines a subtype of non–small-cell lung cancers (NSCLC) that occur mainly in nonsmoking women, that tend to have a distinctly enhanced prognosis, and that typically respond to epidermal growth factor receptor (EGFR)-targeted therapies.[150–152] Similarly, the recent discovery of the *EML4-ALK* fusion identifies yet another subset of NSCLC that is clearly distinct from those that harbor *EGFR* mutations, that have distinct epidemiologic and biologic features, and that respond to ALK inhibitors.[109,153]

The second example is colorectal cancers (CRC), the tumor type for which the genomic landscape has been refined with the highest accuracy. CRCs can be clearly categorized according to the mutational profile of the genes involved in the *KRAS* pathway (Fig. 1.10). It is now known that *KRAS* mutations occur in approximately 40% of CRCs. Another subtype of CRC (approximately 10%) harbors mutations in *BRAF*, the immediate downstream effectors of *KRAS*.[10]

In CRCs and other tumor types, *KRAS* and *BRAF* mutations are known to be mutually exclusive. The mutual exclusivity pattern indicates that these genes operate in the same signaling pathway. Large epidemiologic studies have shown that the prognosis of tumors harboring wild-type *KRAS*/*BRAF* genes is distinct, and typically more favorable, than that of the mutated ones.[154,155] Of note, *KRAS* and *BRAF* mutations have been recently shown to impair responsiveness to the anti-EGFR monoclonal antibodies therapies in CRC patients.[156–158] Clearly distinct subgroups can be genetically identified in both NSCLCs and CRCs with respect to prognosis and response to therapy. It is likely that as soon as the genomic landscapes of other tumor types are defined, molecular subgroups like those described previously will also become defined.

Genotyping tumor tissue in search of somatic genetic alterations for *actionable* information has become routine practice in clinical oncology. The genetic profile of solid tumors is currently obtained from surgical or biopsy specimens. As the techniques

TABLE 1.3

Useful Information for the Description and Management of Cancer

Bioinformatic Tool or Webservices	Database Used	Webservice or Tool	Upload of Data Possible	Gene Search	Chromosomal Region Search	mRNA Expression	SNV	CNV	Methylation	miRNA Expression	Protein	Pathways
cBioPortal for Cancer Genomics	TCGA	Webservice	—	✓	—	✓	✓	✓	—	—	✓	✓
PARADIGM, Broad GDAC Firehose	TCGA	Webservice	✓	✓	—	✓	✓	✓	✓	—	—	✓
WashU Epigenome Browser	ENCODE	Webservice	✓	✓	✓	✓	✓	✓	✓	—	—	✓
UCSC Cancer Genomics Browser	UCSC	Webservice	✓	✓	✓	✓	✓	✓	✓	✓	—	—
The Cancer Genome Workbench	TCGA	Webservice	—	✓	✓	✓	✓	✓	✓	—	—	—
EpiExplorer	ENCODE and ROADMAP	Webservice	✓	✓	✓	✓	✓	✓	✓	—	—	—
EpiGRAPH	ENCODE	Webservice	✓	✓	✓	✓	✓	✓	✓	—	—	—
Catalogue of Somatic Mutations in Cancer (COSMIC)	TCGA and ICGC	Webservice	—	✓	—	—	✓	✓	—	—	—	—
PCmtl, MAGIA, miRvar, CoMeTa, etc.*	GEO and TCGA	Webservice	✓	✓	—	✓	—	—	—	✓	—	✓
ICGC	ICGC	Webservice	—	✓	—	✓	✓	✓	—	—	—	—
Genomatix	User defined	Tool	—	✓	—	✓	✓	✓	✓	—	—	✓
Caleydo	TCGA	Tool	—	✓	✓	✓	✓	✓	✓	✓	—	✓
Integrative Genomics Viewer (IGV)	ENCODE	Tool	—	✓	✓	✓	✓	✓	✓	—	—	—
iCluster and iCluster Plus	User defined	Tool	—	✓	—	✓	—	✓	—	—	—	—

* Web Site with links for integrated analysis of microRNA and mRNA expression.
CNV, copy-number variation; ENCODE, Encyclopedia of DNA Elements; ICGC, the International Cancer Genome Consortium; GDAC, Genomic Data Analysis Center; GEO, Gene Expression Omnibus; miRNA, microRNA; SNV, single-nucleotide variation; TCGA, The Cancer Genome Atlas; USCS, University of California, Santa Cruz;
Based on Plass C, Pfister SM, Lindroth AM, et al. Mutations in regulators of the epigenome and their connections to global chromatin patterns in cancer. *Nat Rev Genet* 2013;14:765–780, Table 1.

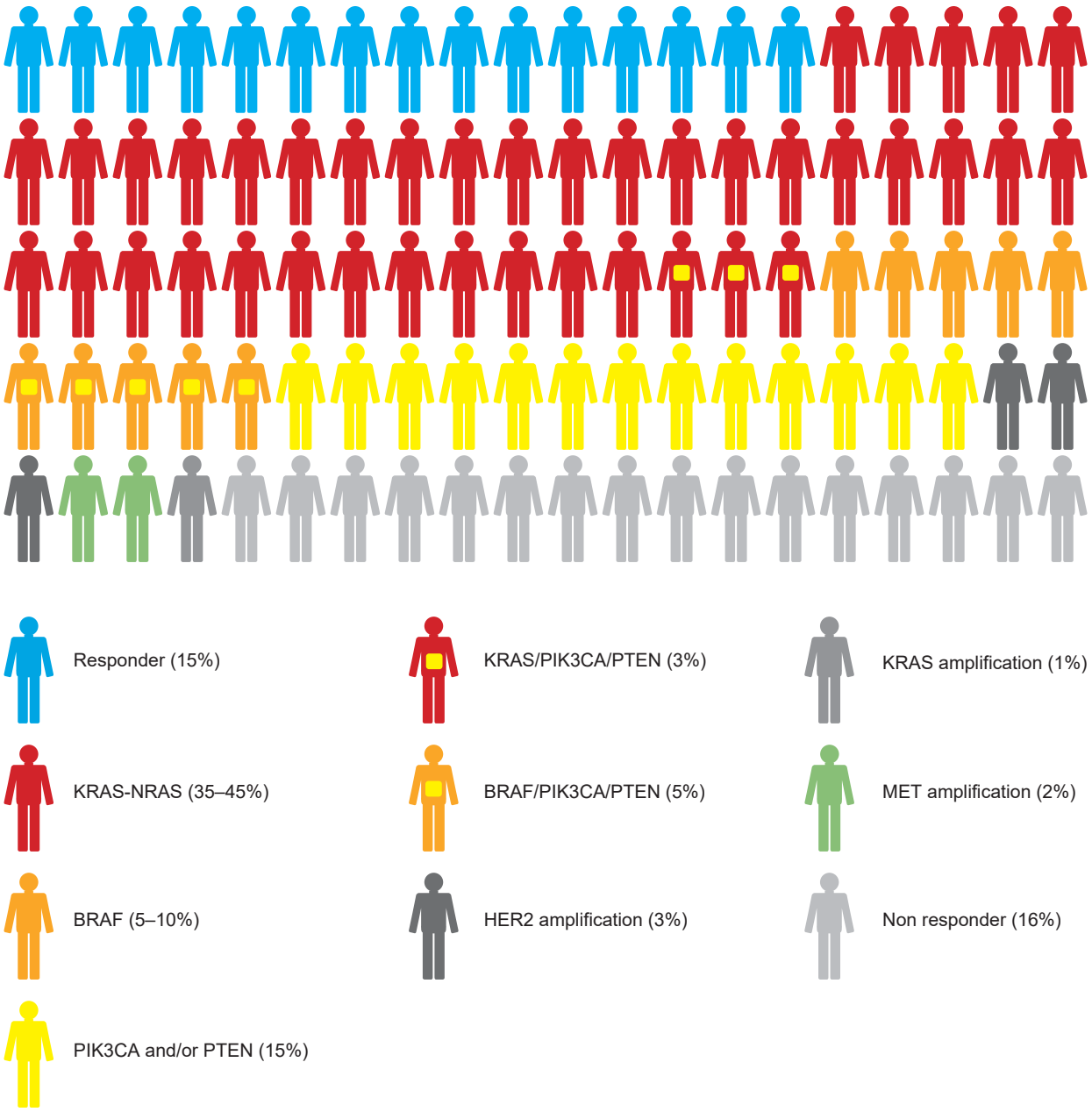

Figure 1.10 Graphic representation of a cohort of 100 patients with colorectal cancer treated with cetuximab or panitumumab. The genetic milieu of individual tumors and their impacts on the clinical response are listed. *KRAS, BRAF,* and *PIK3CA* somatic mutations as well as loss of PTEN protein expression are indicated according to different color codes. Molecular alterations mutually exclusive or coexisting in individual tumors are indicated using different color variants. The relative frequencies at which the molecular alterations occur in colorectal cancers are described. (Redrawn from Bardelli A, Siena S. Molecular mechanisms of resistance to cetuximab and panitumumab in colorectal cancer. *J Clin Oncol* 2010;28:1254–1261.)

that have enabled us to analyze tumor tissues become ever more sophisticated, we have realized the limitations of this approach. As previously discussed, cancers are heterogeneous, with different areas of the same tumor showing different genetic profiles (i.e., intratumoral heterogeneity); likewise, heterogeneity exists between metastases within the same patient (i.e., intermetastatic heterogeneity).[159] A tissue section (or a biopsy) from one part of a solitary tumor will miss the molecular intratumoral as well as intermetastatic heterogeneity. To capture tumor heterogeneity, techniques that are capable of interrogating the genetic landscapes of the overall disease in a single patient are needed.

In 1948, the publication of a manuscript describing the presence of cell-free circulating DNA (cfDNA) in the blood of humans offered—probably without realizing it—unprecedented opportunities in this area.[160] Only recently, the full potential of this seminal discovery has been appreciated. Several groups have reported that the analysis of circulating tumor DNA can, in principle, provide the same genetic information obtained from tumor tissue.[161] The levels of cfDNA are typically higher in cancer patients than healthy individuals, indicating that it is possible to screen for the presence of disease through a simple blood test. Furthermore, the specific detection of tumor-derived cfDNA has been shown to correlate with tumor burden, which changes in response to treatment or surgery.[162–164]

Although the detection of ctDNA has remarkable potential, it is also challenging for several reasons. The first is the need

to discriminate DNA released from tumor cells (ctDNA) from circulating *normal* DNA. Discerning ctDNA from normal cfDNA is aided by the fact that tumor DNA is defined by the presence of mutations. These somatic mutations, commonly single base pair substitutions, are present only in the genomes of cancer cells or precancerous cells and are present in the DNA of normal cells of the same individual. Accordingly, ctDNA offers exquisite specificity as a biomarker. Unfortunately, cfDNA derived from tumor cells often represents a very small fraction (<1%) of the total cfDNA, thus limiting the applicability of the approach. The development and refinement of next-generation sequencing strategies as well as recently developed digital PCR techniques have made it possible to define rare mutant variants in complex mixtures of DNA. Using these approaches, it is possible to detect point mutations, rearrangements, and gene copy number changes in individual genes starting from a few milliliters of plasma.[165] Very recently, several groups have opened a new frontier by showing that exome analyses can also be performed from circulating DNA extracted from the blood of cancer patients.[166]

The detection of tumor-specific genetic alterations in patients' blood (often referred to as *liquid biopsies*) has several applications in the field of oncology, which are summarized as follows. Analyses of cfDNA can be used to genotype tumors when a tissue sample is not available or is difficult to obtain. Circulating tumor DNA fragments contain the identical genetic defects as the tumor themselves, thus the blood can reveal tumor point mutations (*EGFR*, *KRAS*, *BRAF*, *PIK3CA*), rearrangements (e.g., *EML4-ALK*), as well as tumor amplifications (*MET*).[167–169] *Liquid biopsies* may also be useful in monitoring tumor burden—a central aspect in the management of patients with cancer that is typically assessed with imaging. In this regard, several investigational studies have shown that ctDNA can be a surrogate for tumor burden and that, much like viral load changes (e.g., HIV viral load), levels of ctDNA correspond with clinical course. Another application of ctDNA is the detection of minimal residual disease following surgery or therapy with curative intent.[163] Finally, *liquid biopsies* can be used to monitor the genomic drift (clonal evolution) of tumors upon treatment.[166] In this setting, the analysis of ctDNA in plasma samples obtained pretreatment, during, and posttreatment can lead to an understanding of the mechanisms of primary and, especially, acquired resistance to therapies.[170,171]

Importantly, the advances in sequencing technologies have made the idea of personalized treatment of cancer a reality, which is most evident in the field of adoptive cell therapy (ACT). Although already a treatment in use, the ability to use a patient's autologous tumor-infiltrating lymphocytes (TIL) is in position to benefit greatly from advances in sequencing technologies. A recent study demonstrated this when whole-exome data, along with a major histocompatibility complex (MHC)-binding algorithm, were utilized to identify candidate tumor epitopes that are recognized by the patients' TILs.[172] This study should allow for future work in which the information obtained from the direct sequencing of a patient's tumor can quickly be used to generate tumor-reactive T cells that can then be used for a personalized treatment.

In conclusion, the taxonomy of tumors is being rewritten using the presence of genetic lesions as major criteria. Genome-based information will improve the diagnosis and will be used to determine personalized therapeutic regimens based on the genetic landscape of individual tumors.

CANCER GENOMICS AND DRUG RESISTANCE

Cancer genomics has dramatically impacted disease management, because its application is helping researchers determine which patients are likely to benefit from which drug. As discussed in great detail in Chapter 22, good examples for such treatment include targeted therapy using imatinib for chronic myeloid leukemia (CML) patients and the use of gefitinib and erlotinib for NSCLC patients.

The key to the successful development and application of anticancer agents is a better understanding of the effect of the therapeutic regimens and of resistance mechanisms that may develop. In most tumor types, a fraction of patients' tumors are refractory to therapies (intrinsic resistance). Even if an initial response to therapies is obtained, the vast majority of tumors subsequently become refractory (i.e., acquired resistance), and patients eventually succumb to disease progression. Therefore, secondary resistance should be regarded as a key obstacle to treatment progress. The analysis of the cancer genome represents a powerful tool both for the identification of chemotherapeutic signatures as well as to understand resistance mechanisms to therapeutic agents. Examples for each of these are described as follows.

An important application of systematic sequencing experiments is the identification of the effects of chemotherapy on the cancer genome. For example, gliomas that recur after temozolomide treatment have been shown to harbor large numbers of mutations with a signature typical of a DNA alkylating agent.[173,174] Because these alterations were detected using Sanger sequencing, which as described previously has limited sensitivity, the data suggested that the detected alterations were clonal. The model that unfolds from this study indicates that although temozolomide has limited efficacy, almost all of the cells in a glioma respond to the drug. However, a single cell that was resistant to the chemotherapy proliferated and formed a cell clone. Later genomic analyses of the cell clone allowed for the identification of the underlying mutated resistance genes.[173,174]

Single-molecule–targeted therapy is almost always followed by acquired drug resistance.[175–177] Genomic analyses can be successfully exploited to decipher resistance mechanisms to such inhibitors. A few paradigmatic examples are presented as follows, which will be discussed extensively in other chapters. Despite the effectiveness of gefitinib and erlotinib in EGFR mutant cases of NSCLC,[178] drug resistance develops within 6 to 12 months after the initiation of therapy. The underlying reason for this resistance was identified as a secondary mutation in *EGFR* exon 20, T790M, which is detectable in 50% of patients who relapse.[179–181] Importantly, some studies have shown the mutation to be present before the patient was treated with the drug,[182,183] suggesting that exposure to the drug selected for these cells.[184] Because the drug-resistant *EGFR* mutation is structurally analogous to the mutated gatekeeper residue T315I in BCR-ABL, T670I in c-Kit, and L1196M in EML4-ALK, which have been shown previously to confer resistance to imatinib and other kinase inhibitors,[176,185,186] this mechanism of resistance represents a general problem that needs to be overcome.

A recent elegant study, which also represents the use of genomics in understanding drug-resistance mechanisms, focused on the inhibition of activating *BRAF* (V600E) mutations, which occur in 7% of human malignancies and in 60% of melanomas.[9] Clinical trials using PLX4032, a novel class I RAF-selective inhibitor, showed an 80% antitumor response rate in melanoma patients with *BRAF* (V600E) mutations; however, cases of drug resistance were observed.[187] The use of microarray and sequencing technologies showed that, in this case, the resistance was not due to secondary mutations in *BRAF*, but due rather to either upregulation of *PDGFRB* or *NRAS* mutations.[188]

It was, however, the introduction of two anti-EGFR monoclonal antibodies, cetuximab and panitumumab, for the treatment of metastatic colorectal cancer, that provided the largest body of knowledge on the relationship between tumors' genotypes and the response to targeted therapies. The initial clinical analysis

pointed out that only a fraction of metastatic CRC patients benefited from this novel treatment. Different from the NSCLC paradigm, it was found that EGFR mutations do not play a major role in the response. On the contrary, from the initial retrospective analysis, it became clear that somatic *KRAS* mutations, thought to be present in 35% to 45% of metastatic colorectal cancers, are important negative predictors of efficacy in patients who are given panitumumab or cetuximab.[156–158] Among tumors carrying wild-type *KRAS*, mutations of *BRAF* or *PIK3CA*, or a loss of phosphatase and tensin homolog (PTEN) expression may also predict resistance to EGFR-targeted monoclonal antibodies, although the latter biomarkers require further validation before they can be incorporated into clinical practice. From these few examples, it is clear that a future, deeper genomic understanding of targeted drug resistance is crucial to the effective development of additional as well as alternative therapies to overcome this resistance.

PERSPECTIVES OF CANCER GENOME ANALYSIS

The completion of the human genome project has marked a new beginning in biomedical sciences. Because human cancer is a genetic disease, the field of oncology has been one of the first to be impacted by this historic revolution. Knowledge of the sequence and organization of the human genome allows for the systematic analysis of the genetic alterations underlying the origin and evolution of tumors. High-throughput mutational profiling of common tumors, including lung, skin, breast, and colorectal cancers, and the application of next-generation sequencing to whole genome, whole exome, and whole transcriptome of cancer samples has allowed substantial advances in the understanding of this disease by facilitating the detection of all main types of somatic cancer genome alterations. These have also led to historical results, such as the identification of genetic alterations that are likely to be the major drivers of these diseases.

However, the genetic landscape of cancers is by no means complete, and what has been learned so far has raised new and exciting questions that must be addressed. There are still important technical challenges for the detection of somatic mutations.[60] Clinical tumor samples often contain large amounts of nonmalignant cells, which makes the identification of mutations in cancer genomes more challenging when compared with similar analyses of peripheral blood samples for germ-line genome studies. Moreover, the genomic instability inherent to cancer development and progression largely increases the complexity and diversity of genomic alterations of malignant tumors, making it necessary to distinguish between driver and passenger mutations. Likewise, the fact that malignant tumors are genetically heterogeneous and contain several clones simultaneously growing within the same tumor mass raises additional questions regarding the quality of the information currently derived from cancer genomes. Hopefully, in the near future, advances in third-generation sequencing technologies will make it feasible to obtain high-quality sequence data of a genome isolated from a single cell, an aspect of crucial relevance for cancer research.

One of the next imperatives is the definition of the oncogenomic profile of all tumor types. In particular, the less common—although not less lethal—ones are still largely mysterious to scientists and untreatable to clinicians. For some of these diseases, few new therapeutically amenable molecular targets have been discovered in the past years. For example, the identification of drugable genetic lesions associated with pancreatic and ovarian cancers could help define new therapeutic strategies for these aggressive diseases. To achieve this, detailed oncogenomic maps of the corresponding tumors must be drafted. The latter will hopefully be completed in the coming years, thanks to the systematic cancer genome projects that are presently being performed.

Even in the case of common cancers, a lot of genomic profiling efforts still lay ahead. For example, in a significant fraction of breast and lung tumors, the mutations that are likely to be drivers have not yet been found. This is not surprising considering that even in these tumor types only a limited number of samples have been systematically analyzed so far. Therefore, low incidence mutations that could represent potentially key therapeutic targets in a subset of tumors might have escaped detection. Consequently, the scaling up of the mutational profiling to large numbers of specimens for each tumor type is warranted.

Finally, understanding the cellular properties imparted by the hundreds of recently discovered cancer alleles is another area that must be developed. As a matter of fact, compared to the genomic discovery stage, the functional validation of putative novel cancer alleles, despite their potential clinical relevance, is substantially lagging behind. To achieve this, high-throughput functional studies in model systems that accurately recapitulate the genetic alterations found in human cancer must be developed.

To conclude, the eventual goal of profiling the cancer genome is not only to further understand the molecular basis of the disease, but also to discover novel diagnostic and drug targets. One might anticipate that the most immediate application of these new technologies will be noninvasive strategies for early cancer detection. Considering that oncogenic mutations are present only in cancer cells, screening for tumor-derived mutant DNA in patients' blood holds great potential and will progressively substitute current biomarkers, which have poor sensitivity and lack specificity.[171] Further improvements in next-generation sequencing technologies are likely to reduce their cost as well as make these analyses more facile in the future. Once this happens, most cancer patients will undergo in-depth genomic analyses as part of their initial evaluation and throughout their treatment. This will offer more precise diagnostic and prognostic information, which will affect treatment decisions. Although many challenges remain, the information gained from next-generation sequencing platforms is laying a foundation for personalized medicine, in which patients are managed with therapies that are tailored to the specific gene mutations found in their tumors. Ultimately, these should lead to therapeutic successes similar to the ones attained for CML patients with imatinib,[189,190] melanoma patients with PLX4032,[187] and NSCLC patients with gefitinib and erlotinib.[178] Clearly, this is the absolute goal for all of this work.

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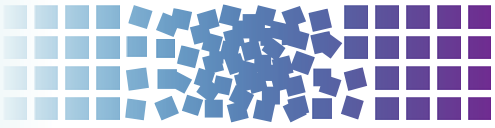
2 Hallmarks of Cancer: An Organizing Principle for Cancer Medicine



Douglas Hanahan and Robert A. Weinberg

INTRODUCTION

The hallmarks of cancer comprise eight biologic capabilities acquired by incipient cancer cells during the multistep development of human tumors. The hallmarks constitute an organizing principle for rationalizing the complexities of neoplastic disease. They include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming energy metabolism, and evading immune destruction. Facilitating the acquisition of these hallmark capabilities are genome instability, which enables mutational alteration of hallmark-enabling genes, and immune inflammation, which fosters the acquisition of multiple hallmark functions. In addition to cancer cells, tumors exhibit another dimension of complexity: They contain a repertoire of recruited, ostensibly normal cells that contribute to the acquisition of hallmark traits by creating the *tumor microenvironment*. Recognition of the widespread applicability of these concepts will increasingly influence the development of new means to treat human cancer.

At the beginning of the new millennium, we proposed that six *hallmarks of cancer* embody an organizing principle that provides a logical framework for understanding the remarkable diversity of neoplastic diseases.[1] Implicit in our discussion was the notion that, as normal cells evolve progressively to a neoplastic state, they acquire a succession of these hallmark capabilities, and that the multistep process of human tumor pathogenesis can be rationalized by the need of incipient cancer cells to acquire the diverse traits that in aggregate enable them to become tumorigenic and, ultimately, malignant.

We noted as an ancillary proposition that tumors are more than insular masses of proliferating cancer cells. Instead, they are complex tissues composed of multiple distinct types of neoplastic and normal cells that participate in heterotypic interactions with one another. We depicted the recruited normal cells, which form tumor-associated stroma, as active participants in tumorigenesis rather than passive bystanders; as such, these stromal cells contribute to the development and expression of certain hallmark capabilities. This notion has been solidified and extended during the intervening period, and it is now clear that the biology of tumors can no longer be understood simply by enumerating the traits of the cancer cells, but instead must encompass the contributions of the *tumor microenvironment* to tumorigenesis. In 2011, we revisited the original hallmarks, adding two new ones to the roster, and expanded on the functional roles and contributions made by recruited stromal cells to tumor biology.[2] Herein we reiterate and further refine the hallmarks-of-cancer perspectives we presented in 2000 and 2011, with the goal of informing students of cancer medicine about the concept and its potential utility for understanding the pathogenesis of human cancer, and the potential relevance of this concept to the development of more effective treatments for this disease.

HALLMARK CAPABILITIES, IN ESSENCE

The eight hallmarks of cancer—distinct and complementary capabilities that enable tumor growth and metastatic dissemination—continue to provide a solid foundation for understanding the biology of cancer (Fig. 2.1). The sections that follow summarize the essence of each hallmark, providing insights into their regulation and functional manifestations.

Sustaining Proliferative Signaling

Arguably, the most fundamental trait of cancer cells involves their ability to sustain chronic proliferation. Normal tissues carefully control the production and release of growth-promoting signals that instruct entry of cells into and progression through the growth-and-division cycle, thereby ensuring proper control of cell number and thus maintenance of normal tissue architecture and function. Cancer cells, by deregulating these signals, become masters of their own destinies. The enabling signals are conveyed in large part by growth factors that bind cell-surface receptors, typically containing intracellular tyrosine kinase domains. The latter proceed to emit signals via branched intracellular signaling pathways that regulate progression through the cell cycle as well as cell growth (that is, increase in cell size); often, these signals influence yet other cell-biologic properties, such as cell survival and energy metabolism.

Remarkably, the precise identities and sources of the proliferative signals operating within normal tissues remain poorly understood. Moreover, we still know relatively little about the mechanisms controlling the release of these mitogenic signals. In part, the study of these mechanisms is complicated by the fact that the growth factor signals controlling cell number and position within normal tissues are thought to be transmitted in a temporally and spatially regulated fashion from one cell to its neighbors; such paracrine signaling is difficult to access experimentally. In addition, the bioavailability of growth factors is regulated by their sequestration in the pericellular space and associated extracellular matrix. Moreover, the actions of these extracellular mitogenic proteins is further controlled by a complex network of proteases, sulfatases, and possibly other enzymes that liberate and activate these factors, apparently in a highly specific and localized fashion.

The mitogenic signaling operating in cancer cells is, in contrast, far better understood.[3–6] Cancer cells can acquire the capability to sustain proliferative signaling in a number of alternative ways: They may produce growth factor ligands themselves, to which they can then respond via the coexpression of cognate receptors, resulting in autocrine proliferative stimulation. Alternatively, cancer cells may send signals to stimulate normal cells within the supporting tumor-associated stroma; the stromal cells then reciprocate by supplying the cancer cells with various growth factors.[7,8] Mitogenic signaling can also be deregulated by elevating the levels of receptor proteins displayed at the cancer cell

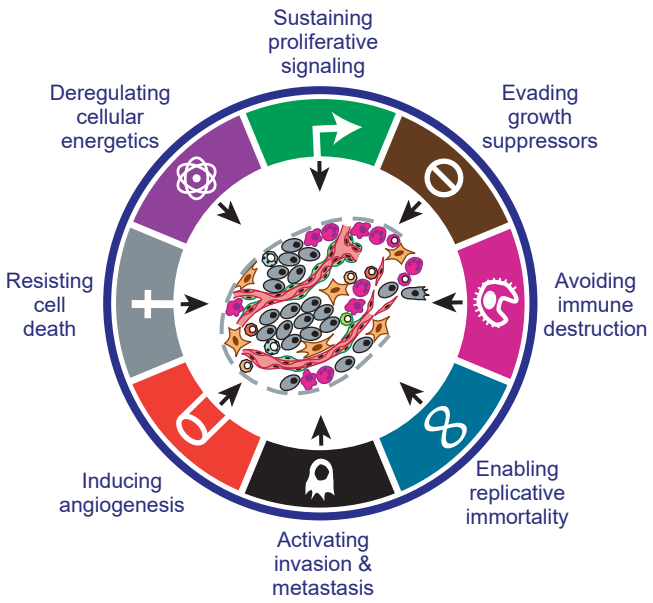


Figure 2.1 The hallmarks of cancer. Eight functional capabilities—the hallmarks of cancer—are thought to be acquired by developing cancers in the course of the multistep carcinogenesis that leads to most forms of human cancer. The order in which these hallmark capabilities are acquired and the relative balance and importance of their contributions to malignant disease appears to vary across the spectrum of human cancers. (Adapted from Hanahan D, Weinberg R. The hallmarks of cancer. *Cell* 2000;100:57–70; Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646–674.)

surface, rendering such cells hyperresponsive to otherwise limiting amounts of growth factor ligands; the same outcome can result from structural alterations in the receptor molecules that facilitate ligand-independent firing.

Independence from externally supplied growth factors may also derive from the constitutive activation of components of intracellular signaling cascades operating downstream of these receptors within cancer cells. These intracellular alterations obviate the need to stimulate cell proliferation pathways by ligand-mediated activation of cell-surface receptors. Of note, because a number of distinct downstream signaling pathways radiate from ligand-stimulated receptors, the activation of one or another of these downstream branches (e.g., the pathway responding to the Ras signal transducer) may only provide a subset of the regulatory instructions transmitted by a ligand-activated receptor.

Somatic Mutations Activate Additional Downstream Pathways

DNA sequencing analyses of cancer cell genomes have revealed somatic mutations in certain human tumors that predict constitutive activation of the signaling circuits, cited previously, that are normally triggered by activated growth factor receptors. The past 3 decades have witnessed the identification in tens of thousands of human tumors of mutant, oncogenic alleles of the *RAS* proto-oncogenes, most of which have sustained point mutations in the 12th codon, which results in RAS proteins that are constitutively active in downstream signaling. Thus, more than 90% of pancreatic adenocarcinomas carry mutant K-*RAS* alleles. More recently, the repertoire of frequently mutated genes has been expanded to include those encoding the downstream effectors of the RAS proteins. For example, we now know that ~40% of human melanomas contain activating mutations affecting the structure of the B-RAF protein, resulting in constitutive signaling through the RAF to the mitogen-activated protein (MAP)–kinase pathway.[9] Similarly, mutations in the catalytic subunit of phosphoinositide 3-kinase (PI3K) isoforms are being detected in an array of tumor types; these mutations typically serve to hyperactivate the PI3K signaling pathway, causing in turn, excess signaling through the crucial Akt/PKB signal transducer.[10,11] The advantages to tumor cells of activating upstream (receptor) versus downstream (transducer) signaling remain obscure, as does the functional impact of cross-talk between the multiple branched pathways radiating from individual growth factor receptors.

Disruptions of Negative-Feedback Mechanisms that Attenuate Proliferative Signaling

Recent observations have also highlighted the importance of negative-feedback loops that normally operate to dampen various types of signaling and thereby ensure homeostatic regulation of the flux of signals coursing through the intracellular circuitry.[12–15] Defects in these negative-feedback mechanisms are capable of enhancing proliferative signaling. The prototype of this type of regulation involves the RAS oncoprotein. The oncogenic effects of mutant RAS proteins do not result from a hyperactivation of its downstream signaling powers; instead, the oncogenic mutations affecting *RAS* genes impair the intrinsic GTPase activity of RAS that normally serves to turn its activity off, ensuring that active signal transmission (e.g., from upstream growth factor receptors) is transient; as such, oncogenic RAS mutations disrupt an autoregulatory negative-feedback mechanism, without which RAS generates chronic proliferative signals.

Analogous negative-feedback mechanisms operate at multiple nodes within the proliferative signaling circuitry. A prominent example involves phosphatase and tensin homolog (PTEN), which counteracts PI3K by degrading its product, phosphatidylinositol 3,4,5-phosphate (PIP_3). Loss-of-function mutations in PTEN amplify PI3K signaling and promote tumorigenesis in a variety of experimental models of cancer; in human tumors, PTEN expression is often lost by the methylation of DNA at specific sites associated with the promoter of the *PTEN* gene, resulting in the shutdown of its transcription.[10,11]

Yet another example involves the mammalian target of rapamycin (mTOR) kinase, a key coordinator of cell growth and metabolism that lies both upstream and downstream of the PI3K pathway. In the circuitry of some cancer cells, mTOR activation results, via negative feedback, in the inhibition of PI3K signaling. Accordingly, when mTOR is pharmacologically inhibited in such cancer cells (e.g., by the drug rapamycin), the associated loss of negative feedback results in increased activity of PI3K and its effector, the Akt/PKB kinase, thereby blunting the antiproliferative effects of mTOR inhibition.[16,17] It is likely that compromised negative feedback loops in this and other signaling pathways will prove to be widespread among human cancer cells, serving as important means by which cancer cells acquire the capability of signaling chronically through these pathways. Moreover, disruption of such normally self-attenuating signaling can contribute to the development of adaptive resistance toward therapeutic drugs targeting mitogenic signaling.

Excessive Proliferative Signaling Can Trigger Cell Senescence

Early studies of oncogene action encouraged the notion that ever-increasing expression of such genes and the signals released by their protein products would result in proportionately increased cancer cell proliferation and, thus, tumor growth. More recent research has undermined this notion, in that it is now apparent that excessively elevated signaling by oncoproteins, such as RAS, MYC, and RAF, can provoke counteracting (protective) responses from cells, such as induction of cell death; alternatively, cancer cells expressing high levels of these oncoproteins may be forced to enter into the nonproliferative but viable state called senescence. These responses contrast with those seen in cells expressing lower levels of these proteins, which permit cells to avoid senescence or cell death and, thus, proliferate.[18–21]

Cells with morphologic features of senescence, including enlarged cytoplasm, the absence of proliferation markers, and the expression of the senescence-induced β-galactosidase enzyme, are abundant in the tissues of mice whose genomes have been reengineered to cause overexpression of certain oncogenes[19,20]; such senescent cells are also prevalent in some cases of human melanoma.[22]

These ostensibly paradoxical responses seem to reflect intrinsic cellular defense mechanisms designed to eliminate cells experiencing excessive levels of certain types of mitogenic signaling. Accordingly, the intensity of oncogenic signaling observed in naturally arising cancer cells may represent compromises between maximal mitogenic stimulation and avoidance of these anti-proliferative defenses. Alternatively, some cancer cells may adapt to high levels of oncogenic signaling by disabling their senescence- or apoptosis-inducing circuitry.

Evading Growth Suppressors

In addition to the hallmark capability of inducing and sustaining positively acting growth-stimulatory signals, cancer cells must also circumvent powerful programs that negatively regulate cell proliferation; many of these programs depend on the actions of tumor suppressor genes. Dozens of tumor suppressors that operate in various ways to limit cell proliferation or survival have been discovered through their inactivation in one or another form of animal or human cancer; many of these genes have been validated as bona fide tumor suppressors through gain- or loss-of-function experiments in mice. The two prototypical tumor suppressor genes encode the retinoblastoma (RB)-associated and TP53 proteins; they operate as central control nodes within two key, complementary cellular regulatory circuits that govern the decisions of cells to proliferate, or alternatively, to activate growth arrest, senescence, or the cell-suicide program known as apoptosis.

The RB protein integrates signals from diverse extracellular and intracellular sources and, in response, decides whether or not a cell should proceed through its growth-and-division cycle.[23–25] Cancer cells with defects in the RB pathway function are thus missing the services of a critical gatekeeper of cell-cycle progression whose absence permits persistent cell proliferation. Whereas RB transduces growth-inhibitory signals that largely originate outside of the cell, TP53 receives inputs from stress and abnormality sensors that function within the cell's intracellular operating systems. For example, if the degree of damage to a cell's genome is excessive, or if the levels of nucleotide pools, growth-promoting signals, glucose, or oxygenation are insufficient, TP53 can call a halt to further cell-cycle progression until these conditions have been normalized. Alternatively, in the face of alarm signals indicating overwhelming or irreparable damage to such cellular systems, TP53 can trigger apoptosis. Of note, the alternative effects of activated TP53 are complex and highly context dependent, varying by cell type as well as by the severity and persistence of conditions of cell-physiologic stress and genomic damage.

Although the two canonical suppressors of proliferation—TP53 and RB—have preeminent importance in regulating cell proliferation, various lines of evidence indicate that each operates as part of a larger network that is wired for functional redundancy. For example, chimeric mice populated throughout their bodies with individual cells lacking a functional *Rb* gene are surprisingly free of proliferative abnormalities, despite the expectation that a loss of RB function should result in unimpeded advance through the cell division cycle by these cells and their lineal descendants; some of the resulting clusters of *Rb*-null cells should, by all rights, progress to neoplasia. Instead, the *Rb*-null cells in such chimeric mice have been found to participate in relatively normal tissue morphogenesis throughout the body; the only neoplasia observed is of pituitary tumors developing late in life.[26] Similarly, *TP53*-null mice develop normally, show largely normal cell and tissue homeostasis, and again develop abnormalities only later in life in the form of leukemias and sarcomas.[27]

Mechanisms of Contact Inhibition and Its Evasion

Four decades of research have demonstrated that the cell-to-cell contacts formed by dense populations of normal cells growing in 2-dimensional culture operate to suppress further cell proliferation, yielding confluent cell monolayers. Importantly, such *contact inhibition* is abolished in various types of cancer cells in culture, suggesting that contact inhibition is an in vitro surrogate of a mechanism that operates in vivo to ensure normal tissue homeostasis that is abrogated during the course of tumorigenesis. Until recently, the mechanistic basis for this mode of growth control remained obscure. Now, however, mechanisms of contact inhibition are beginning to emerge.[28]

One mechanism involves the product of the *NF2* gene, long implicated as a tumor suppressor because its loss triggers a form of human neurofibromatosis. Merlin, the cytoplasmic *NF2* gene product, orchestrates contact inhibition by coupling cell-surface adhesion molecules (e.g., E-cadherin) to transmembrane receptor tyrosine kinases (e.g., the EGF receptor). In so doing, Merlin strengthens the adhesiveness of cadherin-mediated cell-to-cell attachments. Additionally, by sequestering such growth factor receptors, Merlin limits their ability to efficiently emit mitogenic signals.[28–31]

Corruption of the TGF-β Pathway Promotes Malignancy

Transforming growth factor beta (TGF-β is best known for its antiproliferative effects on epithelial cells. The responses of carcinoma cells to TGF-β's proliferation–suppressive effects is now appreciated to be far more elaborate than a simple shutdown of its signaling circuitry.[32–35] In normal cells, exposure to TGF-β blocks their progression through the G1 phase of the cell cycle. In many late-stage tumors, however, TGF-β signaling is redirected away from suppressing cell proliferation and is found instead to activate a cellular program, termed the epithelial-to-mesenchymal transition (EMT), which confers on cancer cells multiple traits associated with high-grade malignancy, as will be discussed in further detail.

Resisting Cell Death

The ability to activate the normally latent apoptotic cell-death program appears to be associated with most types of normal cells throughout the body. Its actions in many if not all multicellular organisms seems to reflect the need to eliminate aberrant cells whose continued presence would otherwise threaten organismic integrity. This rationale explains why cancer cells often, if not invariably, inactivate or attenuate this program during their development.[21,36–38]

Elucidation of the detailed design of the signaling circuitry governing the apoptotic program has revealed how apoptosis is triggered in response to various physiologic stresses that cancer cells experience either during the course of tumorigenesis or as a result of anticancer therapy. Notable among the apoptosis-inducing stresses are signaling imbalances resulting from elevated levels of oncogene signaling and from DNA damage. The regulators of the apoptotic response are divided into two major circuits, one receiving and processing extracellular death-inducing signals (the extrinsic apoptotic program, involving for example the Fas ligand/Fas receptor), and the other sensing and integrating a variety of signals of intracellular origin (the intrinsic program). Each of these circuits culminates in the activation of a normally latent protease (caspase 8 or 9, respectively), which proceeds to initiate a cascade of proteolysis involving effector caspases that are responsible for the execution phase of apoptosis. During this final phase, an apoptotic

cell is progressively disassembled and then consumed, both by its neighbors and by professional phagocytic cells. Currently, the intrinsic apoptotic program is more widely implicated as a barrier to cancer pathogenesis.

The molecular machinery that conveys signals between the apoptotic regulators and effectors is controlled by counterbalancing pro- and antiapoptotic members of the Bcl-2 family of regulatory proteins.[36,37] The archetype, Bcl-2, along with its closest relatives (Bcl-XL, Bcl-W, Mcl-1, A1) are inhibitors of apoptosis, acting in large part by binding to and thereby suppressing two proapoptotic triggering proteins (Bax and Bak); the latter are embedded in the mitochondrial outer membrane. When relieved of inhibition by their antiapoptotic relatives, Bax and Bax disrupt the integrity of the outer mitochondrial membrane, causing the release into the cytosol of proapoptotic signaling proteins, the most important of which is cytochrome C. When the normally sequestered cytochrome C is released, it activates a cascade of cytosolic caspase proteases that proceed to fragment multiple cellular structures, thereby executing the apoptotic death program.[37,39]

Several abnormality sensors have been identified that play key roles in triggering apoptosis.[21,37] Most notable is a DNA damage sensor that acts through the TP53 tumor suppressor[40]; TP53 induces apoptosis by upregulating expression of the proapoptotic, Bcl-2-related Noxa and Puma proteins, doing so in response to substantial levels of DNA breaks and other chromosomal abnormalities. Alternatively, insufficient survival factor signaling (e.g., inadequate levels of interleukin (IL)-3 in lymphocytes or of insulinlike growth factors 1/2 [IGF1/2] in epithelial cells) can elicit apoptosis through another proapoptotic Bcl-2–related protein called Bim. Yet another condition triggering apoptosis involves hyperactive signaling by certain oncoproteins, such as Myc, which acts in part via Bim and other Bcl-2–related proteins.[18,2f,40]

Tumor cells evolve a variety of strategies to limit or circumvent apoptosis. Most common is the loss of TP53 tumor suppressor function, which eliminates this critical damage sensor from the apoptosis-inducing circuitry. Alternatively, tumors may achieve similar ends by increasing the expression of antiapoptotic regulators (Bcl-2, Bcl-XL) or of survival signals (IGF1/2), by downregulating proapoptotic Bcl-2–related factors (Bax, Bim, Puma), or by short-circuiting the extrinsic ligand-induced death pathway. The multiplicity of apoptosis-avoiding mechanisms presumably reflects the diversity of apoptosis-inducing signals that cancer cell populations encounter during their evolution from the normal to the neoplastic state.

Autophagy Mediates Both Tumor Cell Survival and Death

Autophagy represents an important cell-physiologic response that, like apoptosis, normally operates at low, basal levels in cells but can be strongly induced in certain states of cellular stress, the most obvious of which is nutrient deficiency.[41–43] The autophagic program enables cells to break down cellular organelles, such as ribosomes and mitochondria, allowing the resulting catabolites to be recycled and thus used for biosynthesis and energy metabolism. As part of this program, intracellular vesicles (termed autophagosomes) envelope the cellular organelles destined for degradation; the resulting vesicles then fuse with lysosomes in which degradation occurs. In this fashion, low–molecular-weight metabolites are generated that support survival in the stressed, nutrient-limited environments experienced by many cancer cells. When acting in this fashion, autophagy favors cancer cell survival.

However, the autophagy program intersects in more complex ways with the life and death of cancer cells. Like apoptosis, the autophagy machinery has both regulatory and effector components.[41–43] Among the latter are proteins that mediate autophagosome formation and delivery to lysosomes. Of note, recent research has revealed intersections between the regulatory circuits governing autophagy, apoptosis, and cellular homeostasis. For example, the signaling pathway involving PI3K, AKT, and mTOR, which is stimulated by survival signals to block apoptosis, similarly inhibits autophagy; when survival signals are insufficient, the PI3K signaling pathway is downregulated, with the result that autophagy and/or apoptosis may be induced.[41,42,44,45]

Another interconnection between these two programs resides in the Beclin-1 protein, which has been shown by genetic studies to be necessary for the induction of autophagy.[41–44] Beclin-1 is a member of the Bcl-2 family of apoptotic regulatory proteins, and its BH3 domain allows it to bind the Bcl-2/Bcl-XL proteins. Stress sensor–coupled BH3-containing proteins (e.g. Bim, Noxa) can displace Beclin-1 from its association with Bcl-2/Bcl-XL, enabling the liberated Beclin-1 to trigger autophagy, much as they can release proapoptotic Bax and Bak to trigger apoptosis. Hence, stress-transducing Bcl-2–related proteins can induce apoptosis and/or autophagy depending on the physiologic state of the cell.

Genetically altered mice bearing inactivated alleles of the *Beclin-1* gene or of certain other components of the autophagy machinery exhibit increased susceptibility to cancer.[42,46] These results suggest that the induction of autophagy can serve as a barrier to tumorigenesis that may operate independently of or in concert with apoptosis. For example, excessive activation of the autophagy program may cause cells to devour too many of their own critical organelles, such that cell growth and division are crippled. Accordingly, autophagy may represent yet another barrier that needs to be circumvented by incipient cancer cells during multistep tumor development.[41,46]

Perhaps paradoxically, nutrient starvation, radiotherapy, and certain cytotoxic drugs can induce elevated levels of autophagy that apparently protect cancer cells.[45–48] Moreover, severely stressed cancer cells have been shown to shrink via autophagy to a state of reversible dormancy.[46,49] This particular survival response may enable the persistence and eventual regrowth of some late-stage tumors following treatment with potent anticancer agents. Together, observations like these indicate that autophagy can have dichotomous effects on tumor cells and, thus, tumor progression.[46,47] An important agenda for future research will involve clarifying the genetic and cell-physiologic conditions that determine when and how autophagy enables cancer cells to survive or, alternatively, causes them to die.

Necrosis Has Proinflammatory and Tumor-Promoting Potential

In contrast to apoptosis, in which a dying cell contracts into an almost invisible corpse that is soon consumed by its neighbors, necrotic cells become bloated and explode, releasing their contents into the local tissue microenvironment. A body of evidence has shown that cell death by necrosis, like apoptosis, is an organized process under genetic control, rather than being a random and undirected process.[50–52]

Importantly, necrotic cell death releases proinflammatory signals into the surrounding tissue microenvironment, in contrast to apoptosis, which does not. As a consequence, necrotic cells can recruit inflammatory cells of the immune system,[51,53,54] whose dedicated function is to survey the extent of tissue damage and remove associated necrotic debris. In the context of neoplasia, however, multiple lines of evidence indicate that immune inflammatory cells can be actively tumor-promoting by fostering angiogenesis, cancer cell proliferation, and invasiveness (discussed in subsequent sections). Additionally, necrotic cells can release bioactive regulatory factors, such as IL1α, which can directly stimulate neighboring viable cells to proliferate, with the potential, once again, to facilitate neoplastic progression.[53] Consequently, necrotic cell death, while seemingly beneficial in counterbalancing cancer-associated hyperproliferation, may ultimately do more damage to the patient than good.

Enabling Replicative Immortality

Cancer cells require unlimited replicative potential in order to generate macroscopic tumors. This capability stands in marked contrast to the behavior of the cells in most normal cell lineages in the body, which are only able to pass through a limited number of successive cell growth-and-division cycles. This limitation has been associated with two distinct barriers to proliferation: *replicative senescence*, a typically irreversible entrance into a nonproliferative but viable state, and *crisis*, which involves cell death. Accordingly, when cells are propagated in culture, repeated cycles of cell division lead first to induction of replicative senescence and then, for those cells that succeed in circumventing this barrier, to the crisis phase, in which the great majority of cells in the population die. On rare occasion, cells emerge from a population in crisis and exhibit unlimited replicative potential. This transition has been termed immortalization, a trait that most established cell lines possess by virtue of their ability to proliferate in culture without evidence of either senescence or crisis.

Multiple lines of evidence indicate that telomeres protecting the ends of chromosomes are centrally involved in the capability for unlimited proliferation.[55–58] The telomere-associated DNA, composed of multiple tandem hexanucleotide repeats, shortens progressively in the chromosomes of nonimmortalized cells propagated in culture, eventually losing the ability to protect the ends of chromosomal DNA from end-to-end fusions; such aberrant fusions generate unstable dicentric chromosomes, whose resolution during the anaphase of mitosis results in a scrambling of karyotype and entrance into crisis that threatens cell viability. Accordingly, the length of telomeric DNA in a cell dictates how many successive cell generations its progeny can pass through before telomeres are largely eroded and have consequently lost their protective functions.

Telomerase, the specialized DNA polymerase that adds telomere repeat segments to the ends of telomeric DNA, is almost absent in nonimmortalized cells but is expressed at functionally significant levels in the great majority (~90%) of spontaneously immortalized cells, including human cancer cells. By extending telomeric DNA, telomerase is able to counter the progressive telomere erosion that would otherwise occur in its absence. The presence of telomerase activity, either in spontaneously immortalized cells or in the context of cells engineered to express the enzyme, is correlated with a resistance to induction of both senescence and crisis/apoptosis; conversely, the suppression of telomerase activity leads to telomere shortening and to activation of one or the other of these proliferative barriers.

The two barriers to proliferation—replicative senescence and crisis/apoptosis—have been rationalized as crucial anticancer defenses that are hardwired into our cells and are deployed to impede the outgrowth of clones of preneoplastic and, frankly, neoplastic cells. According to this thinking, most incipient neoplasias exhaust their endowment of replicative doublings and are stopped in their tracks by either of these barriers. The eventual immortalization of rare variant cells that proceed to form tumors has been attributed to their ability to maintain telomeric DNA at lengths sufficient to avoid triggering either senescence or apoptosis, which is achieved most commonly by upregulating the expression of telomerase or, less frequently, via an alternative recombination-based (ALT) telomere maintenance mechanism.[59] Hence, telomere shortening has come to be viewed as a clocking device that determines the limited replicative potential of normal cells and, thus, one that must be overcome by cancer cells.

Reassessing Replicative Senescence

The senescent state induced by oncogenes, as described previously, is remarkably similar to that induced when cells are explanted from living tissue and introduced into culture, the latter being the replicative senescence just discussed. Importantly, the concept of replication-induced senescence as a general barrier requires refinement and reformulation. Recent experiments have revealed that the induction of senescence in certain cultured cells can be delayed and possibly eliminated by the use of improved cell culture conditions, suggesting that recently explanted primary cells may be intrinsically able to proliferate unimpeded in culture up the point of crisis and the associated induction of apoptosis triggered by critically shortened telomeres.[60–63] This result indicates that telomere shortening does not necessarily induce senescence prior to crisis. Additional insight comes from experiments in mice engineered to lack telomerase; this work has revealed that shortening telomeres can shunt premalignant cells into a senescent state that contributes (along with apoptosis) to attenuated tumorigenesis in mice genetically destined to develop particular forms of cancer.[58] Such telomerase-null mice with highly eroded telomeres exhibit multiorgan dysfunction and abnormalities that provide evidence of both senescence and apoptosis, perhaps similar to the senescence and apoptosis observed in cell culture.[58,64] Thus, depending on the cellular context, the proliferative barrier of telomere shortening can be manifested by the induction of senescence and/or apoptosis.

Delayed Activation of Telomerase May Both Limit and Foster Neoplastic Progression

There is now evidence that clones of incipient cancer cells in spontaneously arising tumors experience telomere loss-induced crisis relatively early during the course of multistep tumor progression due to their inability to express significant levels of telomerase. Thus, extensively eroded telomeres have been documented in premalignant growths through the use of fluorescence in situ hybridization (FISH), which has also revealed the end-to-end chromosomal fusions that signal telomere failure and crisis.[65,66] These results suggest that such incipient cancer cells have passed through a substantial number of successive telomere-shortening cell divisions during their evolution from fully normal cells of origin. Accordingly, the development of some human neoplasias may be aborted by telomere-induced crisis long before they have progressed to become macroscopic, frankly neoplastic growths.

A quite different situation is observed in cells that have lost the TP53-mediated surveillance of genomic integrity and, thereafter, experience critically eroded telomeres. The loss of the TP53 DNA damage sensor can enable such cells to avoid apoptosis that would otherwise be triggered by the DNA damage resulting from dysfunctional telomeres. Instead, such cells lacking TP53 continue to divide, suffering repeated cycles of interchromosomal fusion and subsequent breakage at mitosis. Such breakage-fusion-bridge (BFB) cycles result in deletions and amplifications of chromosomal segments, evidently serving to mutagenize the genome, thereby facilitating the generation and subsequent clonal selection of cancer cells that have acquired mutant oncogenes and tumor suppressor genes.[58,67] One infers, however, that the clones of cancer cells that survive this telomere collapse must eventually acquire the ability to stabilize and thus protect their telomeres via the activation of telomerase or the ALT mechanism noted previously.

These considerations present an interesting dichotomy: Although dysfunctional telomeres are an evident barrier to chronic proliferation, they can also facilitate the genomic instability that generates hallmark-enabling mutations, as will be discussed further. Both mechanisms may be at play in certain forms of carcinogenesis in the form of transitory telomere deficiency prior to telomere stabilization. Circumstantial support for this concept of transient telomere deficiency in facilitating malignant progression has come from comparative analyses of premalignant and malignant lesions in the human breast.[68,69] The premalignant lesions did not express significant levels of telomerase and were marked by telomere shortening and chromosomal aberrations. In contrast, overt carcinomas exhibited telomerase expression concordantly with the reconstruction of longer telomeres and the fixation of the

aberrant karyotypes that would seem to have been acquired after telomere failure but before the acquisition of telomerase activity. When portrayed in this way, the delayed acquisition of telomerase function serves to generate tumor-promoting mutations, whereas its subsequent expression stabilizes the mutant genome and confers the unlimited replicative capacity that cancer cells require in order to generate clinically apparent tumors.

Inducing Angiogenesis

Like normal tissues, tumors require sustenance in the form of nutrients and oxygen as well as an ability to evacuate metabolic wastes and carbon dioxide. The tumor-associated neovasculature, generated by the process of angiogenesis, addresses these needs. During embryogenesis, the development of the vasculature involves the birth of new endothelial cells and their assembly into tubes (vasculogenesis) in addition to the sprouting (angiogenesis) of new vessels from existing ones. Following this morphogenesis, the normal vasculature becomes largely quiescent. In the adult, as part of physiologic processes such as wound healing and female reproductive cycling, angiogenesis is turned on, but only transiently. In contrast, during tumor progression, an *angiogenic switch* is almost always activated and remains on, causing normally quiescent vasculature to continually sprout new vessels that help sustain expanding neoplastic growths.[70]

A compelling body of evidence indicates that the angiogenic switch is governed by countervailing factors that either induce or oppose angiogenesis.[71,72] Some of these angiogenic regulators are signaling proteins that bind to stimulatory or inhibitory cell-surface receptors displayed by vascular endothelial cells. The well-known prototypes of angiogenesis inducers and inhibitors are vascular endothelial growth factor-A (VEGF-A) and thrombospondin-1 (Tsp-1), respectively.

The VEGF-A gene encodes ligands that are involved in orchestrating new blood vessel growth during embryonic and postnatal development, in the survival of endothelial cells in already-formed vessels, and in certain physiologic and pathologic situations in the adult. VEGF signaling via three receptor tyrosine kinases (VEGFR1–3) is regulated at multiple levels, reflecting this complexity of purpose. VEGF gene expression can be upregulated both by hypoxia and by oncogene signaling.[73–75] Additionally, VEGF ligands can be sequestered in the extracellular matrix in latent forms that are subject to release and activation by extracellular matrix-degrading proteases (e.g., matrix metallopeptidase 9 [MMP-9]).[76] In addition, other proangiogenic proteins, such as members of the fibroblast growth factor (FGF) family, have been implicated in sustaining tumor angiogenesis.[71] TSP-1, a key counterbalance in the angiogenic switch, also binds transmembrane receptors displayed by endothelial cells and thereby triggers suppressive signals that can counteract proangiogenic stimuli.[77]

The blood vessels produced within tumors by an unbalanced mix of proangiogenic signals are typically aberrant: Tumor neovasculature is marked by precocious capillary sprouting, convoluted and excessive vessel branching, distorted and enlarged vessels, erratic blood flow, microhemorrhaging, leaking of plasma into the tissue parenchyma, and abnormal levels of endothelial cell proliferation and apoptosis.[78,79]

Angiogenesis is induced surprisingly early during the multistage development of invasive cancers both in animal models and in humans. Histologic analyses of premalignant, noninvasive lesions, including dysplasias and in situ carcinomas arising in a variety of organs, have revealed the early tripping of the angiogenic switch.[70,80] Historically, angiogenesis was envisioned to be important only when rapidly growing macroscopic tumors had formed, but more recent data indicate that angiogenesis also contributes to the microscopic premalignant phase of neoplastic progression, further cementing its status as an integral hallmark of cancer.

Gradations of the Angiogenic Switch

Once angiogenesis has been activated, tumors exhibit diverse patterns of neovascularization. Some tumors, including highly aggressive types such as pancreatic ductal adenocarcinomas, are hypovascularized and replete with stromal deserts that are largely avascular and indeed may even be actively antiangiogenic.[81] In contrast, many other tumors, including human renal and pancreatic neuroendocrine carcinomas, are highly angiogenic and, consequently, densely vascularized.[82,83]

Collectively, such observations suggest an initial tripping of the angiogenic switch during tumor development, which is followed by a variable intensity of ongoing neovascularization, the latter being controlled by a complex biologic rheostat that involves both the cancer cells and the associated stromal microenvironment.[71,72] Of note, the switching mechanisms can vary, even though the net result is a common inductive signal (e.g., VEGF). In some tumors, dominant oncogenes operating within tumor cells, such as *Ras* and *Myc*, can upregulate the expression of angiogenic factors, whereas in others, such inductive signals are produced indirectly by immune inflammatory cells, as will be discussed.

Endogenous Angiogenesis Inhibitors Present Natural Barriers to Tumor Angiogenesis

A variety of secreted proteins have been reported to have the capability to help shut off normally transitory angiogenesis, including thrombospondin-1 (TSP-1), fragments of plasmin (angiostatin) and type 18 collagen (endostatin), along with another dozen candidate antiangiogenic proteins.[77,84–88] Most are proteins, and many are derived by proteolytic cleavage of structural proteins that are not themselves angiogenic regulators.

A number of these endogenous inhibitors of angiogenesis can be detected in the circulation of normal mice and humans. Genes that encode several endogenous angiogenesis inhibitors have been deleted from the mouse germ line without untoward developmental or physiologic effects; however, the growth of autochthonous and implanted tumors is enhanced as a consequence.[84,85,88] By contrast, if the circulating levels of an endogenous inhibitor are genetically increased (e.g., via overexpression in transgenic mice or in xenotransplanted tumors), tumor growth is impaired.[85,88] Interestingly, wound healing and fat deposition are impaired or accelerated by elevated or ablated expression of such genes.[89,90] The data suggest that, under normal conditions, endogenous angiogenesis inhibitors serve as physiologic regulators modulating the transitory angiogenesis that occurs during tissue remodeling and wound healing; they may also act as intrinsic barriers to the induction and/or persistence of angiogenesis by incipient neoplasias.

Pericytes Are Important Components of the Tumor Neovasculature

Pericytes have long been known as supporting cells that are closely apposed to the outer surfaces of the endothelial tubes in normal tissue vasculature, where they provide important mechanical and physiologic support to the endothelial cells. Microscopic studies conducted in recent years have revealed that pericytes are associated, albeit loosely, with the neovasculature of most, if not all, tumors.[91–93] More importantly, mechanistic studies (discussed subsequently) have revealed that pericyte coverage is important for the maintenance of a functional tumor neovasculature.

A Variety of Bone Marrow-Derived Cells Contribute to Tumor Angiogenesis

It is now clear that a repertoire of cell types originating in the bone marrow play crucial roles in pathologic angiogenesis.[94–97] These include cells of the innate immune system—notably, macrophages, neutrophils, mast cells, and myeloid progenitors—that assemble

at the margins of such lesions or infiltrate deeply within them; the tumor-associated inflammatory cells can help to trip the angiogenic switch in quiescent tissue and sustain ongoing angiogenesis associated with tumor growth. In addition, they can help protect the vasculature from the effects of drugs targeting endothelial cell signaling.[98] Moreover, several types of bone marrow–derived *vascular progenitor cells* have been observed to have migrated into neoplastic lesions and become intercalated into the existing neovasculature, where they assumed the roles of either pericytes or endothelial cells.[92,99,100]

Activating Invasion and Metastasis

The multistep process of invasion and metastasis has been schematized as a sequence of discrete steps, often termed the invasion–metastasis cascade.[101,102] This depiction portrays a succession of cell-biologic changes, beginning with local invasion, then intravasation by cancer cells into nearby blood and lymphatic vessels, transit of cancer cells through the lymphatic and hematogenous systems, followed by the escape of cancer cells from the lumina of such vessels into the parenchyma of distant tissues (extravasation), the formation of small nests of cancer cells (micrometastases), and finally, the growth of micrometastatic lesions into macroscopic tumors, this last step being termed *colonization*. These steps have largely been studied in the context of carcinoma pathogenesis. Indeed, when viewed through the prism of the invasion–metastasis cascade, the diverse tumors of this class appear to behave in similar ways.

During the malignant progression of carcinomas, the neoplastic cells typically develop alterations in their shape as well as their attachment to other cells and to the extracellular matrix (ECM). The best-characterized alteration involves the loss by carcinoma cells of E-cadherin, a key epithelial cell-to-cell adhesion molecule. By forming adherens junctions between adjacent epithelial cells, E-cadherin helps to assemble epithelial cell sheets and to maintain the quiescence of the cells within these sheets. Moreover, increased expression of E-cadherin has been well established as an antagonist of invasion and metastasis, whereas a reduction of its expression is known to potentiate these behaviors. The frequently observed downregulation and occasional mutational inactivation of the E-cadherin–encoding gene, *CDH1*, in human carcinomas provides strong support for its role as a key suppressor of the invasion–metastasis hallmark capability.[103,104]

Notably, the expression of genes encoding other cell-to-cell and cell-to-ECM adhesion molecules is also significantly altered in the cells of many highly aggressive carcinomas, with those favoring cytostasis typically being downregulated. Conversely, adhesion molecules normally associated with the cell migrations that occur during embryogenesis and inflammation are often upregulated. For example, N-cadherin, which is normally expressed in migrating neurons and mesenchymal cells during organogenesis, is upregulated in many invasive carcinoma cells, replacing the previously expressed E-cadherin.[104]

Research into the capability for invasion and metastasis has accelerated dramatically over the past decade as powerful new research tools, and refined experimental models have become available. Although still an emerging field replete with major unanswered questions, significant progress has been made in delineating important features of this complex hallmark capability. An admittedly incomplete representation of these advances is highlighted as follows.

The Epithelial-to-Mesenchymal Transition Program Broadly Regulates Invasion and Metastasis

A developmental regulatory program, termed the EMT, has become implicated as a prominent means by which neoplastic epithelial cells can acquire the abilities to invade, resist apoptosis, and disseminate.[105–110] By co-opting a process involved in various steps of embryonic morphogenesis and wound healing, carcinoma cells can concomitantly acquire multiple attributes that enable invasion and metastasis. This multifaceted EMT program can be activated transiently or stably, and to differing degrees, by carcinoma cells during the course of invasion and metastasis.

A set of pleiotropically acting transcriptional factors (TF), including Snail, Slug, Twist, and Zeb1/2, orchestrate the EMT and related migratory processes during embryogenesis; most were initially identified by developmental genetics. These transcriptional regulators are expressed in various combinations in a number of malignant tumor types. Some of these EMT-TFs have been shown in experimental models of carcinoma formation to be causally important for programming invasion; others have been found to elicit metastasis when experimentally expressed in primary tumor cells.[105,111–114] Included among the cell-biologic traits evoked by these EMT-TFs are loss of adherens junctions and associated conversion from a polygonal/epithelial to a spindly/fibroblastic morphology, concomitant with expression of secreted matrix-degrading enzymes, increased motility, and heightened resistance to apoptosis, which are implicated in the processes of invasion and metastasis. Several of these transcription factors can directly repress E-cadherin gene expression, thereby releasing neoplastic epithelial cells from this key suppressor of motility and invasiveness.[115]

The available data suggest that EMT-TFs regulate one another as well as overlapping sets of target genes. Results from developmental genetics indicate that contextual signals received from neighboring cells in the embryo are involved in triggering expression of these transcription factors in cells that are destined to pass through an EMT[111]; in an analogous fashion, heterotypic interactions of cancer cells with adjacent tumor-associated stromal cells have been shown to induce expression of the malignant cell phenotypes that are known to be choreographed by one or more of these EMT-TFs.[116,117] Moreover, cancer cells at the invasive margins of certain carcinomas can be seen to have undergone an EMT, suggesting that these cancer cells are subject to microenvironmental stimuli distinct from those received by cancer cells located in the cores of these lesions.[118] Although the evidence is still incomplete, it would appear that EMT-TFs are able to orchestrate most steps of the invasion–metastasis cascade, except perhaps the final step of colonization, which involves adaptation of cells originating in one tissue to the microenvironment of a foreign, potentially inhospitable tissue.

We still know rather little about the various manifestations and temporal stability of the mesenchymal state produced by an EMT. Indeed, it seems increasingly likely that many human carcinoma cells only experience a *partial EMT*, in which they acquire mesenchymal markers while retaining many preexisting epithelial ones. Although the expression of EMT-TFs has been observed in certain nonepithelial tumor types, such as sarcomas and neuroectodermal tumors, their roles in programming malignant traits in these tumors are presently poorly documented. Additionally, it remains to be determined whether aggressive carcinoma cells invariably acquire their malignant capabilities through activation of components of the EMT program, or whether alternative regulatory programs can also enable expression of these traits.

Heterotypic Contributions of Stromal Cells to Invasion and Metastasis

As mentioned previously, cross-talk between cancer cells and cell types of the neoplastic stroma is involved in the acquired capabilities of invasiveness and metastasis.[94,119–121] For example, mesenchymal stem cells (MSC) present in the tumor stroma have been found to secrete CCL5/RANTES in response to signals released by cancer cells; CCL5 then acts reciprocally on the cancer cells to stimulate invasive behavior.[122] In other work, carcinoma cells secreting IL-1 have been shown to induce MSCs to synthesize a spectrum of other cytokines that proceed thereafter to promote activation of the EMT program in the carcinoma cells; these

effectors include IL-6, IL-8, growth-regulated oncogene alpha (GRO-α), and prostaglandin E2.[123]

Macrophages at the tumor periphery can foster local invasion by supplying matrix-degrading enzymes such as metalloproteinases and cysteine cathepsin proteases[76,120,124,125]; in one model system, the invasion-promoting macrophages are activated by IL-4 produced by the cancer cells.[126] And in an experimental model of metastatic breast cancer, tumor-associated macrophages (TAM) supply epidermal growth factor (EGF) to breast cancer cells, while the cancer cells reciprocally stimulate the macrophages with colony stimulating factor 1 (CSF-1). Their concerted interactions facilitate intravasation into the circulatory system and metastatic dissemination of the cancer cells.[94,127]

Observations like these indicate that the phenotypes of high-grade malignancy do not arise in a strictly cell-autonomous manner, and that their manifestation cannot be understood solely through analyses of signaling occurring within tumor cells. One important implication of the EMT model, still untested, is that the ability of carcinoma cells in primary tumors to negotiate most of the steps of the invasion–metastasis cascade may be acquired in certain tumors without the requirement that these cells undergo additional mutations beyond those that were needed for primary tumor formation.

Plasticity in the Invasive Growth Program

The role of contextual signals in inducing an invasive growth capability (often via an EMT) implies the possibility of reversibility, in that cancer cells that have disseminated from a primary tumor to more distant tissue sites may no longer benefit from the activated stroma and the EMT-inducing signals that they experienced while residing in the primary tumor. In the absence of ongoing exposure to these signals, carcinoma cells may revert in their new tissue environment to a noninvasive state. Thus, carcinoma cells that underwent an EMT during initial invasion and metastatic dissemination may reverse this metamorphosis, doing so via a mesenchymal-to-epithelial transition (MET). This plasticity may result in the formation of new tumor colonies of carcinoma cells exhibiting an organization and histopathology similar to those created by carcinoma cells in the primary tumor that never experienced an EMT.[128]

Distinct Forms of Invasion May Underlie Different Cancer Types

The EMT program regulates a particular type of invasiveness that has been termed *mesenchymal*. In addition, two other distinct modes of invasion have been identified and implicated in cancer cell invasion.[129,130] *Collective invasion* involves phalanxes of cancer cells advancing en masse into adjacent tissues and is characteristic of, for example, squamous cell carcinomas. Interestingly, such cancers are rarely metastatic, suggesting that this form of invasion lacks certain functional attributes that facilitate metastasis. Less clear is the prevalence of an *amoeboid* form of invasion,[131,132] in which individual cancer cells show morphologic plasticity, enabling them to slither through existing interstices in the ECM rather than clearing a path for themselves, as occurs in both the mesenchymal and collective forms of invasion. It is presently unresolved whether cancer cells participating in the collective and amoeboid forms of invasion employ components of the EMT program, or whether entirely different cell-biologic programs are responsible for choreographing these alternative invasion programs.

Another emerging concept, noted previously, involves the facilitation of cancer cell invasion by inflammatory cells that assemble at the boundaries of tumors, producing the ECM-degrading enzymes and other factors that enable invasive growth.[76,94,120,133] These functions may obviate the need of invading cancer cells to produce these proteins through activation of EMT programs. Thus, rather than synthesizing these proteases themselves, cancer cells may secrete chemoattractants that recruit proinvasive inflammatory cells; the latter then proceed to produce matrix-degrading enzymes that enable invasive growth.

The Daunting Complexity of Metastatic Colonization

Metastasis can be broken down into two major phases: the physical dissemination of cancer cells from the primary tumor to distant tissues, and the adaptation of these cells to foreign tissue microenvironments that results in successful colonization (i.e., the growth of micrometastases into macroscopic tumors). The multiple steps of dissemination would seem to lie within the purview of the EMT and similarly acting migratory programs. Colonization, however, is not strictly coupled with physical dissemination, as evidenced by the presence in many patients of myriad micrometastases that have disseminated but never progress to form macroscopic metastatic tumors.[101,102,134–136]

In some types of cancer, the primary tumor may release systemic suppressor factors that render such micrometastases dormant, as revealed clinically by explosive metastatic growth soon after resection of the primary growth.[87,137] In others, however, such as breast cancer and melanoma, macroscopic metastases may erupt decades after a primary tumor has been surgically removed or pharmacologically destroyed. These metastatic tumor growths evidently reflect dormant micrometastases that have solved, after much trial and error, the complex problem of adaptation to foreign tissue microenvironments, allowing subsequent tissue colonization.[135,136,138] Implicit here is the notion that most disseminated cancer cells are likely to be poorly adapted, at least initially, to the microenvironment of the tissue in which they have landed. Accordingly, each type of disseminated cancer cell may need to develop its own set of ad hoc solutions to the problem of thriving in the microenvironment of one or another foreign tissue.[139]

One can infer from such natural histories that micrometastases may lack certain hallmark capabilities necessary for vigorous growth, such as the ability to activate angiogenesis. Indeed, the inability of certain experimentally generated dormant micrometastases to form macroscopic tumors has been ascribed to their failure to activate tumor angiogenesis.[135,140] Additionally, recent experiments have shown that nutrient starvation can induce intense autophagy that causes cancer cells to shrink and adopt a state of reversible dormancy. Such cells may exit this state and resume active growth and proliferation when permitted by changes in tissue microenvironment, such as increased availability of nutrients, inflammation from causes such as infection or wound healing, or other local abnormalities.[49,141] Other mechanisms of micrometastatic dormancy may involve antigrowth signals embedded in normal tissue ECM[138] and tumor-suppressing actions of the immune system.[135,142]

Metastatic dissemination has long been depicted as the last step in multistep primary tumor progression; indeed, for many tumors, that is likely the case, as illustrated by recent genome sequencing studies that provide genetic evidence for clonal evolution of pancreatic ductal adenocarcinoma to a metastatic stage.[143–145] Importantly, however, recent results have revealed that some cancer cells can disseminate remarkably early, dispersing from apparently noninvasive premalignant lesions in both mice and humans.[146,147] Additionally, micrometastases can be spawned from primary tumors that are not obviously invasive but possess a neovasculature lacking in luminal integrity.[148] Although cancer cells can clearly disseminate from such preneoplastic lesions and seed the bone marrow and other tissues, their capability to colonize these sites and develop into pathologically significant macrometastases remains unproven. At present, we view this early metastatic dissemination as a demonstrable phenomenon in mice and humans, the clinical significance of which is yet to be established.

Having developed such a tissue-specific colonizing ability, the cells in metastatic colonies may proceed to disseminate further, not only to new sites in the body, but also back to the primary

tumors in which their ancestors arose. Accordingly, tissue-specific colonization programs that are evident among certain cells within a primary tumor may originate not from classical tumor progression occurring entirely within the primary lesion, but instead from immigrants that have returned home.[149] Such reseeding is consistent with the aforementioned studies of human pancreatic cancer metastasis.[143–145] Stated differently, the phenotypes and underlying gene expression programs in focal subpopulations of cancer cells within primary tumors may reflect, in part, the reverse migration of their distant metastatic progeny.

Implicit in this *self-seeding* process is another notion: The supportive stroma that arises in a primary tumor and contributes to its acquisition of malignant traits provides a hospitable site for reseeding and colonization by circulating cancer cells released from metastatic lesions.

Clarifying the regulatory programs that enable metastatic colonization represents an important agenda for future research. Substantial progress is being made, for example, in defining sets of genes (*metastatic signatures*) that correlate with and appear to facilitate the establishment of macroscopic metastases in specific tissues.[139,146,150–152] Importantly, metastatic colonization almost certainly requires the establishment of a permissive tumor microenvironment composed of critical stromal support cells. For these reasons, the process of colonization is likely to encompass a large number of cell-biologic programs that are, in aggregate, considerably more complex and diverse than the preceding steps of metastatic dissemination that allow carcinoma cells to depart from primary tumors to sites of lodging and extravasation throughout the body.

Reprogramming Energy Metabolism

The chronic and often uncontrolled cell proliferation that represents the essence of neoplastic disease involves not only deregulated control of cell proliferation but also corresponding adjustments of energy metabolism in order to fuel cell growth and division. Under aerobic conditions, normal cells process glucose, first to pyruvate via glycolysis in the cytosol and thereafter via oxidative phosphorylation to carbon dioxide in the mitochondria. Under anaerobic conditions, glycolysis is favored and relatively little pyruvate is dispatched to the oxygen-consuming mitochondria. Otto Warburg first observed an anomalous characteristic of cancer cell energy metabolism[153–155]: Even in the presence of oxygen, cancer cells can reprogram their glucose metabolism, and thus their energy production, leading to a state that has been termed *aerobic glycolysis*.

The existence of this metabolic specialization operating in cancer cells has been substantiated in the ensuing decades. A key signature of aerobic glycolysis is upregulation of glucose transporters, notably GLUT1, which substantially increases glucose import into the cytoplasm.[156–158] Indeed, markedly increased uptake and utilization of glucose has been documented in many human tumor types, most readily by noninvasively visualizing glucose uptake using positron-emission tomography (PET) with a radiolabeled analog of glucose (^{18}F-fluorodeoxyglucose [FDG]) as a reporter.

Glycolytic fueling has been shown to be associated with activated oncogenes (e.g., *RAS*, *MYC*) and mutant tumor suppressors (e.g., *TP53*),[18,156,157,159] whose alterations in tumor cells have been selected primarily for their benefits in conferring the hallmark capabilities of cell proliferation, subversion of cytostatic controls, and attenuation of apoptosis. This reliance on glycolysis can be further accentuated under the hypoxic conditions that operate within many tumors: The hypoxia response system acts pleiotropically to upregulate glucose transporters and multiple enzymes of the glycolytic pathway.[156,157,160] Thus, both the Ras oncoprotein and hypoxia can independently increase the levels of the HIF1α and HIF2α hypoxia-response transcription factors, which in turn upregulate glycolysis.[160–162]

The reprogramming of energy metabolism is seemingly counterintuitive, in that cancer cells must compensate for the ~18-fold lower efficiency of ATP production afforded by glycolysis relative to mitochondrial oxidative phosphorylation. According to one long-forgotten[163] and a recently revived and refined hypothesis,[164] increased glycolysis allows the diversion of glycolytic intermediates into various biosynthetic pathways, including those generating nucleosides and amino acids. In turn, this facilitates the biosynthesis of the macromolecules and organelles required for assembling new cells. Moreover, Warburg-like metabolism seems to be present in many rapidly dividing embryonic tissues, once again suggesting a role in supporting the large-scale biosynthetic programs that are required for active cell proliferation.

Interestingly, some tumors have been found to contain two subpopulations of cancer cells that differ in their energy-generating pathways. One subpopulation consists of glucose-dependent (Warburg-effect) cells that secrete lactate, whereas cells of the second subpopulation preferentially import and utilize the lactate produced by their neighbors as their main energy source, employing part of the citric acid cycle to do so.[165–168] These two populations evidently function symbiotically: The hypoxic cancer cells depend on glucose for fuel and secrete lactate as waste, which is imported and preferentially used as fuel by their better oxygenated brethren. Although this provocative mode of intratumoral symbiosis has yet to be generalized, the cooperation between lactate-secreting and lactate-utilizing cells to fuel tumor growth is in fact not an invention of tumors, but rather again reflects the co-opting of a normal physiologic mechanism, in this case one operative in muscle[165,167,168] and the brain.[169] Additionally, it is becoming apparent that oxygenation, ranging from normoxia to hypoxia, is not necessarily static in tumors, but instead fluctuates temporally and regionally,[170] likely as a result of the instability and chaotic organization of the tumor-associated neovasculature.

Finally, the notion of the Warburg effect needs to be refined for most if not all tumors exhibiting aerobic glycolysis. The effect does not involve a switching off oxidative phosphorylation concurrent with activation of glycolysis, the latter then serving as the sole source of energy. Rather, cancer cells become highly adaptive, utilizing both mitochondrial oxidative phosphorylation and glycolysis in varying proportions to generate fuel (ATP) and biosynthetic precursors needed for chronic cell proliferation. Finally, this capability for reprograming energy metabolism, dubbed to be an *emerging hallmark* in 2011,[2] is clearly intertwined with the hallmarks conveying deregulated proliferative signals and evasion of growth suppressors, as discussed earlier. As such, its status as a discrete, independently acquired hallmark remains unclear, despite growing appreciation of its importance as a crucial component of the neoplastic growth state.

Evading Immune Destruction

The eighth hallmark reflects the role played by the immune system in antagonizing the formation and progression of tumors. A long-standing theory of immune surveillance posited that cells and tissues are constantly monitored by an ever alert immune system, and that such immune surveillance is responsible for recognizing and eliminating the vast majority of incipient cancer cells and, thus, nascent tumors.[171,172] According to this logic, clinical detectable cancers have somehow managed to avoid detection by the various arms of the immune system, or have been able to limit the extent of immunologic killing, thereby evading eradication.

The role of defective immunologic monitoring of tumors would seem to be validated by the striking increases of certain cancers in immune-compromised individuals.[173] However, the great majority of these are virus-induced cancers, suggesting that much of the control of this class of cancers normally depends on reducing viral burden in infected individuals, in part through eliminating virus-infected cells. These observations, therefore, shed little light on

the possible role of the immune system in limiting formation of the >80% of tumors of nonviral etiology. In recent years, however, an increasing body of evidence, both from genetically engineered mice and from clinical epidemiology, suggests that the immune system operates as a significant barrier to tumor formation and progression, at least in some forms of non–virus-induced cancer.[174–177]

When mice genetically engineered to be deficient for various components of the immune system were assessed for the development of carcinogen-induced tumors, it was observed that tumors arose more frequently and/or grew more rapidly in the immunodeficient mice relative to immune-competent controls. In particular, deficiencies in the development or function of either $CD8^+$ cytotoxic T lymphocytes (CTL), $CD4^+$ T_H1 helper T cells, or natural killer (NK) cells, each led to demonstrable increases in tumor incidence. Moreover, mice with combined immunodeficiencies in both T cells and NK cells were even more susceptible to cancer development. The results indicated that, at least in certain experimental models, both the innate and adaptive cellular arms of the immune system are able to contribute significantly to immune surveillance and, thus, tumor eradication.[142,178]

In addition, transplantation experiments have shown that cancer cells that originally arose in immunodeficient mice are often inefficient at initiating secondary tumors in syngeneic immunocompetent hosts, whereas cancer cells from tumors arising in immunocompetent mice are equally efficient at initiating transplanted tumors in both types of hosts.[142,178] Such behavior has been interpreted as follows: Highly immunogenic cancer cell clones are routinely eliminated in immunocompetent hosts—a process that has been referred to as *immunoediting*—leaving behind only weakly immunogenic variants to grow and generate solid tumors. Such weakly immunogenic cells can thereafter successfully colonize both immunodeficient and immunocompetent hosts. Conversely, when arising in immunodeficient hosts, the immunogenic cancer cells are not selectively depleted and can, instead, prosper along with their weakly immunogenic counterparts. When cells from such nonedited tumors are serially transplanted into syngeneic recipients, the immunogenic cancer cells are rejected when they confront, for the first time, the competent immune systems of their secondary hosts.[179] (Unanswered in these particular experiments is the question of whether the chemical carcinogens used to induce such tumors are prone to generate cancer cells that are especially immunogenic.)

Clinical epidemiology also increasingly supports the existence of antitumoral immune responses in some forms of human cancer.[180–182] For example, patients with colon and ovarian tumors that are heavily infiltrated with CTLs and NK cells have a better prognosis than those who lack such abundant killer lymphocytes.[176,177,182,183] The case for other cancers is suggestive but less compelling and is the subject of ongoing investigation. Additionally, some immunosuppressed organ transplant recipients have been observed to develop donor-derived cancers, suggesting that in ostensibly tumor-free organ donors, the cancer cells were held in check in a dormant state by a functional immune system,[184] only to launch into proliferative expansion once these *passenger cells* in the transplanted organ found themselves in immunocompromised patients who lack the physiologically important capabilities to mount immune responses that would otherwise hold latent cancer cells in check or eradicate them.

Still, the epidemiology of chronically immunosuppressed patients does not indicate significantly increased incidences of the major forms of nonviral human cancers, as noted previously. This might be taken as an argument against the importance of immune surveillance as an effective barrier to tumorigenesis and tumor progression. We note, however, that HIV and pharmacologically immunosuppressed patients are predominantly immunodeficient in the T- and B-cell compartments and thus do not present with the multicomponent immunologic deficiencies that have been produced in the genetically engineered mutant mice lacking both NK cells and CTLs. This leaves open the possibility that such patients still have residual capability for mounting an anticancer immunologic defense that is mediated by NK and other innate immune cells.

In truth, the previous discussions of cancer immunology simplify tumor–host immunologic interactions, because highly immunogenic cancer cells may well succeed in evading immune destruction by disabling components of the immune system that have been dispatched to eliminate them. For example, cancer cells may paralyze infiltrating CTLs and NK cells by secreting TGF-β or other immunosuppressive factors.[32,185,186] Alternatively, cancer cells may express immunosuppressive cell-surface ligands, such as PD-L1, that prevent activation of the cytotoxic mechanisms of the CTLs. These PD-L1 molecules serve as ligands for the PD-1 receptors displayed by the CTLs, together exemplifying a system of *checkpoint* ligands and receptors that serve to constrain immune responses in order to avoid autoimmunity.[187–189] Yet other localized immunosuppressive mechanisms operate through the recruitment of inflammatory cells that can actively suppress CTL activity, including regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSC).[174,190–193]

In summary, these eight hallmarks each contribute qualitatively distinct capabilities that seem integral to most lethal forms of human cancer. Certainly, the balance and relative importance of their respective contributions to disease pathogenesis will vary among cancer types, and some hallmarks may be absent or of minor importance in some cases. Still, there is reason to postulate their generality and, thus, their applicability to understanding the biology of human cancer. Next, we turn to the question of how these capabilities are acquired during the multistep pathways through which cancers develop, focusing on two facilitators that are commonly involved.

TWO UBIQUITOUS CHARACTERISTICS FACILITATE THE ACQUISITION OF HALLMARK CAPABILITIES

We have defined the hallmarks of cancer as acquired functional capabilities that allow cancer cells to survive, proliferate, and disseminate. Their acquisition is made possible by two *enabling characteristics* (Fig. 2.2). Most prominent is the development of genomic instability in cancer cells, which generates random mutations, including chromosomal rearrangements, among which are rare genetic changes that can orchestrate individual hallmark capabilities. A second enabling characteristic involves the inflammatory state of premalignant and frankly malignant lesions. A variety of cells of the innate and adaptive immune system infiltrate neoplasias, some of which serve to promote tumor progression through various means.

An Enabling Characteristic: Genome Instability and Mutation

Acquisition of the multiple hallmarks enumerated previously depends in large part on a succession of alterations in the genomes of neoplastic cells. Basically, certain mutant genotypes can confer selective advantage to particular subclones among proliferating nests of incipient cancer cells, enabling their outgrowth and eventual dominance in a local tissue environment. Accordingly, multistep tumor progression can be portrayed as a succession of clonal expansions, most of which are triggered by the chance acquisition of an enabling mutation.

Indeed, it is apparent that virtually every human cancer cell genome carries mutant alleles of one or several growth-regulating genes, underscoring the central importance of these genetic alterations in driving malignant progression.[194] Still, we note that many heritable phenotypes—including, notably, inactivation of tumor suppressor genes—can be acquired through epigenetic

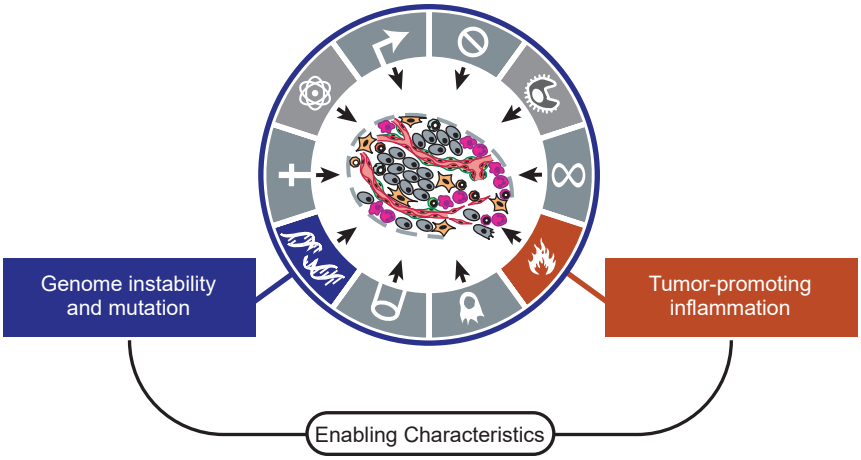


Figure 2.2 Enabling characteristics. Two ostensibly generic characteristics of cancer cells and the neoplasias they create are involved in the acquisition of the hallmark capabilities. First and foremost, the impairment of genome maintenance systems in aberrantly proliferating cancer cells enables the generation of mutations in genes that contribute to multiple hallmarks. Secondarily, neoplasias invariably attract cells of the innate immune system that are programmed to heal wounds and fight infections; these cells, including macrophages, neutrophils, and partially differentiated myeloid cells, can contribute functionally to acquisition of many of the hallmark capabilities. (Adapted from Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646–674.)

mechanisms, such as DNA methylation and histone modifications.[195–198] Thus, many clonal expansions may also be triggered by heritable nonmutational changes affecting the regulation of gene expression. At present, the relative importance of genetic versus heritable epigenetic alterations to the various clonal expansions remains unclear, and likely, varies broadly amongst the catalog of human cancer types.

The extraordinary ability of genome maintenance systems to detect and resolve defects in the DNA ensures that rates of spontaneous mutation in normal cells of the body are typically very low, both in quiescent cells and during cell division. The genomes of most cancer cells, by contrast, are replete with these alterations, reflecting loss of genomic integrity with concomitantly increased rates of mutation. This heightened mutability appears to accelerate the generation of variant cells, facilitating the selection of those cells whose advantageous phenotypes enable their clonal expansion.[199,200] This mutability is achieved through increased sensitivity to mutagenic agents, through a breakdown in one or several components of the genomic maintenance machinery, or both. In addition, the accumulation of mutations can be accelerated by aberrations that compromise the surveillance systems that normally monitor genomic integrity and force such genetically damaged cells into either quiescence, senescence, or apoptosis.[201–203] The role of TP53 is central here, leading to its being called the *guardian of the genome*.[204]

A diverse array of defects affecting various components of the DNA-maintenance machinery, referred to as the *caretakers* of the genome,[205] have been documented. The catalog of defects in these caretaker genes includes those whose products are involved in (1) detecting DNA damage and activating the repair machinery, (2) directly repairing damaged DNA, and (3) inactivating or intercepting mutagenic molecules before they have damaged the DNA.[199,201,202,206–208] From a genetic perspective, these caretaker genes behave much like tumor suppressor genes, in that their functions are often lost during the course of tumor progression, with such losses being achieved either through inactivating mutations or via epigenetic repression. Mutant copies of many of these caretaker genes have been introduced into the mouse germ line, resulting, not unexpectedly, in increased cancer incidence, thus supporting their involvement in human cancer development.[209]

In addition, research over the past decade has revealed another major source of tumor-associated genomic instability. As described earlier, the loss of telomeric DNA in many tumors generates karyotypic instability and associated amplification and deletion of chromosomal segments.[58] When viewed in this light, telomerase is more than an enabler of the hallmark capability for unlimited replicative potential. It must also be added to the list of critical caretakers responsible for maintaining genome integrity.

Advances in the molecular–genetic analysis of cancer cell genomes have provided the most compelling demonstrations of function-altering mutations and of ongoing genomic instability during tumor progression. One type of analysis—comparative genomic hybridization (CGH)—documents the gains and losses of gene copy number across the cell genome. In many tumors, the pervasive genomic aberrations revealed by CGH provide clear evidence for loss of control of genome integrity. Importantly, the recurrence of specific aberrations (both amplifications and deletions) at particular locations in the genome indicates that such sites are likely to harbor genes whose alteration favors neoplastic progression.[210]

More recently, with the advent of efficient and economical DNA sequencing technologies, higher resolution analyses of cancer cell genomes have become possible. Early studies are revealing distinctive patterns of DNA mutations in different tumor types (see: http://cancergenome.nih.gov/). In the not-too-distant future, the sequencing of entire cancer cell genomes promises to clarify the importance of ostensibly random mutations scattered across cancer cell genomes.[194] Thus, the use of whole genome resequencing offers the prospect of revealing recurrent genetic alterations (i.e., those found in multiple independently arising tumors) that in aggregate represent only minor proportions of the tumors of a given type. The recurrence of such mutations, despite their infrequency, may provide clues about the regulatory pathways playing causal roles in the pathogenesis of the tumors under study.

These surveys of cancer cell genomes have shown that the specifics of genome alteration vary dramatically between different tumor types. Nonetheless, the large number of already documented genome maintenance and repair defects, together with abundant evidence of widespread destabilization of gene copy number and nucleotide sequence, persuade us that instability of the genome is inherent to the cancer cells forming virtually all types of human tumors. This leads, in turn, to the conclusion that the defects in genome maintenance and repair are selectively advantageous and, therefore, instrumental for tumor progression, if only because they accelerate the rate at which evolving premalignant cells can accumulate favorable genotypes. As such, genome instability is clearly an *enabling characteristic* that is causally associated with the acquisition of hallmark capabilities.

An Enabling Characteristic: Tumor-Promoting Inflammation

Among the cells recruited to the stroma of carcinomas are a variety of cell types of the immune system that mediate various inflammatory functions. Pathologists have long recognized that some (but not all) tumors are densely infiltrated by cells

of both the innate and adaptive arms of the immune system, thereby mirroring inflammatory conditions arising in nonneoplastic tissues.[211] With the advent of better markers for accurately identifying the distinct cell types of the immune system, it is now clear that virtually every neoplastic lesion contains immune cells present at densities ranging from subtle infiltrations detectable only with cell type–specific antibodies to gross inflammations that are apparent even by standard histochemical staining techniques.[183] Historically, such immune responses were largely thought to reflect an attempt by the immune system to eradicate tumors, and indeed, there is increasing evidence for antitumoral responses to many tumor types with an attendant pressure on the tumor to evade immune destruction,[174,176,177,183] as discussed earlier.

By 2000, however, there were also clues that tumor-associated inflammatory responses can have the unanticipated effect of facilitating multiple steps of tumor progression, thereby helping incipient neoplasias to acquire hallmark capabilities. In the ensuing years, research on the intersections between inflammation and cancer pathogenesis has blossomed, producing abundant and compelling demonstrations of the functionally important tumor-promoting effects that immune cells—largely of the innate immune system—have on neoplastic progression.[19,53,94,174,212,213] Inflammatory cells can contribute to multiple hallmark capabilities by supplying signaling molecules to the tumor microenvironment, including growth factors that sustain proliferative signaling; survival factors that limit cell death; proangiogenic factors; extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion, and metastasis; and inductive signals that lead to activation of EMT and other hallmark-promoting programs.[53,94,116,212,213]

Importantly, localized inflammation is often apparent at the earliest stages of neoplastic progression and is demonstrably capable of fostering the development of incipient neoplasias into full-blown cancers.[94,214] Additionally, inflammatory cells can release chemicals—notably, reactive oxygen species—that are actively mutagenic for nearby cancer cells, thus accelerating their genetic evolution toward states of heightened malignancy.[53] As such, inflammation by selective cell types of the immune system is demonstrably an *enabling characteristic* for its contributions to the acquisition of hallmark capabilities. The cells responsible for this enabling characteristic are described in the following section.

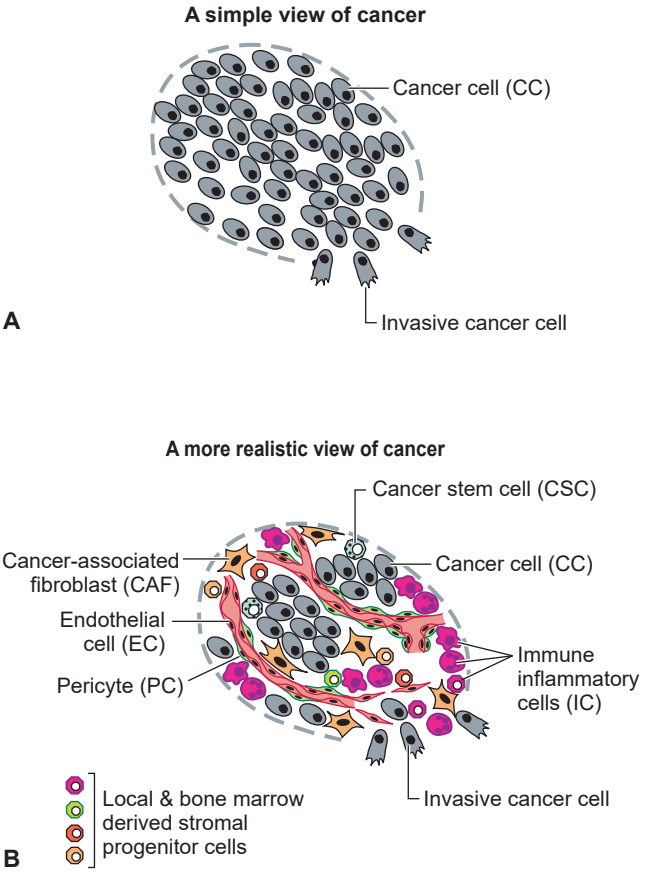


Figure 2.3 Tumors as outlaw organs. Research aimed at understanding the biology of tumors has historically focused on the cancer cells, which constitute the drivers of neoplastic disease. This view of tumors as nothing more than masses of cancer cells **(A)** ignores an important reality, that cancer cells recruit and corrupt a variety of normal cell types that form the tumor-associated stroma. Once formed, the stroma acts reciprocally on the cancer cells, affecting almost all of the traits that define the neoplastic behavior of the tumor as a whole **(B).** The assemblage of heterogeneous populations of cancer cells and stromal cells is often referred to as the tumor microenvironment (TME). (Adapted from Hanahan D, Weinberg R. The hallmarks of cancer. *Cell* 2000;100:57–70; Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646–674.)

THE CONSTITUENT CELL TYPES OF THE TUMOR MICROENVIRONMENT

Over the past 2 decades, tumors have increasingly been recognized as tissues whose complexity approaches and may even exceed that of normal healthy tissues. This realization contrasts starkly with the earlier, reductionist view of a tumor as nothing more than a collection of relatively homogeneous cancer cells, whose entire biology could be understood by elucidating the cell-autonomous properties of these cells (Fig. 2.3A). Rather, assemblages of diverse cell types associated with malignant lesions are increasingly documented to be functionally important for the manifestation of symptomatic disease (Fig. 2.3B). When viewed from this perspective, the biology of a tumor can only be fully understood by studying the individual specialized cell types within it. We enumerate as follows a set of accessory cell types recruited directly or indirectly by neoplastic cells into tumors, where they contribute in important ways to the biology of many tumors, and we discuss the regulatory mechanisms that control their individual and collective functions. Most of these observations stem from the study of carcinomas, in which the neoplastic epithelial cells constitute a compartment (the parenchyma) that is clearly distinct from the mesenchymal cells forming the tumor-associated stroma.

Cancer-Associated Fibroblasts

Fibroblasts are found in various proportions across the spectrum of carcinomas, in many cases constituting the preponderant cell population of the tumor stroma. The term *cancer-associated fibroblasts* (CAFs) subsumes at least two distinct cell types: (1) cells with similarities to the fibroblasts that create the structural foundation supporting most normal epithelial tissues, and (2) myofibroblasts, whose biologic roles and properties differ markedly from those of the widely distributed tissue-derived fibroblasts. Myofibroblasts are identifiable by their expression of α-smooth muscle actin (αSMA). They are rare in most healthy epithelial tissues, although certain tissues, such as the liver and pancreas, contain appreciable numbers of αSMA-expressing cells. Myofibroblasts transiently increase in abundance in wounds and are also found in sites of chronic inflammation. Although beneficial to tissue repair, myofibroblasts are problematic in chronic inflammation, in that they contribute to the pathologic fibrosis observed in tissues such as the lung, kidney, and liver.

Recruited myofibroblasts and variants of normal tissue-derived fibroblastic cells have been demonstrated to enhance tumor phenotypes, notably cancer cell proliferation, angiogenesis, invasion,

and metastasis. Their tumor-promoting activities have largely been defined by transplantation of cancer-associated fibroblasts admixed with cancer cells into mice, and more recently by genetic and pharmacologic perturbation of their functions in tumor-prone mice.[8,121,133,215–219] Because they secrete a variety of ECM components, cancer-associated fibroblasts are implicated in the formation of the desmoplastic stroma that characterizes many advanced carcinomas. The full spectrum of functions contributed by both subtypes of cancer-associated fibroblasts to tumor pathogenesis remains to be elucidated.

Endothelial Cells

Prominent among the stromal constituents of the TME are the endothelial cells forming the tumor-associated vasculature. Quiescent tissue capillary endothelial cells are activated by *angiogenic* regulatory factors to produce a neovasculature that sustains tumor growth concomitant with continuing endothelial cell proliferation and vessel morphogenesis. A network of interconnected signaling pathways involving ligands of signal-transducing receptors (e.g., the Angiopoeitin-1/2, Notch ligands, Semaphorin, Neuropilin, Robo, and Ephrin-A/B) is now known to be involved in regulating quiescent versus activated angiogenic endothelial cells, in addition to the aforementioned counterbalancing VEGF and TSP signals. This network of signaling pathways has been functionally implicated in developmental and tumor-associated angiogenesis, further illustrating the complex regulation of endothelial cell phenotypes.[220–224]

Other avenues of research are revealing distinctive gene expression profiles of tumor-associated endothelial cells and identifying cell-surface markers displayed on the luminal surfaces of normal versus tumor endothelial cells.[78,225,226] Differences in signaling, in transcriptome profiles, and in vascular *ZIP codes* will likely prove to be important for understanding the conversion of normal endothelial cells into tumor-associated endothelial cells. Such knowledge may lead, in turn, to opportunities to develop novel therapies that exploit these differences in order to selectively target tumor-associated endothelial cells. Additionally, the activated (*angiogenic*) tumor vasculature has been revealed as a barrier to efficient intravasation and a functional suppressor of cytotoxic T cells,[227] and thus, tumor endothelial cells can contribute to the hallmark capability for evading immune destruction. As such, another emerging concept is to normalize rather than ablate them, so as to improve immunotherapy[190] as well as delivery of chemotherapy.[228]

Closely related to the endothelial cells of the circulatory system are those forming lymphatic vessels.[229] Their role in the tumor-associated stroma, specifically in supporting tumor growth, is poorly understood. Indeed, because of high interstitial pressure within solid tumors, intratumoral lymphatic vessels are typically collapsed and nonfunctional; in contrast, however, there are often functional, actively growing (*lymphangiogenic*) lymphatic vessels at the periphery of tumors and in the adjacent normal tissues that cancer cells invade. These associated lymphatics likely serve as channels for the seeding of metastatic cells in the draining lymph nodes that are commonly observed in a number of cancer types. Recent results that are yet to be generalized suggest an alternative role for the activated (i.e., lymphangiogenic) lymphatic endothelial cells associated with tumors, not in supporting tumor growth like the blood vessels, but in inducing (via VEGF-C–mediated signaling) a lymphatic tissue microenvironment that suppresses immune responses ordinarily marshaled from the draining lymph nodes.[230] As such, the real value to a tumor from activating the signaling circuit involving the ligand VEGF-C and its receptor VEGFR3 may be to facilitate the evasion of antitumor immunity by abrogating the otherwise immunostimulatory functions of draining lymphatic vessels and lymph nodes, with the collateral effect of inducing lymphatic endothelial cells to form the new lymphatic vessels that are commonly detected in association with tumors.

Pericytes

Pericytes represent a specialized mesenchymal cell type that are closely related to smooth muscle cells, with fingerlike projections that wrap around the endothelial tubing of blood vessels. In normal tissues, pericytes are known to provide paracrine support signals to the quiescent endothelium. For example, Ang-1 secreted by pericytes conveys antiproliferative stabilizing signals that are received by the Tie2 receptors expressed on the surface of endothelial cells. Some pericytes also produce low levels of VEGF that serve a trophic function in endothelial homeostasis.[93,231] Pericytes also collaborate with the endothelial cells to synthesize the vascular basement membrane that anchors both pericytes and endothelial cells and helps vessel walls to withstand the hydrostatic pressure created by the blood.

Genetic and pharmacologic perturbation of the recruitment and association of pericytes has demonstrated the functional importance of these cells in supporting the tumor endothelium.[93,217,231] For example, the pharmacologic inhibition of signaling through the platelet-derived growth factor (PDGF) receptor expressed by tumor pericytes and bone marrow–derived pericyte progenitors results in reduced pericyte coverage of tumor vessels, which in turn destabilizes vascular integrity and function.[91,217,231] Interestingly, and in contrast, the pericytes of normal vessels are not prone to such pharmacologic disruption, providing another example of the differences in the regulation of normal quiescent and tumor vasculature. An intriguing hypothesis, still to be fully substantiated, is that tumors with poor pericyte coverage of their vasculature may be more prone to permit cancer cell intravasation into the circulatory system, thereby enabling subsequent hematogenous dissemination.[91,148]

Immune Inflammatory Cells

Infiltrating cells of the immune system are increasingly accepted to be generic constituents of tumors. These inflammatory cells operate in conflicting ways: Both tumor-antagonizing and tumor-promoting leukocytes can be found in various proportions in most, if not all, neoplastic lesions. Evidence began to accumulate in the late 1990s that the infiltration of neoplastic tissues by cells of the immune system serves, perhaps counterintuitively, to promote tumor progression. Such work traced its conceptual roots back to the observed association of tumor formation with sites of chronic inflammation. Indeed, this led some to liken tumors to "wounds that do not heal."[211,232] In the course of normal wound healing and the resolution of infections, immune inflammatory cells appear transiently and then disappear, in contrast to their persistence in sites of chronic inflammation, where their presence has been associated with a variety of tissue pathologies, including fibrosis, aberrant angiogenesis, and as mentioned, neoplasia.[53,233]

We now know that immune cells play diverse and critical roles in fostering tumorigenesis. The roster of tumor-promoting inflammatory cells includes macrophage subtypes, mast cells, and neutrophils, as well as T and B lymphocytes.[96,97,119,133,212,234,235] Studies of these cells are yielding a growing list of tumor-promoting signaling molecules that they release, which include the tumor growth factor EGF, the angiogenic growth factors VEGF-A/-C, other proangiogenic factors such as FGF2, plus chemokines and cytokines that amplify the inflammatory state. In addition, these cells may produce proangiogenic and/or proinvasive matrix-degrading enzymes, including MMP-9 and other MMPs, cysteine cathepsin proteases, and heparanase.[94,96] Consistent with the expression of these diverse signals, tumor-infiltrating inflammatory cells have been shown to induce and help sustain tumor angiogenesis, to stimulate cancer cell proliferation, to facilitate tissue invasion, and to support the metastatic dissemination and seeding of cancer cells.[94,96,97,119,120,234–237]

In addition to fully differentiated immune cells present in tumor stroma, a variety of partially differentiated myeloid progenitors have been identified in tumors.[96] Such cells represent intermediaries between circulating cells of bone marrow origin and the differentiated immune cells typically found in normal and inflamed tissues. Importantly, these progenitors, like their more differentiated derivatives, have demonstrable tumor-promoting activity. Of particular interest, a class of tumor-infiltrating myeloid cells has been shown to suppress CTL and NK cell activity, having been identified as MDSCs that function to block the attack on tumors by the adaptive (i.e., CTL) and innate (i.e., NK) arms of the immune system.[94,133,193] Hence, recruitment of certain myeloid cells may be doubly beneficial for the developing tumor, by directly promoting angiogenesis and tumor progression, while at the same time affording a means of evading immune destruction.

These conflicting roles of the immune system in confronting tumors would seem to reflect similar situations that arise routinely in normal tissues. Thus, the immune system detects and targets infectious agents through cells of the adaptive immune response. Cells of the innate immune system, in contrast, are involved in wound healing and in clearing dead cells and cellular debris. The balance between the conflicting immune responses within particular tumor types (and indeed in individual patients' tumors) is likely to prove critical in determining the characteristics of tumor growth and the stepwise progression to stages of heightened aggressiveness (i.e., invasion and metastasis). Moreover, there is increasing evidence supporting the proposition that this balance can be modulated for therapeutic purposes in order to redirect or reprogram the immune response to focus its functional capabilities on destroying tumors.[133,238,239]

Stem and Progenitor Cells of the Tumor Stroma

The various stromal cell types that constitute the tumor microenvironment may be recruited from adjacent normal tissue—the most obvious reservoir of such cell types. However, in recent years, bone marrow (BM) has increasingly been implicated as a key source of tumor-associated stromal cells.[99,100,240–243] Thus, mesenchymal stem and progenitor cells can be recruited into tumors from BM, where they may subsequently differentiate into the various well-characterized stromal cell types. Some of these recent arrivals may also persist in an undifferentiated or partially differentiated state, exhibiting functions that their more differentiated progeny lack.

The BM origins of stromal cell types have been demonstrated using tumor-bearing mice in which the BM cells (and thus their disseminated progeny) have been selectively labeled with reporters such as green fluorescent protein (GFP). Although immune inflammatory cells have been long known to derive from BM, more recently progenitors of endothelial cells, pericytes, and several subtypes of cancer-associated fibroblasts have also been shown to originate from BM in various mouse models of cancer.[100,240–243] The prevalence and functional importance of endothelial progenitors for tumor angiogenesis is, however, currently unresolved.[99,242] Taken together, these various lines of evidence indicate that tumor-associated stromal cells may be supplied to growing tumors by the proliferation of preexisting stromal cells or via recruitment of BM-derived stem/progenitor cells.

In summary, it is evident that virtually all cancers, including even the *liquid tumors* of hematopoietic malignancies, depend not only on neoplastic cells for their pathogenic effects, but also on diverse cell types recruited from local and distant tissue sources to assemble specialized, supporting tumor microenvironments. Importantly, the composition of stromal cell types supporting a particular cancer evidently varies considerably from one tumor type to another; even within a particular type, the patterns and abundance can be informative about malignant grade and prognosis. The inescapable conclusion is that cancer cells are not fully autonomous, and rather depend to various degrees on stromal cells of the tumor microenvironment, which can contribute functionally to seven of the eight hallmarks of cancer (Fig. 2.4).

Heterotypic Signaling Orchestrates the Cells of the Tumor Microenvironment

Every cell in our bodies is governed by an elaborate intracellular signaling circuit—in effect, its own microcomputer. In cancer cells, key subcircuits in this integrated circuit are reprogrammed so as to activate and sustain hallmark capabilities. These changes are induced by mutations in the cells' genomes, by epigenetic alterations affecting gene expression, and by the receipt of a diverse array of signals from the tumor microenvironment. Figure 2.5A illustrates some of the circuits that are reprogrammed to enable cancer cells to proliferate chronically, to avoid proliferative brakes and cell death, and to become invasive and metastatic. Similarly, the intracellular integrated circuits that regulate the actions of stromal cells are also evidently reprogrammed. Current evidence suggests that stromal cell reprogramming is primarily affected by extracellular cues and epigenetic alterations in gene expression, rather than gene mutation.

Given the alterations in the signaling within both neoplastic cells and their stromal neighbors, a tumor can be depicted as a network of interconnected (cellular) microcomputers. This dictates that a complete elucidation of a particular tumor's biology will require far more than an elucidation of the aberrantly functioning integrated circuits within its neoplastic cells. Accordingly, the rapidly growing catalog of the function-enabling genetic mutations within cancer cell genomes[194] provides only one dimension to this problem. A reasonably complete, graphical depiction of the network of microenvironmental signaling interactions remains far beyond our reach, because the great majority of signaling molecules and their circuitry are still to be identified. Instead, we provide a hint of such interactions in Figure 2.5B. These few well-established examples are intended to exemplify a signaling network of remarkable complexity that is of critical importance to tumor pathogenesis.

Coevolution of the Tumor Microenvironment During Carcinogenesis

The tumor microenvironment described previously is not static during multistage tumor development and progression, thus creating another dimension of complexity. Rather, the abundance and functional contributions of the stromal cells populating neoplastic lesions will likely vary during progression in two respects. First, as the neoplastic cells evolve, there will be a parallel coevolution occurring in the stroma, as indicated by the shifting composition of stroma-associated cell types. Second, as cancer cells enter into different locations, they encounter distinct stromal microenvironments. Thus, the microenvironment in the interior of a primary tumor will likely be distinct both from locally invasive breakout lesions and from the one encountered by disseminated cells in distant organs (Fig. 2.6A). This dictates that the observed histopathologic progression of a tumor reflects underlying changes in heterotypic signaling between tumor parenchyma and stroma.

We envision back-and-forth reciprocal interactions between the neoplastic cells and the supporting stromal cells that change during the course of multistep tumor development and progression, as depicted in Figure 2.6B. Thus, incipient neoplasias begin the interplay by recruiting and activating stromal cell types that assemble into an initial preneoplastic stroma, which in turn responds reciprocally by enhancing the neoplastic phenotypes of the nearby cancer cells. The cancer cells, in response, may then undergo further genetic evolution, causing them to feed signals back to the stroma. Ultimately, signals originating in the stroma of primary tumors enable cancer cells to invade normal adjacent tissues and disseminate, seeding distant tissues and, with low efficiency, metastatic colonies (see Fig. 2.6B).

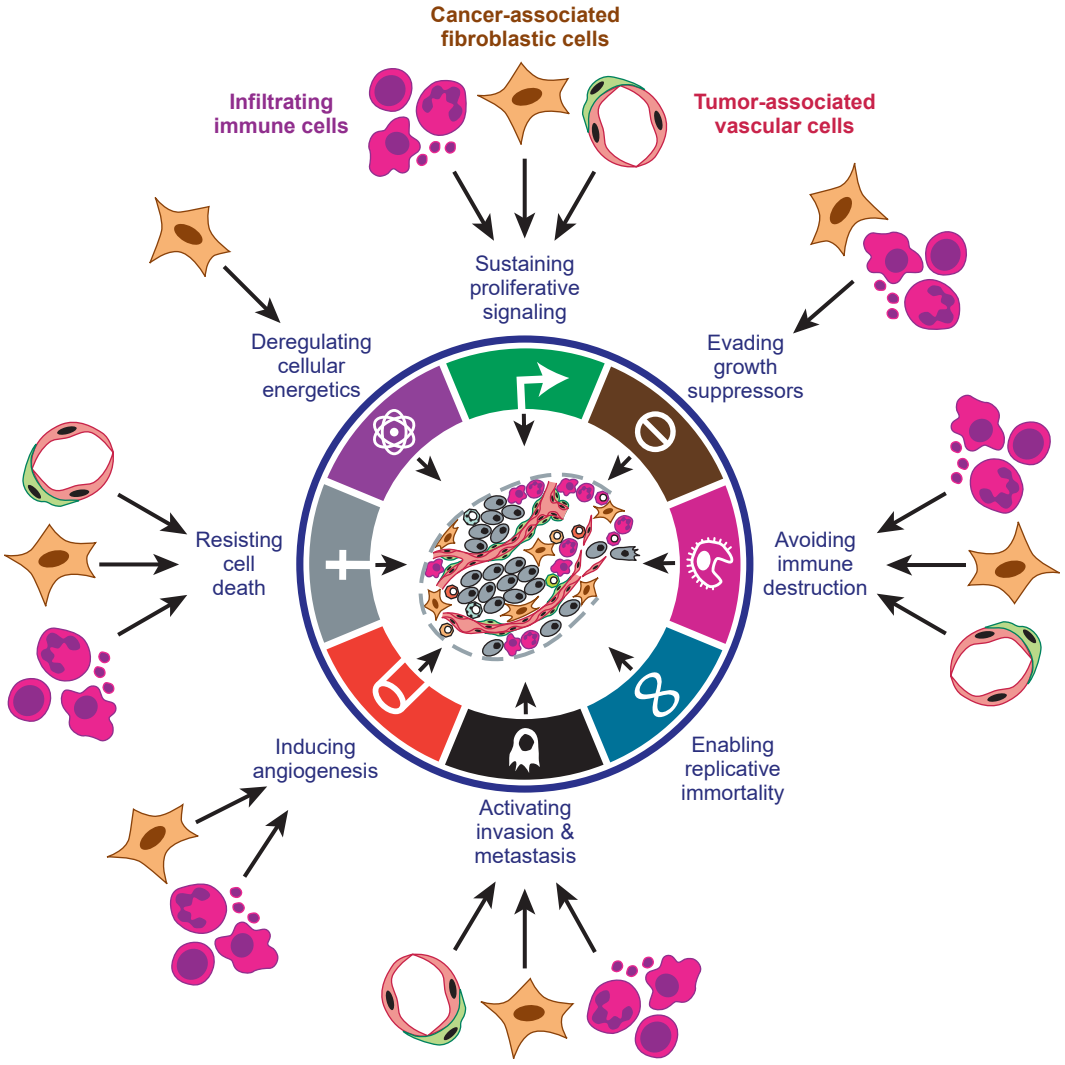


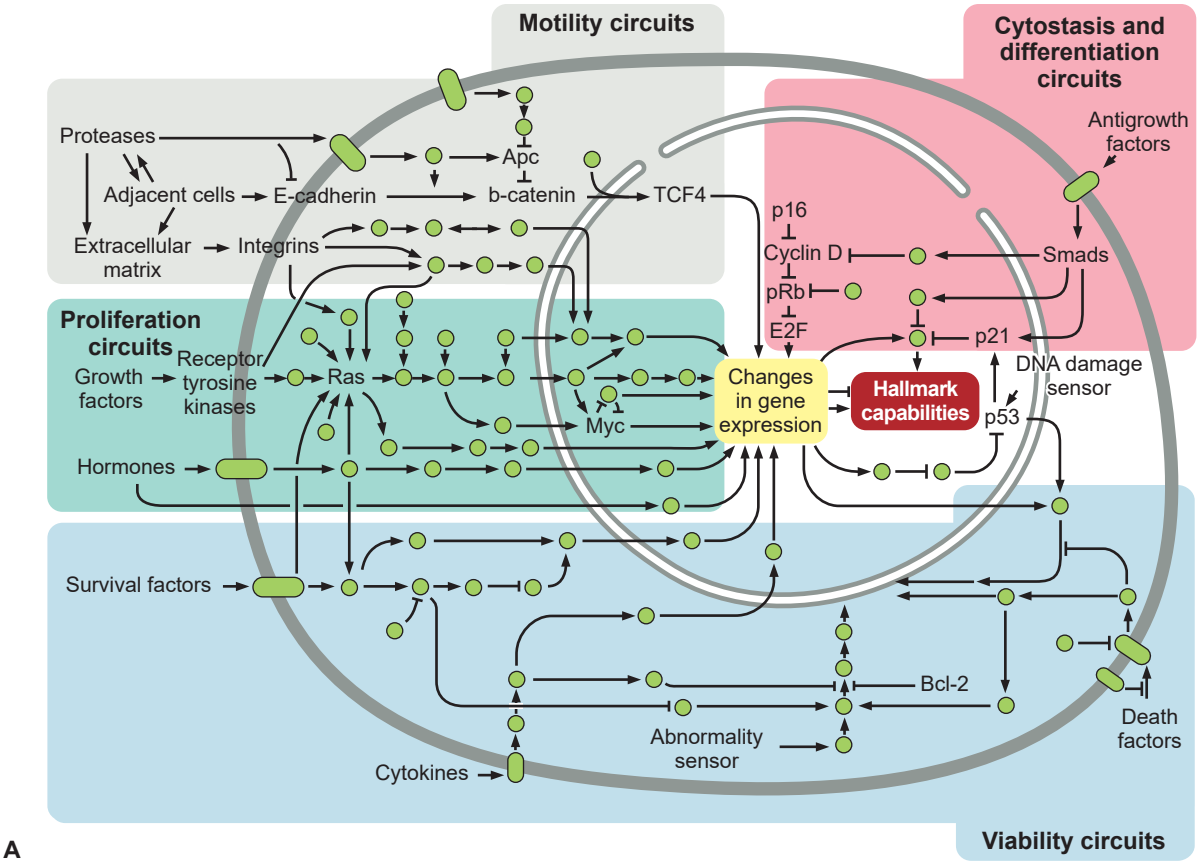
Figure 2.4 Diverse contributions of stromal cells to the hallmarks of cancer. Of the eight hallmark capabilities acquired by cancer cells, seven depend on contributions by stromal cells forming the tumor microenvironment.[2,213] The stromal cells can be divided into three general classes: infiltrating immune cells, cancer-associated fibroblastic cells, and tumor-associated vascular cells. The association of these corrupted cell types with the acquisition of individual hallmark capabilities has been documented through a variety of experimental approaches that are often supported by descriptive studies in human cancers. The relative importance of each of these stromal cell classes to a particular hallmark varies according to tumor type and stage of progression. (Adapted from Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell* 2012;21:309–322.)

The circulating cancer cells that are released from primary tumors leave a microenvironment supported by this coevolved stroma. Upon landing in a distant organ, however, disseminated cancer cells must find a means to grow in a quite different tissue microenvironment. In some cases, newly seeded cancer cells must survive and expand in naïve, fully normal tissue microenvironments. In other cases, the newly encountered tissue microenvironments may already be supportive of such disseminated cancer cells, having been preconditioned prior to their arrival. Such permissive sites have been referred to as *premetastatic niches*.[146,244,245] These supportive niches may already preexist in distant tissues for various physiologic reasons,[101] including the actions of circulating factors dispatched systemically by primary tumors.[245]

The fact that signaling interactions between cancer cells and their supporting stroma are likely to evolve during the course of multistage primary tumor development and metastatic colonization clearly complicates the goal of fully elucidating the mechanisms of cancer pathogenesis. For example, this complexity poses challenges to systems biologists seeking to chart the crucial regulatory networks that orchestrate malignant progression, because much of the critical signaling is not intrinsic to cancer cells and instead operates through the interactions that these cells establish with their neighbors.

Cancer Cells, Cancer Stem Cells, and Intratumoral Heterogeneity

Cancer cells are the foundation of the disease. They initiate neoplastic development and drive tumor progression forward, having acquired the oncogenic and tumor suppressor mutations that define cancer as a genetic disease. Traditionally, the cancer cells within tumors have been portrayed as reasonably homogeneous cell populations until relatively late in the course of tumor progression, when hyperproliferation combined with increased genetic instability spawn genetically distinct clonal subpopulations. Reflecting such clonal heterogeneity, many human tumors are histopathologically diverse, containing regions demarcated by various



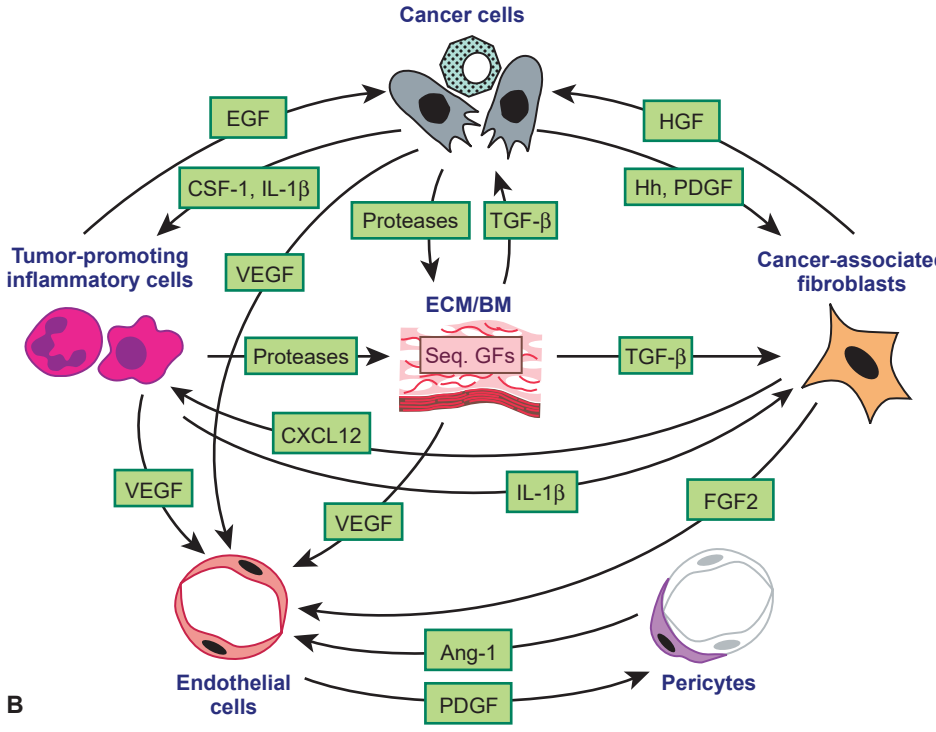


Figure 2.5 Reprogramming intracellular circuits and cell-to-cell signaling pathways dictates tumor inception and progression. An elaborate integrated circuit operating within normal cells is reprogrammed to regulate the hallmark capabilities acquired by cancer cells **(A)** and by associated stromal cells. Separate subcircuits, depicted here in differently colored fields, are specialized to orchestrate distinct capabilities. At one level, this depiction is simplistic, because there is considerable cross-talk between such subcircuits. More broadly, the integrated circuits operating inside cancer cells and stromal cells are interconnected via a complex network of signals transmitted by the various cells in the tumor microenvironment (in some cases via the extracellular matrix *[ECM]* and basement membranes *[BM]* they synthesize), of which a few signals are exemplified **(B).** HGF, hepatocyte growth factor for the cMet receptor; Hh, hedgehog ligand for the Patched (PTCH) receptor; Seq. GF, growth factors sequested in the ECM/BM. (Adapted from Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646–674.)

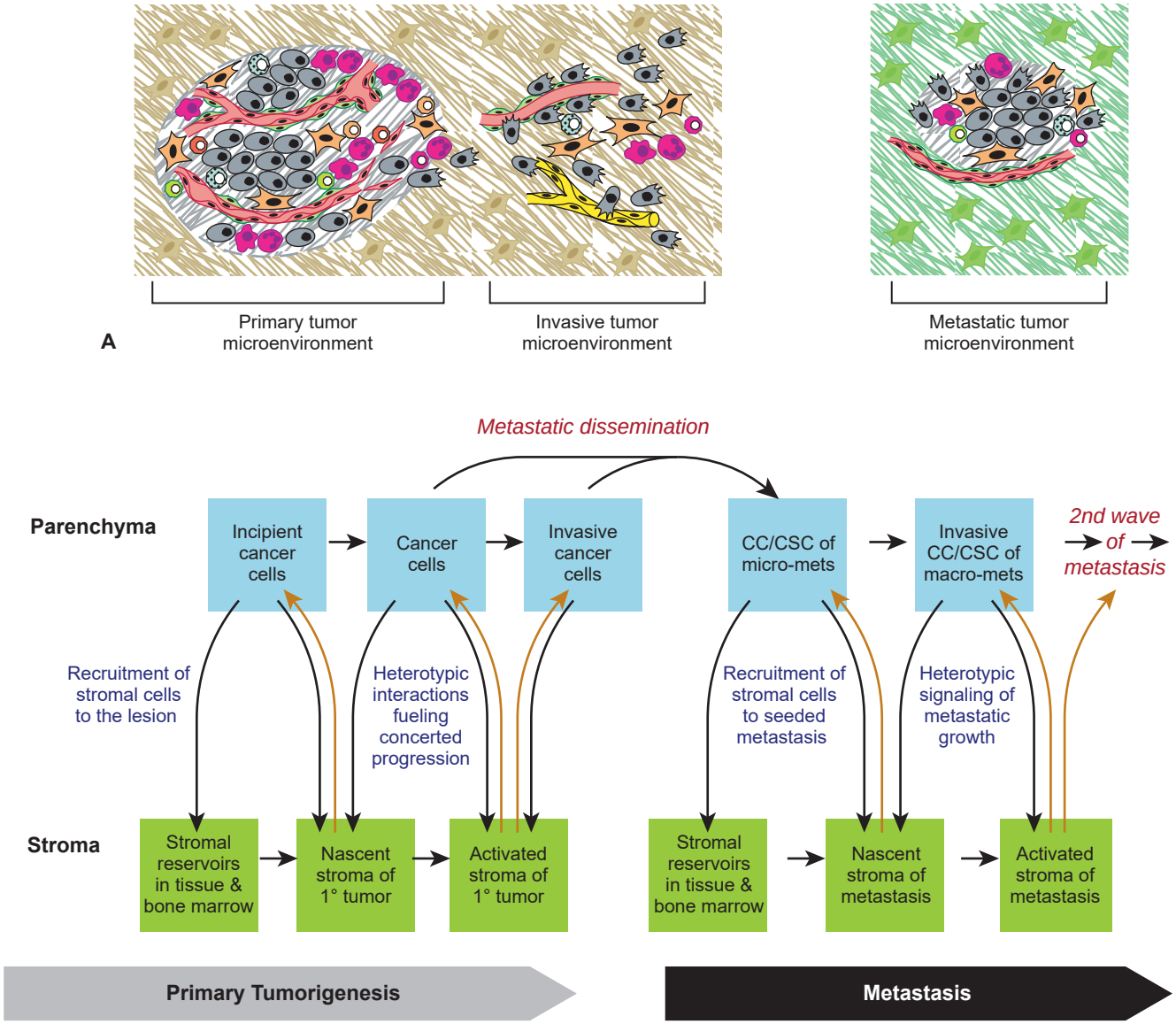


Figure 2.6 The dynamic variation and coevolution of the tumor microenvironment during the lesional progression of cancer. **(A)** Interactions between multiple stromal cell types and heterogeneously evolving mutant cancer cells create a succession of tumor microenvironments that change dynamically as tumors are initiated, invade normal tissues, and thereafter seed and colonize distant tissues. The abundance, histologic organization, and characteristics of the stromal cell types and associated extracellular matrix *(hatched background)* evolves during progression, thereby enabling primary, invasive, and then metastatic growth. **(B)** Importantly, the signaling networks depicted in Figure 2.5 involving cancer cells and their stromal collaborators change during tumor progression as a result of reciprocal signaling interactions between these various cells. CC, cancer cell; CSC, cancer stem cell; mets, metastases. (Adapted from Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646–674.)

degrees of differentiation, proliferation, vascularity, and invasiveness. In recent years, however, evidence has accumulated pointing to the existence of a new dimension of intratumor heterogeneity and a hitherto unappreciated subclass of neoplastic cells within tumors, termed cancer stem cells (CSC).

CSCs were initially implicated in the pathogenesis of hematopoietic malignancies,[246,247] and years later, were identified in solid tumors, in particular breast carcinomas and neuroectodermal tumors.[248,249] The fractionation of cancer cells on the basis of cell-surface markers has yielded subpopulations of neoplastic cells with a greatly enhanced ability, relative to the corresponding majority populations of non-CSCs, to seed new tumors upon implantation in immunodeficient mice. These, often rare, tumor-initiating cells have proven to share transcriptional profiles with certain normal tissue stem cells, thus justifying their designation as stemlike.

Although the evidence is still fragmentary, CSCs may prove to be a constituent of many, if not most tumors, albeit being present with highly variable abundance. CSCs are defined operationally through their ability to efficiently seed new tumors upon implantation into recipient host mice.[250–253] This functional definition is often complemented by profiling the expression of certain CSC-associated markers that are typically expressed by the normal stem cells in the corresponding normal tissues of origin.[249] Importantly, recent in vivo lineage-tracing experiments have provided an additional functional test of CSCs by demonstrating their ability to spawn large numbers of progeny, including non-CSCs within tumors.[250] At the same time, these experiments have provided the most compelling evidence to date that CSCs exist, and that they can be defined functionally through tests that do not depend on the implantation of tumor cells into appropriate mouse hosts.

The origins of CSCs within a solid tumor have not been clarified and, indeed, may well vary from one tumor type to another.[250,251,254] In some tumors, normal tissue stem cells may serve as the cells of origin that undergo oncogenic transformation to yield CSCs; in others, partially differentiated transit-amplifying cells, also termed progenitor cells, may suffer the initial oncogenic transformation, thereafter assuming more stemlike characters. Once primary tumors have formed, the CSCs, like their normal counterparts, may self-renew as well as spawn more differentiated derivatives. In the case of neoplastic CSCs, these descendant cells form the great bulk of many tumors and thus are responsible for creating many tumor-associated phenotypes. It remains to be established whether multiple distinct classes of increasingly neoplastic stem cells form during the inception and subsequent multistep progression of tumors, ultimately yielding the CSCs that have been described in fully developed cancers.

Recent research has interrelated the acquisition of CSC traits with the EMT transdifferentiation program discussed previously.[250,255] The induction of this program in certain model systems can induce many of the defining features of stem cells, including self-renewal ability and the antigenic phenotypes associated with both normal and cancer stem cells. This concordance suggests that the EMT program may not only enable cancer cells to physically disseminate from primary tumors, but can also confer on such cells the self-renewal capability that is crucial to their subsequent role as founders of new neoplastic colonies at sites of dissemination.[256] If generalized, this connection raises an important corollary hypothesis: The heterotypic signals that trigger an EMT, such as those released by an activated, inflammatory stroma, may also be important in creating and maintaining CSCs.

An increasing number of human tumors are reported to contain subpopulations with the properties of CSCs, as defined operationally through their efficient tumor-initiating capabilities upon xenotransplantation into mice. Nevertheless, the importance of CSCs as a distinct phenotypic subclass of neoplastic cells remains a matter of debate, as does their oft cited rarity within tumors.[254,257–259] Indeed, it is plausible that the phenotypic plasticity operating within tumors may produce bidirectional interconversion between CSCs and non-CSCs, resulting in dynamic variation in the relative abundance of CSCs.[250,260] Such plasticity could complicate a definitive measurement of their characteristic abundance. Analogous plasticity is already implicated in the EMT program, which can be engaged reversibly.[261]

These complexities notwithstanding, it is already evident that this new dimension of tumor heterogeneity holds important implications for successful cancer therapies. Increasing evidence in a variety of tumor types suggests that cells exhibiting the properties of CSCs are more resistant to various commonly used chemotherapeutic treatments.[255,262,263] Their persistence following initial treatment may help to explain the almost inevitable disease recurrence occurring after apparently successful debulking of human solid tumors by radiation and various forms of chemotherapy. Moreover, CSCs may well prove to underlie certain forms of tumor dormancy, whereby latent cancer cells persist for years or even decades after initial surgical resection or radio/chemotherapy, only to suddenly erupt and generate life-threatening disease. Hence, CSCs represent a double threat in that they are more resistant to therapeutic killing, and at the same time, are endowed with the ability to regenerate a tumor once therapy has been halted.

This phenotypic plasticity implicit in the CSC state may also enable the formation of functionally distinct subpopulations within a tumor that support overall tumor growth in various ways. Thus, an EMT can convert epithelial carcinoma cells into mesenchymal, fibroblast-like cancer cells that may well assume the duties of CAFs in some tumors (e.g., pancreatic ductal adenocarcinoma).[264] Intriguingly, several recent reports that have yet to be thoroughly validated in terms of generality, functional importance, or prevalence have documented the ability of glioblastoma cells (or possibly their associated CSC subpopulations) to transdifferentiate into endothelial-like cells that can substitute for bona fide host-derived endothelial cells in forming a tumor-associated neovasculature.[265–267] These examples suggest that certain tumors may induce some of their own cancer cells to undergo various types of metamorphoses in order to generate stromal cell types needed to support tumor growth and progression, rather than relying on recruited host cells to provide the requisite hallmark-enabling functions.

Another form of phenotypic variability resides in the genetic heterogeneity of cancer cells within a tumor. Genomewide sequencing of cancer cells microdissected from different sectors of the same tumor[145] has revealed striking intratumoral genetic heterogeneity. Some of this genetic diversity may be reflected in the long recognized histologic heterogeneity within individual human tumors. Thus, genetic diversification may produce subpopulations of cancer cells that contribute distinct and complementary capabilities, which then accrue to the common benefit of overall tumor growth, progression, and resistance to therapy, as described earlier. Alternatively, such heterogeneity may simply reflect the genetic chaos that arises as tumor cell genomes become increasingly destabilized.

THERAPEUTIC TARGETING OF THE HALLMARKS OF CANCER

We do not attempt here to enumerate the myriad therapies that are currently under development or have been introduced of late into the clinic. Instead, we consider how the description of hallmark principles is likely to inform therapeutic development at present and may increasingly do so in the future. Thus, the rapidly growing armamentarium of therapeutics directed against specific molecular targets can be categorized according to their respective effects on one or more hallmark capabilities, as illustrated in the examples presented in Figure 2.7. Indeed, the observed efficacy of these drugs represents, in each case, a validation of a particular capability: If a capability is truly critical to the biology of tumors, then its inhibition should impair tumor growth and progression.

Unfortunately, however, the clinical responses elicited by these targeted therapies have generally been transitory, being followed all too often by relapse. One interpretation, which is supported by growing experimental evidence, is that each of the core hallmark capabilities is regulated by a set of partially redundant signaling pathways. Consequently, a targeted therapeutic agent inhibiting one key pathway in a tumor may not completely eliminate a hallmark capability, allowing some cancer cells to survive with residual function until they or their progeny eventually adapt to the selective pressure imposed by the initially applied therapy. Such adaptation can reestablish the expression of the functional capability, permitting renewed tumor growth and clinical relapse. Because the number of parallel signaling pathways supporting a given hallmark must be limited, it may become possible to therapeutically cotarget all of these supporting pathways, thereby preventing the development of adaptive resistance.

Another dimension of the plasticity of tumors under therapeutic attack is illustrated by the unanticipated responses to antiangiogenic therapy, in which cancer cells reduce their dependence on this hallmark capability by increasing their dependence on another. Thus, many observers anticipated that potent inhibition of angiogenesis would starve tumors of vital nutrients and oxygen, forcing them into dormancy and possibly leading to their dissolution.[86,87,268] Instead, the clinical responses to antiangiogenic therapies have been found to be transitory, followed by relapse, implicating adaptive or evasive resistance mechanisms.[220,269–271] One such mechanism of evasive resistance, observed in certain preclinical models of antiangiogenic therapy, involves reduced dependence on continuing angiogenesis by increasing the activity of two other capabilities: invasiveness and metastasis.[269–271] By invading nearby and distant tissues, initially hypoxic cancer cells gain access to normal,

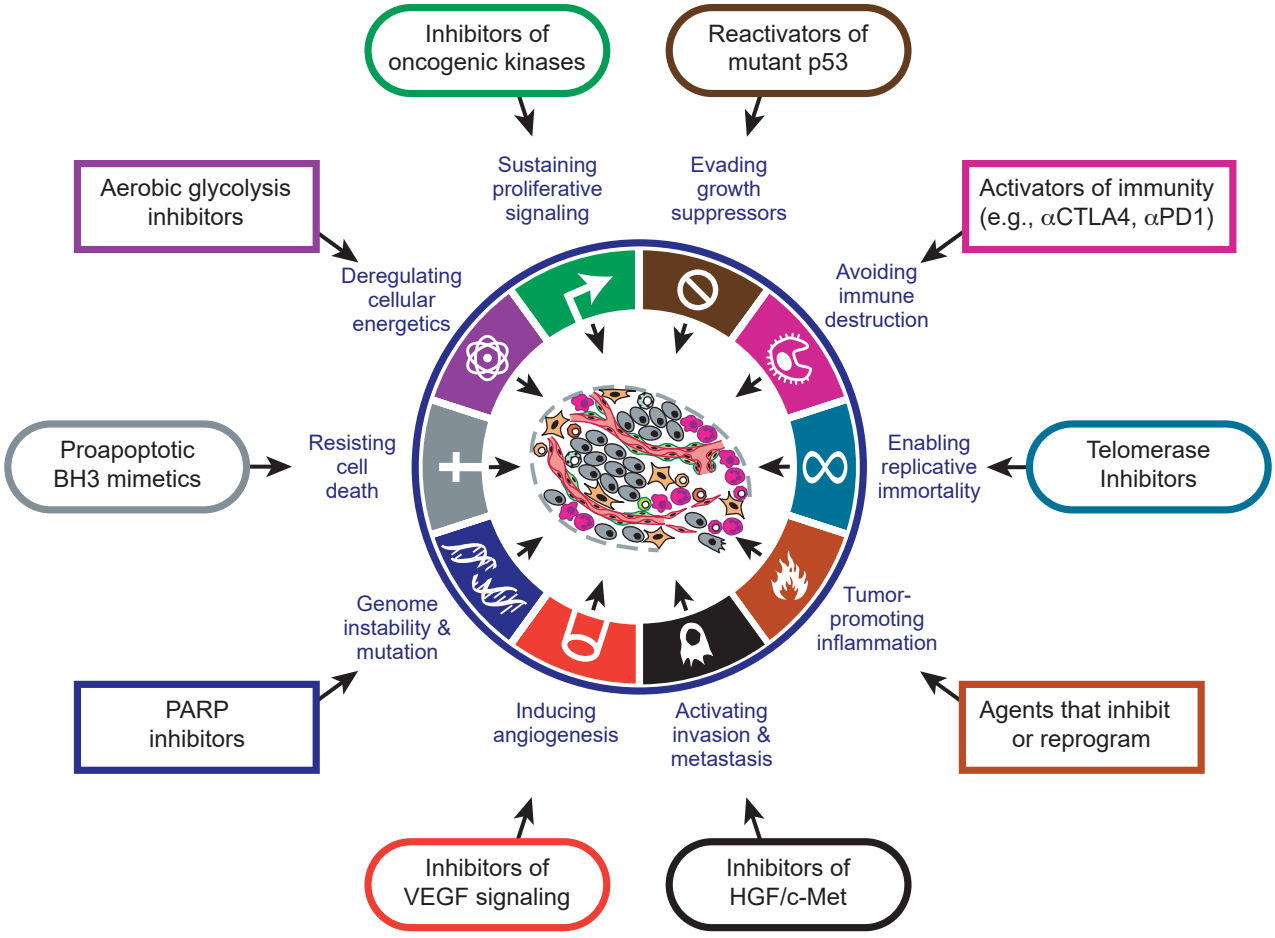


Figure 2.7 Therapeutic targeting of the hallmarks of cancer. Drugs that interfere with each of the hallmark capabilities and hallmark-enabling processes have been developed and are in preclinical and/or clinical testing, and in some cases, approved for use in treating certain forms of human cancer. A focus on antagonizing specific hallmark capabilities is likely to yield insights into developing novel, highly effective therapeutic strategies. PARP, poly ADP ribose polymerase. (Adapted from Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646–674.)

preexisting tissue vasculature. The initial clinical validation of this adaptive/evasive resistance is apparent in the increased invasion and local metastasis seen when human glioblastomas are treated with antiangiogenic therapies.[272–274] The applicability of this lesson to other human cancers has yet to be established.

Analogous adaptive shifts in dependence on other hallmark traits may also limit the efficacy of analogous hallmark-targeting therapies. For example, the deployment of apoptosis-inducing drugs may induce cancer cells to hyperactivate mitogenic signaling, enabling them to compensate for the initial attrition triggered by such treatments. Such considerations suggest that drug development and the design of treatment protocols will benefit from incorporating the concepts of functionally discrete hallmark capabilities and of the multiple biochemical pathways involved in supporting each of them. For these reasons, we envisage that attacking multiple hallmark capabilities with hallmark-targeting drugs (see Fig. 2.7), in carefully considered combinations, sequences, and temporal regimens,[275] will result in increasingly effective therapies that produce more durable clinical responses.

CONCLUSION AND A VISION FOR THE FUTURE

Looking ahead, we envision significant advances in our understanding of invasion and metastasis during the coming decade. Similarly, the role of altered energy metabolism in malignant growth will be elucidated, including a resolution of whether this metabolic reprogramming is a discrete capability separable from the core hallmark of chronically sustained proliferation. We are excited about the new frontier of immunotherapy, which will be empowered to leverage detailed knowledge about the regulation of immune responses in order to develop pharmacologic tools that can modulate them therapeutically for the purpose of effectively and sustainably attacking tumors and, most importantly, their metastases.

Other areas are currently in rapid flux. In recent years, elaborate molecular mechanisms controlling transcription through chromatin modifications have been uncovered, and there are clues that specific shifts in chromatin configuration occur during the acquisition of certain hallmark capabilities.[195,196] Functionally significant epigenetic alterations seem likely to be factors not only in the cancer cells, but also in the altered cells of the tumor-associated stroma. At present, it is unclear whether an elucidation of these epigenetic mechanisms will materially change our overall understanding of the means by which hallmark capabilities are acquired, or simply add additional detail to the regulatory circuitry that is already known to govern them.

Similarly, the discovery of hundreds of distinct regulatory microRNAs has already led to profound changes in our understanding of the molecular control mechanisms that operate in health and disease. By now, dozens of microRNAs have been implicated in various tumor phenotypes.[276,277] Still, these only scratch the surface of the true complexity, because the functions of hundreds of microRNAs known to be present in our cells and to

be altered in expression levels in different forms of cancer remain total mysteries. Here again, we are unclear whether future progress will cause fundamental shifts in our understanding of the pathogenic mechanisms of cancer, or only add detail to the elaborate regulatory circuits that have already been mapped out.

Finally, the existing diagrams of heterotypic interactions between the multiple distinct cell types that collaborate to produce malignant tumors are still rudimentary. We anticipate that, in another decade, the signaling pathways describing the intercommunication between these various cell types within tumors will be charted in far greater detail and clarity, eclipsing our current knowledge. And, as before,[1,2] we continue to foresee cancer research as an increasingly logical science, in which myriad phenotypic complexities are manifestations of an underlying organizing principle.

ACKNOWLEDGMENT

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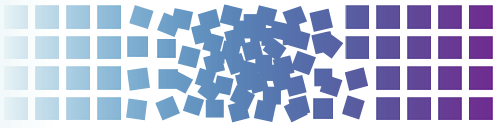
3 Molecular Methods in Cancer



Larissa V. Furtado, Jay L. Hess, and Bryan L. Betz

APPLICATIONS OF MOLECULAR DIAGNOSTICS IN ONCOLOGY

Molecular diagnostics is increasingly impacting a number of areas of cancer care delivery including diagnosis, prognosis, in predicting response to particular therapies, and in minimal residual disease monitoring. Each of these depends on detection or measurement of one or more disease-specific molecular biomarkers representing abnormalities in genetic or epigenetic pathways controlling cellular proliferation, differentiation, or cell death (Table 3.1). In addition, molecular diagnostics is beginning to play a role in predicting host metabolism of drugs—for example, in predicting fast versus slow thiopurine metabolizers using polymorphisms in the thiopurine methyltransferase (TPMT) allele and in use in dosing patients with thiopurine drugs.[1] Molecular diagnostics has also had a major impact on assessing an engraftment after bone marrow transplantation and in tissue typing for bone marrow and solid organ transplantation.

The ideal cancer biomarker is only associated with the disease and not the normal state. The utility of the biomarker largely depends on what the clinical effect the biomarker predicts for, how large the effect is, and how strong the evidence is for the effect. For clinical application, biomarkers need a high level of *analytic validity*, *clinical validity*, and *clinical utility*. Analytic validity refers to the ability of the overall testing process to accurately detect and, in many cases, measure the biomarker. Clinical validity is the ability of a biomarker to predict a particular disease behavior or response to therapy. Clinical utility, arguably the most difficult to assess, addresses whether the information available from the biomarker is actually beneficial for patient care.

Biomarkers can take many forms including *chromosomal translocations* and *other chromosomal rearrangements*, *gene amplification*, *copy number variation*, *point mutations*, *single nucleotide polymorphisms*, *changes in gene expression* (including micro RNAs), and *epigenetic alterations*. Most biomarkers in widespread use represent either gain of function or loss of function alterations in key signaling pathways. Those that occur early and at a high frequency in tumors tend to be *driver mutations*, whose function is important for the cancer cell's proliferation and/or survival. These are particularly useful as biomarkers because they often represent important therapeutic targets. However, cancer cells accumulate many genetic alterations, called *passenger mutations*, which tend to occur at a lower frequency overall and in a subset of a heterogeneous population of tumor cells that may contribute to the cancer phenotype but are not absolutely essential.[2] Distinguishing passenger from driver mutations using various functional assays has become a major focus of translational research in cancer. The same biomarker may have utility in a variety of settings. For example, the detection of the *BCR-ABL1* translocation, pathognomonic for chronic myelogenous leukemia (CML), is used for establishing the diagnosis, for the selection of therapy, and for monitoring for minimal residual disease during and after therapy.

Some of the most heavily used genetic biomarkers in cancer, particularly in hematologic malignancies, are *chromosomal translocations*. For certain diseases such as CML, detection of the *BCR-ABL1* translocation or in Burkitt lymphoma the immunoglobulin gene-*MYC* translocation is required, according to current World Health Organization (WHO) guidelines, to make the diagnosis. Identification of translocations is important in the diagnosis and subtyping of acute leukemias (e.g., detection of *PML-RARA* and variant translocations in acute promyelocytic leukemia) and is also extremely important for the diagnosis of sarcomas such as Ewing sarcoma. The discovery of chromosomal translocations, such as the *TMPRSS-ETS* in prostate cancer and *ALK* translocations in non–small-cell lung cancer, portends an importance of detecting translocations in solid tumors.[3] Chromosomal translocations, especially for hematologic malignancies, have been traditionally detected by classical karyotyping. This approach has limitations; in particular, it requires viable, dividing cells, which are often not readily available from solid tumor biopsies. In addition, a significant proportion of chromosomal translocations are not detectable by conventional karyotyping. For example, 5% to 10% of CML cases lack detectable t(9;22) by G banding. Such "cryptic" translocations require other approaches for detection, which are to be discussed, including *fluorescent in situ hybridization (FISH)*, *polymerase chain reaction (PCR)*, as well as *nucleic acid sequencing-based methods*.

In certain settings, it can be helpful to detect if a population of cells is clonal. For example, in some lymphoid infiltrates, the cells are well differentiated and it can be difficult to determine whether these represent a reactive or neoplastic infiltrate. If dispersed, cells are available and these could be analyzed by flow cytometer to detect whether a monotypic population expressing either immunoglobulin kappa or lambda light chains is present. In theory, immunohistochemical staining (IHC) for immunoglobulin light chains could be used to assess clonality; however, in practice this is done with more sensitivity using RNA in situ hybridization for immunoglobulin kappa and lambda light chain transcripts. The most sensitive way to detect clonality in a B-cell population is to analyze the size of the break point cluster region that arises as a result of VDJ recombination by *PCR*. Reactive B cells will show a distribution in the size of the VDJ recombination for the *IGH* or *IGK* or *IGL*, whereas clonal cells will show a predominant band that represents the size of the VDJ region of the dominant clone. Similarly, sometimes it can be difficult to distinguish neoplastic from reactive T-cell infiltrates. Given the large number of T-cell antigen receptors, it is not as simple to detect clonality by IHC or flow cytometry in T-cell proliferations. One approach is to use aberrant loss of T-cell antigen expression to aid in the diagnosis of T-cell neoplasms. Another is to detect clonal rearrangement of the VDJ region of the T-cell receptor gamma (*TCR*γ) gene, which can be done by PCR on both fresh and formalin-fixed paraffin-embedded (FFPE) tissue.

Gene amplification is another important mechanism in cancer that has been found to have high utility in a subset of cancers. *MYCN* amplification occurs in approximately 40% of undifferentiated or poorly differentiated neuroblastoma subtypes,[4,5] either appearing as double minute chromosomes or homogeneously

TABLE 3.1

Genomic Alterations as Putative Predictive Biomarkers for Cancer Therapy

Genes	Pathways	Aberration Type	Disease Examples	Putative or Proven Drugs
PIK3CA,[51,52] *PIK3R1*,[53] *PIK3R2*, *AKT1*, *AKT2*, and *AKT3*[54,55]	Phosphoinositide 3-kinase (PI3K)	Mutation or amplification	Breast, colorectal, and endometrial cancer	■ PI3K inhibitors ■ AKT inhibitors
PTEN[56]	PI3K	Deletion	Numerous cancers	■ PI3K inhibitors
MTOR,[57] *TSC1*,[58] and *TSC2*[59]	mTOR	Mutation	Tuberous sclerosis and bladder cancer	■ mTOR inhibitors
RAS family (*HRAS*, *NRAS*, *KRAS*), *BRAF*,[60] and *MEK1*	RAS–MEK	Mutation, rearrangement, or amplification	Numerous cancers, including melanoma and prostate cancers	■ RAF inhibitors ■ MEK inhibitors ■ PI3K inhibitors
Fibroblast growth factor receptor 1 (*FGFR1*), *FGFR2*, *FGFR3*, *FGFR4*[36]	FGFR	Mutation, amplification, or rearrangement	Myeloma, sarcoma, and bladder, breast, ovarian, lung, endometrial, and myeloid cancers	■ FGFR inhibitors ■ FGFR antibodies
Epidermal growth factor receptor (*EGFR*)	EGFR	Mutation, deletion, or amplification	Lung and gastrointestinal cancer	■ EGFR inhibitors ■ EGFR antibodies
ERBB2[61]	ERBB2	Amplification or mutation	Breast, bladder, gastric, and lung cancers	■ ERBB2 inhibitors ■ ERBB2 antibodies
SMO[62,63] and *PTCH1*[64]	Hedgehog	Mutation	Basal cell carcinoma	■ Hedgehog inhibitor
MET[65]	MET	Amplification or mutation	Bladder, gastric, and renal cancers	■ MET inhibitors ■ MET antibodies
JAK1, *JAK2*, *JAK3*,[66] *STAT1*, *STAT3*	JAK–STAT	Mutation or rearrangement	Leukemia and lymphoma	■ JAK–STAT inhibitors ■ STAT decoys
Discoidin domain-containing receptor 2 (*DDR2*)	RTK	Mutation	Lung cancer	■ Some tyrosine kinase inhibitors
Erythropoietin receptor (*EPOR*)	JAK–STAT	Rearrangement	Leukemia	■ JAK–STAT inhibitors
Interleukin-7 receptor (*IL-7R*)	JAK–STAT	Mutation	Leukemia	■ JAK–STAT inhibitors
Cyclin-dependent kinases (*CDKs*[67]; *CDK4*, *CDK6*, *CDK8*), *CDKN2A*, and cyclin D1 (*CCND1*)	CDK	Amplification, mutation, deletion, or rearrangement	Sarcoma, colorectal cancer, melanoma, and lymphoma	■ CDK inhibitors
ABL1	ABL	Rearrangement	Leukemia	■ ABL inhibitors
Retinoic acid receptor-α (*RARA*)	RARα	Rearrangement	Leukemia	■ All-trans retinoic acid
Aurora kinase A (*AURKA*)[68]	Aurora kinases	Amplification	Prostate and breast cancers	■ Aurora kinase inhibitors
Androgen receptor (*AR*)[69]	Androgen	Mutation, amplification, or splice variant	Prostate cancer	■ Androgen synthesis inhibitors ■ Androgen receptor inhibitors
FLT3[70]	FLT3	Mutation or deletion	Leukemia	■ FLT3 inhibitors
MET	MET–HGF	Mutation or amplification	Lung and gastric cancers	■ MET inhibitors
Myeloproliferative leukemia (*MPL*)	THPO, JAK–STAT	Mutation	Myeloproliferative neoplasms	■ JAK–STAT inhibitors
MDM2[71]	MDM2	Amplification	Sarcoma and adrenal carcinomas	■ MDM2 antagonist
KIT[72]	KIT	Mutation	GIST, mastocytosis, and leukemia	■ KIT inhibitors
PDGFRA and *PDGFRB*	PDGFR	Deletion, rearrangement, or amplification	Hematologic cancer, GIST, sarcoma, and brain cancer	■ PDGFR inhibitors
Anaplastic lymphoma kinase (*ALK*)[9,37,73,74]	ALK	Rearrangement or mutation	Lung cancer and neuroblastoma	■ ALK inhibitors

(continued)

TABLE 3.1

Genomic Alterations as Putative Predictive Biomarkers for Cancer Therapy *(continued)*

Genes	Pathways	Aberration Type	Disease Examples	Putative or Proven Drugs
RET	RET	Rearrangement or mutation	Lung and thyroid cancers	■ RET inhibitors
ROS1[75]	ROS1	Rearrangement	Lung cancer and cholangiocarcinoma	■ ROS1 inhibitors
NOTCH1 and *NOTCH2*	Notch	Rearrangement or mutation	Leukemia and breast cancer	■ Notch signalling pathway inhibitors

PIK3CA, PI3K catalytic subunit-α; *PIK3R1*, PI3K regulatory subunit 1; *PI3K*, phosphoinositide 3-kinase; *AKT*, v-akt murine thymoma viral oncogene homolog; *PTEN*, phosphatase and tensin homolog; mTOR, mechanistic target of rapamycin; *TSC1*, tuberous sclerosis 1 protein; RAS–MEK, rat sarcoma; *MEK*, MAPK/ERK (mitogen-activated protein kinase/extracellular signal-regulated kinase) kinase; RAF, v-raf murine sarcoma viral oncogene homolog; *ERBB2*, also known as *HER2*; *SMO*, smoothened homolog; *PTCH1*, patched homolog; *MET*, hepatocyte growth factor receptor; *JAK*, Janus kinase; *THPO*, thrombopoietin; *STAT*, signal transducer and activator of transcription; RTK, receptor tyrosine kinase; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; ABL, Abelson murine leukemia viral oncogene homolog 1; *FLT3*, FMS-like tyrosine kinase 3; *HGF*, hepatocyte growth factor; *MDM2*, mouse double minute 2; *KIT*, v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog; GIST, gastrointestinal stromal tumor; PDGFR, platelet-derived growth factor receptor; *ROS1*, v-ros avian UR2 sarcoma virus oncogene homolog.

staining regions. *MYCN* amplification is a very strong predictor of poor outcomes, particularly in patients with localized (stage 1 or stage 2) disease or in infants with stage 4S metastatic disease, where fewer than half of patients survive beyond 5 years.[6]

Use of *other chromosome abnormalities* has been largely limited to the diagnosis and prognostication of hematologic disorders. Roughly half of all myelodysplastic disorders show cytogenetically detectable chromosomal abnormalities, such as monosomy 5 or 7, partial chromosomal loss (5q-, 7q-), or complex chromosomal abnormalities. Certain abnormalities in isolation (e.g., 5q-) have a favorable prognosis, whereas many others (e.g., "complex" karyotypes with three or more abnormalities) carry a worse prognosis. Differences in ploidy have proven to be useful predictors in pediatric acute lymphocytic leukemia (ALL), with hyperdiploid cases (>50 chromosomes) showing a distinctly more favorable course compared with hypodiploid or near diploid cases.[7] Overall, DNA ploidy can be assessed by flow cytometry. Specific chromosomal copy number alterations can be detected by *conventional karyotyping*, *array hybridization methods*, or *FISH*.

Copy number variation (CNV) represents the most common type of structural chromosomal alteration. Regions affected by CNVs range from approximately 1 kilobase to several megabases that are either amplified or deleted. It is estimated that about 0.4% of the genomes of healthy individuals differ in copy number.[8] CNVs resulting in deletion of genes such as *BRCA1*, *BRCA2*, *APC*, mismatch repair genes, and *TP53* have been implicated in a wide range of highly penetrant cancers.[9,10] CNVs can be detected by a variety of means including *FISH*, *comparative or array genomic hybridization*, or *virtual karyotyping* using *single nucleotide polymorphism (SNP) arrays*. Increasingly, CNV is detected using *next-generation sequencing*.

Large-scale sequencing of tumors has identified many *mutations* that are of potential prognostic and therapeutic significance. As will be discussed further, a wide range of strategies is available for the detection of point mutations (Fig. 3.1). It is important to

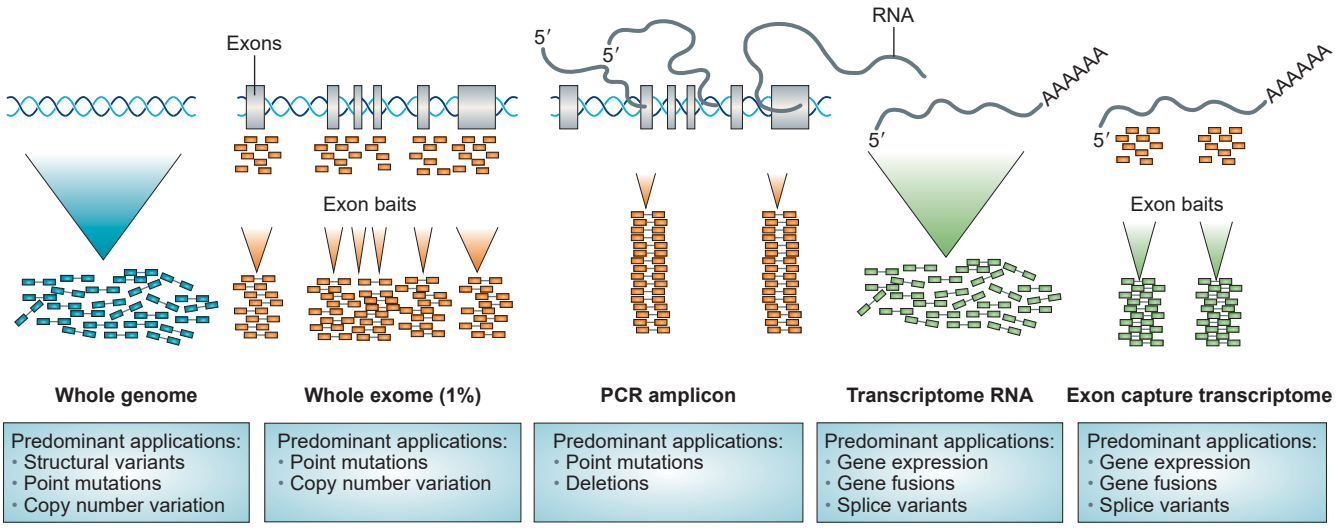


Figure 3.1 Strategies for the detection of mutations, translocations, and other structural genomic abnormalities in cancer. Whole genome sequencing, which involves determining the entire sequence of both introns and exons, is not only the most comprehensive, but also the most laborious and expensive approach. Exome sequencing uses *baits* to capture either the entire exome (roughly 20,000 genes [about 1% of the genome]) or else a subset of genes of interest. Amplicon-based sequencing uses PCR or other amplification techniques to amplify targets of interest for sequencing. Transcriptome sequencing, also known as RNAseq, is based on sequencing expressed RNA and can be used to detect not only mutations, but also translocations, other structural abnormalities, as well as differences in expression levels. This can be combined with exome capture techniques for a higher sensitivity analysis of genes of particular interest. (Reprinted by permission from Macmillan Publishers Limited: Nature Reviews Drug Discovery, Simon, R. and Rowchodhury, S. 12:358–369, 2013, ©2013.)

recognize that many nucleotide variations occur at any given allele in populations. Formally, the term *polymorphism* is used to describe genetic differences present in ≥1% of the human population, whereas *mutation* describes less frequent differences. However, in practice, *polymorphism* is often used to describe a nonpathogenic genetic change, and mutation a deleterious change, regardless of their frequencies.

Mutations can be classified according to their effect in the structure of a gene. The most common of these disease-associated alterations are single nucleotide substitutions (point mutations); however, many deletions, insertions, gene rearrangements, gene amplification, and copy number variations have been identified that have clinical significance. Point mutations may affect promoters, splicing sites, or coding regions. Coding region mutations can be classified into three kinds, depending on the impact on the codon: *missense mutation*, a nucleotide change leads to the substitution of an amino acid to another; *nonsense mutation*, a nucleotide substitution causes premature termination of codons with protein truncation; and *silent mutation*, a nucleotide change does not change the coded amino acid.

Loss of function mutations, either through point mutations or deletions in tumor suppression genes such as *APC* and *TP53*, are the most common mutations in cancers. Tumor suppression genes require two-hit (biallelic) mutations that inactivate both copies of the gene in order to allow tumorigenesis to occur. The first hit is usually an inherited or somatic point mutation, and the second hit is assumed to be an acquired deletion mutation that deletes the second copy of the tumor suppression gene. Promoter methylation of tumor suppressor genes is an alternative route to tumorigenesis that, to date, has not been commonly employed for molecular diagnostics.

Oncogenes originate from the deregulation of genes that normally encode for proteins associated with cell growth, differentiation, apoptosis, and signal transduction (proto-oncogenes, [e.g., *BRAF* and *KRAS*]). Proto-oncogenes generally require only one gain of function or activating mutation to become oncogenic. Common mutation types that result in proto-oncogene activation include point mutations, gene amplifications, and chromosomal translocations. One example is mutations in the epidermal growth factor receptor (*EGFR*) that occur in lung cancer, which are almost exclusively seen in nonmucinous bronchoalveolar carcinomas. Somatic mutations of *EGFR* constitutively activate the receptor tyrosine kinase (TK). Importantly, responsiveness of tumors harboring these mutations to the inhibitor gefitinib is highly coordinated with a mutation of the EGFR TK domain.[11,12]

One of the challenges with using mutations as biomarkers is that there can be many nucleotide alterations that affect a given gene. For example, there are over 100 known different point mutations in *EGFR* reported in non–small-cell lung cancer. Many of these mutations occur at low frequency and have an unknown clinical significance.[13,14] Another important concept is that the same driver oncogene may be mutated in a variety of different tumors. For example, lung cancers harbor a number of other different alterations that are common in other solid tumors, which generally occur at lower frequencies than *EGFR* mutations such as *KRAS*, *BRAF*, and *HER2*. Some lung cancers have translocations involving the *ALK* kinase gene. *ALK*, interestingly, is also activated by point mutations in a neuroblastoma as by translocation in anaplastic large cell lymphoma (Fig. 3.2). Hence, a therapy targeted to a genetic alteration in one cancer may demonstrate efficacy in other cancers.

The detection of mutations is also important in the evaluation of chemotherapy resistance. Roughly a third of CML patients are resistant to the frontline ABL1 kinase inhibitor imatinib, either at the time of initial treatment or, more commonly, secondarily. In cases of primary failure or secondary failure, over 100 different *ABL1* mutations have been identified, including particularly common ones such as T315I and P loop mutations. While some

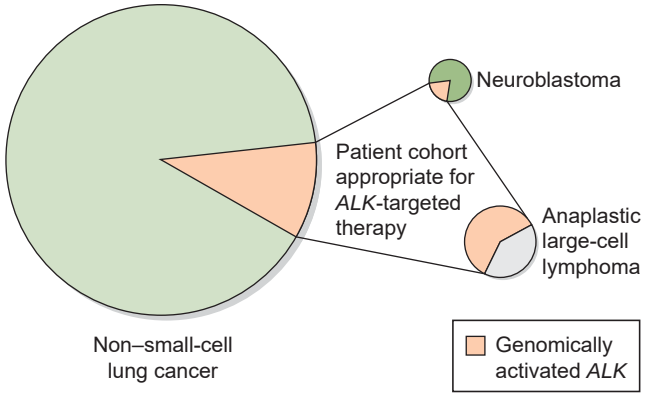


Figure 3.2 Activating genomic alterations occur in a variety of tumor types. *ALK* translocations, mutations, and amplifications occur in non–small-cell lung cancer, neuroblastomas, and in anaplastic large cell lymphomas. Such recurrent alterations in cancer, together with effective inhibitors of these pathways, are transforming oncologic therapies from organ-specific to pathway-specific interventions and are driving the use of molecular diagnostics in a wider range of tumor types (Reprinted with permission. © 2009 American Society of Clinical Oncology. All rights reserved. From McDermott, U. and Settleman, J. *J Clin Oncol* 2009;27:5650–5659.)

mutations, such as Y253H, respond to second generation TK inhibitors (TKI), others, such as the T315I mutation, are noteworthy because they confer resistance not only to imatinib, but also to nilotinib and dasatinib.

Mutations are also used as important predictive biomarkers (Table 3.1). Two of the most notable examples are the use of the *BRCA1* and *BRCA2* mutation analysis for women with a strong family history of breast cancer. Over 200 mutations (loss of function point mutations, small deletions, or insertions) occur in *BRCA* genes, which are distributed across the genes necessitating full sequencing for their detection. The overall prevalence of these occur in about 0.1% of the general population.[15,16] The lifetime risk of breast cancer for women carrying *BRCA1* mutations is in the range of 47% to 66%, whereas for *BRCA2* mutations, it is in the range of 40% to 57%.[17,18] In addition, the risk of other tumors including ovarian, fallopian, and pancreatic cancer is also increased. Detection of *BRCA1* and *BRCA2* mutations is, therefore, important for cancer prevention and risk reduction.

THE CLINICAL MOLECULAR DIAGNOSTICS LABORATORY: RULES AND REGULATIONS

Laboratories in the United States that perform molecular diagnostic testing are categorized as high-complexity laboratories under the Clinical Laboratory Improvement Amendments of 1988 (CLIA).[19] The CLIA program sets the minimum administrative and technical standards that must be met in order to ensure quality laboratory testing. Most laboratories in the United States that perform clinical testing in humans are regulated under CLIA. CLIA-certified laboratories must be accredited by professional organizations such as the Joint Commission, the College of American Pathologists, or another agency officially approved by the Centers for Medicare & Medicaid Services (CMS), and must comply with CLIA standards and guidelines for quality assurance. Although the regulation of laboratory services is in the U.S. Food and Drug Administration's (FDA) jurisdiction, the FDA has historically exercised enforcement discretion. Therefore, FDA approval is not currently required for clinical implementation of molecular tests as long as other regulations are met.[20,21]

SPECIMEN REQUIREMENTS FOR MOLECULAR DIAGNOSTICS

Samples typically received for molecular oncology testing include blood, bone marrow aspirates and biopsies, fluids, organ-specific fresh tissues in saline or tissue culture media such as Roswell Park Memorial Institute (RPMI), FFPE tissues, and cytology cell blocks. Molecular tests can be ordered electronically or through written requisition forms, but never through verbal requests only. All samples submitted for molecular testing need to be appropriately identified. Sample type, quantity, and specimen handling and transport requirements should conform to the laboratory's stated requirements in order to ensure valid test results.

Blood and bone marrow samples should be drawn into anticoagulated tubes. The preferred anticoagulant for most molecular assays is ethylenediaminetetraacetic acid (EDTA; lavender). Other acceptable collection tubes include ACD (yellow) solutions A and B. Heparinized tubes are not preferred for most molecular tests because heparin inhibits the polymerase enzyme utilized in PCR, which may lead to assay failure. Blood and bone marrow samples can be transported at ambient temperature. Blood samples should never be frozen prior to separation of cellular elements because this causes hemolysis, which interferes with DNA amplification. Fluids should be transported on ice. Tissues should be frozen (preferred method) as soon as possible and sent on dry ice to minimize degradation. Fresh tissues in RPMI should be sent on ice or cold packs. Cells should be kept frozen and sent on dry ice; DNA samples can be sent at ambient temperature or on ice.

For FFPE tissue blocks, typical collection and handling procedures include cutting 4 to 6 microtome sections of 10-micron thickness each on uncoated slides, air-drying unstained sections at room temperature, and staining one of the slides with hematoxylin and eosin (H&E). A board-certified pathologist reviews the H&E slides to ensure the tissue block contains a sufficient quantity of neoplastic tumor cells, and circles an area on the H&E slide that will be used as a template to guide macrodissection or microdissection of the adjacent, unstained slides. The pathologist also provides an estimate of the percentage of neoplastic cells in the area that will be tested, which should exceed the established limit of detection (LOD) of the assay.

MOLECULAR DIAGNOSTICS TESTING PROCESS

The workflow of a molecular test begins with receipt and accessioning of the specimen in the clinical molecular diagnostics laboratory followed by extraction of the nucleic acid (DNA or RNA), test setup, detection of analyte (e.g., PCR products), data analysis, and result reporting to the patient medical record (Fig. 3.3).

An extraction of intact, moderately high-quality DNA is essential for molecular assays. For DNA extraction, the preferred age for blood, bone marrow, and fluid samples is less than 5 days; for frozen or fixed tissue, it is indefinite; and for fresh tissue, it is overnight. Although there is no age limit for the use of a fixed and embedded tissue specimen for analysis, older specimens may yield a lower quantity and quality of DNA. Because RNA is significantly more labile than DNA, the preferred age for blood and bone marrow is less than 48 hours (from time of collection). Tissue samples intended for an RNA analysis should be promptly processed in fresh state, snap frozen, or preserved with RNA stabilizing agents for transport.

Dedicated areas, equipment, and materials are designated for various stages of DNA and RNA extraction procedures. DNA and RNA isolation can be done by manual or automated methods. Currently, most clinical laboratories employ commercial protocols based on liquid- or solid-phase extractions. Nucleated cells are isolated from biological samples prior to nucleic acid extraction.

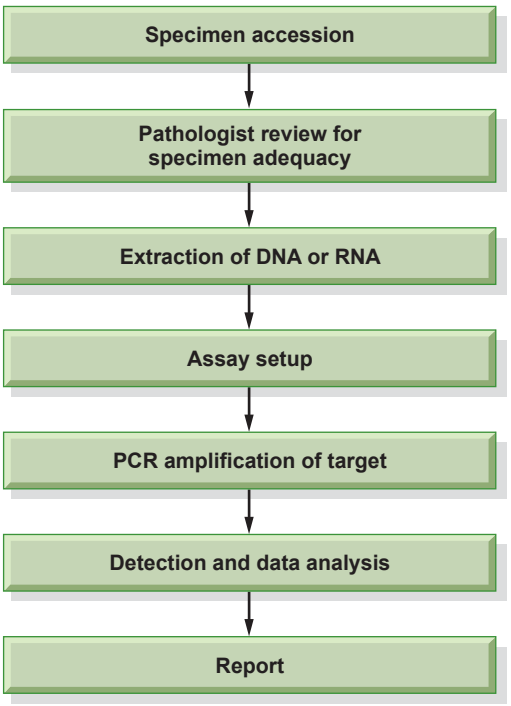


Figure 3.3 Simplified workflow of clinical molecular diagnostic testing.

White blood cells (WBC) can be isolated from blood and bone marrow samples by different methods. One method involves lysing the red blood cells with an ammonium chloride solution, which yields the total WBC population and other nucleated cells present. Another method involves a gradient preparation with a Ficoll solution, which yields the mononuclear cell population only. Sections of FFPE tissue blocks are prepared for DNA extraction by first removing the paraffin and disrupting the cell membranes with proteinase K digestion. Fresh and frozen tissues also undergo proteinase K digestion prior to nucleic acid extraction. DNA isolation protocols consist of several steps, including cell lysis, DNA purification by salting out the proteins and other debris (nonorganic method), or by solvent extractions of the proteins with phenol and chloroform solutions (organic method). The DNA is then precipitated out of the solution with isopropanol or ethanol. The pellet is washed with 70% to 80% ethanol and then solubilized in buffer, such as Tris-EDTA solution. Proteinase K can be added to assist in the disruption and to prevent nonspecific degradation of the DNA. RNase is sometimes added to eliminate contaminating RNA. The DNA yield is quantitated spectrophotometrically, and the DNA sample integrity is visually checked, if necessary, on an agarose gel followed by ethidium bromide staining. Intact DNA appears as a high–molecular-weight single band, whereas degraded DNA is identified as a smear of variably sized fragments. After extraction, the DNA is stored at 4°C prior to use in a PCR assay, and is then stored at −70°C after completion of the assay. Because the DNA extracted from formalin-fixed tissue is degraded to a variable extent, an analysis of the extraction product by gel electrophoresis is not informative. Yield and integrity of the extracted DNA is best assessed by an amplification control to ensure that the quality and quantity of input DNA is adequate to yield a valid result.

RNA isolation steps are similar to the ones described previously for DNA extraction. However, RNA is inherently less stable than DNA due to its single-strand conformation and susceptibility to degradation by RNase, which is ubiquitous in the environment. To ensure preservation of target RNA, special precautions are required, including the use of diethylpyrocarbonate (DEPC) water in all reagents used in RNA procedures, and special decontamination

of work area and pipettes to prevent RNase contamination. The extracted RNA is usually degraded to a variable extent so that the analysis of the extraction product by gel electrophoresis is not informative. The quality of the RNA and its suitability for use in a reverse transcriptase polymerase chain reaction (RT-PCR)–based assay is assessed most appropriately by the demonstration of a positive result in an assay designed to detect the RNA transcripts for a "housekeeping gene," such as *ABL1* or *GAPDH*. Any RNA sample in which the 260/280-nm absorption ratio is below 1.9 or greater than 2.0 may contain contaminants and must be cleaned prior to analysis.

Following nucleic acid extraction, the assay is set up according to written procedures established during validation/verification of the assay by qualified laboratory staff. Dedicated areas, equipment, and materials are designated for various stages of the test (e.g., extraction, pre-PCR and post-PCR for amplification-based assays). For each molecular oncology test, appropriated positive and negative control specimens are included to each run as a matter of routine quality assessment. A no template (blank) control, containing the complete reaction mixture except for nucleic acids, is also included in amplification-based assays to evaluate for amplicon contamination in the assay reagents that may lead to inaccurate results. The controls are processed in the same manner as patient samples to ensure that established performance characteristics are being met for each step of the assay (extraction, amplification, and detection). All assay controls and overall performance of the run must be examined prior to interpretation of sample results. Following acceptance of the controls, results are electronically entered into reports. The final report is reviewed and signed by the laboratory director or a qualified designee who meets the same qualifications as the director, as defined by CLIA (see previous).

TECHNOLOGIES

Several traditional and emerging techniques are currently available for mutation detection in cancer (Table 3.2). In the era of personalized medicine, molecular oncology assays are rapidly moving from a mutational analysis of single genes toward a multigene panel analysis. As the number of "actionable" mutations such as *ALK*, *EGFR*, *BRAF*, and others increase, the use of next-generation sequencing platforms is expected to become much more widespread. Both traditional and emerging testing approaches have advantages and disadvantages that need to be balanced before a test platform is implemented into practice.

An important consideration when adding a new oncology test in the clinical laboratory menu is to define the intended use of the assay (e.g., diagnosis, prognosis, prediction of therapy response). The clinical utility of the assay, appropriate types of specimens, the spectrum of possible mutations that can be found in the genomic region of interest, and available methods for testing should also be determined. The laboratory director and ordering physicians should also discuss the estimated test volume, optimal reporting format, and required turnaround time for the proposed new test.[21–23]

Polymerase Chain Reaction

Polymerase chain reaction (PCR)[24,25] is widely used in all molecular diagnostics laboratories for the rapid amplification of targeted DNA sequences. The reaction includes the specimen template DNA, forward and reverse primers (18 to 24 oligonucleotides long), Taq DNA polymerase, and each of the four nucleotides bases (dATP, dTTP, dCTP, dGTP). During PCR, selected genomic sequences undergo repetitive temperature cycling (sequential heat and cooling) that allows for *denaturation* of double-stranded DNA template, *annealing* of the primers to the targeted complementary sequences on the template, and *extension* of new strands of DNA by Taq polymerase from nucleotides, using the primers as the starting point. Each cycle doubles the copy number of PCR templates for the next round of polymerase activity, resulting in an exponential amplification of the selected target sequence. The PCR products (amplicons) are detected by electrophoresis or in real-time systems simultaneously to the amplification reaction (see real-time PCR, which follows).

PCR is specifically designed to work on DNA templates because the Taq polymerase does not recognize RNA as a starting material. Nonetheless, PCR can be adapted to RNA testing by including a reverse transcription step to convert a RNA sequence into its cognate cDNA sequence before the PCR reaction is performed (see reverse-transcription PCR, which follows). Multiplex PCR reactions can also be designed with multiple primers for simultaneous amplification of multiple genomic targets. PCR is a highly sensitive and specific technique that can be employed in different capacities for the detection of point mutations, small deletions, insertions and duplications, as well as gene rearrangements and clonality assessment. Limits of detection can reach 0.1% mutant allele or lower, which is important for the detection of somatic mutations in oncology because tumor specimens are usually composed of a mixture of tumor and normal cells. Reverse transcription PCR can also be used for the relative quantification of target RNA in minimal residual disease testing, such as *BCR-ABL1* transcripts in CML. Another advantage of PCR is its ability to amplify small amounts of low quality FFPE-derived DNA. However, applications of PCR can be limited because it cannot amplify across large or highly repetitive genomic regions. Also, the PCR reaction can be inhibited by heparin or melanin if present in the extracted DNA, which may lead to assay failure. Finally, the risk of false positives due to specimen or amplicon contamination is an important issue when using PCR-based techniques; therefore, stringent laboratory procedures, as described previously, are used to minimize contamination. With the exception of hybridization assays, such as fluorescence in situ hybridization and genomic microarrays, PCR is the necessary initial step in all current molecular oncology assays.

Targeted Mutation Analysis Methods

Real-Time PCR (q-PCR)

In real-time PCR (q-PCR), the polymerase chain reaction is performed with a PCR reporter that is usually a fluorescent double-stranded DNA binding dye or a fluorescent reporter probe. The intensity of the fluorescence produced at each amplification cycle is monitored in real time, and both quantification and detection of targeted sequences is accomplished in the reaction tube as the PCR amplification proceeds.

The intensity of the fluorescent signal for a given DNA fragment (wild type or mutant) is correlated with its quantity, based on the PCR cycle in which the fluorescence rises above the background (crossing threshold [Ct] or crossing point [Cp]).[26] The Ct value can be used for qualitative or quantitative analysis. Qualitative assays use the Ct as a cutoff for determining "presence" or "absence" of a given target in the reaction. A qualitative analysis by q-PCR is particularly useful for a targeted detection of point mutations that are located in mutational hotspots. Examples include the *JAK2* V617F mutation, which is located within exon 14, and is found in several myeloproliferative neoplasms (polycythemia vera, essential thrombocythemia, and primary myelofibrosis),[27] and the *BRAF* V600E,[28] which is located within exon 15, and is found in various cancer types including melanomas and thyroid and lung cancers.

For a quantitative analysis, the Ct of standards with known template concentration is used to generate a standard curve to which Ct values of unknown samples are compared. The concentration of the unknown samples is then extrapolated from values from the standard curve. The quantity of amplicons produced in a PCR reaction is proportional to the prevalence of the targeted sequence;

TABLE 3.2

Molecular Methods in Oncology

Method	Advantages	Disadvantages	Analytic Sensitivity	Examples of Applications in Oncology
Real-time PCR (q-PCR) Allele-specific PCR (AS-PCR) Reverse transcriptase PCR (RT-PCR)	Flexible platforms that permit detection of a variety of conserved hotspot mutations including nucleotide substitutions, small length mutations (deletions, insertions), and translocations High sensitivity is beneficial for residual disease testing and specimens with limited tumor content Adaptable to quantitative assays	Detects only specific targeted mutations/ chromosomal translocations Not suitable for variable mutations May not determine the exact change in nucleotide sequence	Very high	*KRAS, BRAF,* and *EGFR* mutations in solid tumors *JAK2* V617F and *MPL* mutations in myeloproliferative neoplasms *KIT* D816V mutation in systemic mastocytosis and AML Quantitation of *BCR-ABL1* and *PML-RARA* transcripts for residual disease monitoring in CML and APL, respectively
Fragment analysis	Detects small to medium insertions and deletions Detects variable insertions and deletions regardless of specific alteration Provides semiquantitative information regarding mutation level	Does not determine the exact change in nucleotide sequence Does not detect single nucleotide substitution mutations Limited multiplex capability	High	*NPM1* insertion mutations in AML *FLT3* internal tandem duplications in AML *JAK2* exon 12 insertions and deletions in PV *EGFR* exon 19 deletions in NSCLC
FISH	Detects chromosomal translocation, gene amplification, and deletion Morphology of tumor is preserved, allowing for a more accurate interpretation of heterogeneous samples	High cost Unable to detect small insertions and deletions Limited multiplex capability Does not determine the exact breakpoint and change in nucleotide sequence	High	*IGH/BCL2* translocation detection in follicular lymphoma and in a subset of diffuse large B-cell lymphoma *ALK* translocation in NSCLC *EWSR1* translocation in soft tissue tumors *HER2* amplification in breast cancer 1p/19q deletion in oligodendroglioma
High-resolution melting (HRM) curve analysis	Qualitative detection of variable single nucleotide substitutions and small insertions and deletions	Does not determine the exact mutation Result interpretation may require testing via an alternate technology Limited multiplex capability	Medium	*KRAS* and *BRAF* mutations in solid tumors *JAK2* exon 12 mutations in PV
Sanger sequencing	Detects variable single nucleotide substitutions and small insertions and deletions Provides semiquantitative information about mutation level Current gold standard for mutation detection	Low throughput Low analytic sensitivity limits application in specimens with low tumor burden Does not detect copy number changes or large (>500 bp) insertions and deletions	Low	*KIT* mutations in GIST and melanoma *CEBPA* mutations in AML *EGFR* mutations in NSCLC
Pyrosequencing	Higher analytical sensitivity than Sanger sequencing Detects variable single nucleotide substitutions and small insertions and deletions Provides quantitative information about mutation level	Short read lengths limit analysis to mutational hotspots Low throughput	Medium	*KRAS* and *BRAF* mutations in solid tumors
Single nucleotide extension assay (SNaPshot)	Simultaneous detection of targeted nucleotide substitution mutations Multiplex capability	Detects only targeted mutations	High	Small gene panels (3–10) for melanoma, NSCLC, breast cancer, and metastatic colorectal cancer

(continued)

TABLE 3.2

Molecular Methods in Oncology *(continued)*

Method	Advantages	Disadvantages	Analytic Sensitivity	Examples of Applications in Oncology
Next-generation sequencing (NGS)	Quantitative detection of variable single nucleotide substitutions, small insertions and deletions, chromosomal translocations, and gene copy number variations Highly multiplexed High throughput	Requires costly investment in instrumentation and bioinformatics Technology is rapidly evolving Higher error rates for insertion and deletion mutations Limited ability to sequence GC-rich regions	High	Small to large gene panels (3–500) for solid tumor and hematologic malignancies
Genomic microarray	Simultaneous detection of copy number variation and LOH (SNP array)	Limited application to FFPE tissue Does not detect balanced translocations May not detect low-level mutant allele burden	Medium	Analysis of recurrent copy number variation and LOH in chronic lymphocytic leukemia and myeloproliferative neoplasms

AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; APL, acute promyelocytic leukemia; PV, polycythemia vera; NSCLC, non–small-cell lung carcinoma; GIST, gastrointestinal stromal tumor; GC, guanine-cytosine; LOH, loss of heterozygosity; FFPE, formalin-fixed paraffin-embedded.

therefore, samples with a higher template concentration reaches the Ct at earlier PCR cycles than one with a low concentration of the amplified target. Quantitative q-PCR has high analytical sensitivity for the detection of low mutant allele burden. For that reason, this method has been widely utilized for monitoring minimal residual disease.

Allele-Specific PCR

Allele-specific PCR (AS-PCR) is a variant of conventional PCR. The method is based on the principle that Taq polymerase is incapable of catalyzing chain elongation in the presence of a mismatch between the 3′ end of the primer and the template DNA. Selective amplification by AS-PCR is achieved by designing a forward primer that matches the mutant sequence at the 3′ end primer. A second mismatch within the primer can be introduced at the adjacent -1 or -2 position to decrease the efficiency of mismatched amplification products. This will minimize the chance of amplifying and, therefore, detecting the wild-type target. AS-PCR is usually performed as two PCR reactions: one employing a forward primer specific for the mutant sequence, the other using a forward primer specific for the correspondent wild-type sequence. In this case, a common reverse primer is used for both reactions. Following amplification, the PCR products are detected by electrophoresis (capillary or agarose gel) or in q-PCR systems. The detection of adequate PCR product in the wild-type amplification reaction is important to control for adequate specimen quality and quantity, particularly when the specimen is negative in the mutation-specific PCR reaction.

AS-PCR is particularly useful for the detection of targeted point mutations. Multiplex AS-PCR reactions can be designed for the simultaneous detection of multiple mutations by including several mutation-specific primers. The method has high analytical sensitivity and specificity and can be easily deployed in most clinical laboratories. However, an important limitation is that this approach will not detect mutations other than those for which specific primers are designed. Therefore, it is utilized for highly recurrent mutations that occur at specific locations within genes, rather than for the detection of variable mutations that may occur throughout a gene.

Examples of AS-PCR applications in oncology include the detection of *JAK2* V617F and *MPL* mutations in myeloproliferative neoplasms (primary myelofibrosis, essential thrombocythemia, and/or polycythemia vera),[29] the *BRAF* V600E mutation,[30] and *KIT* D816V mutations in cases of systemic mastocytosis and in acute myelogenous leukemia (AML).

Reverse Transcriptase PCR

RT-PCR is utilized for the detection and quantification of RNA transcripts. The first step for all amplification-based assays that use RNA as a starting material is reverse transcription of RNA into cDNA, because RNA is not a suitable substrate for Taq polymerase. In RT-PCR, RNA is isolated and reverse transcribed into cDNA by using a reverse transcriptase enzyme and one of the following: (1) random hexamer primers, which anneal randomly to RNA and reverse transcribe all RNA in the cell; (2) oligo dT primers, which anneal to the polyA tail of mRNA and reverse transcribe only mRNA; or (3) gene-specific primers that reverse transcribe only the target of interest. PCR is subsequently performed on the cDNA with forward and reverse primers specific to the gene(s) of interest. The RT-PCR products may then be analyzed by capillary electrophoresis or in real-time systems as in a standard PCR reaction.

RT-PCR is commonly used for detecting gene fusions during translocation analysis because breakpoints frequently occur within the intron of each partner gene and the precise intronic breakpoint locations may be variable. This variability complicates the design of primers used in DNA-based PCR assays. RT-PCR tests are advantageous because mature mRNA has intronic sequence spliced out, allowing for simplified primer design within the affected exon of each partner gene. In this setting, RT-PCR is useful in tests where both translocation partners are recurrent and only one or a few exons are involved in each partner gene. For instance, 95% of acute promyelocytic leukemia (APL) cases harbor the reciprocal t(15;17) chromosomal translocation and these breakpoints always occur within intron 2 of the *RARA* gene. By contrast, three distinct chromosome 15 breakpoints are involved, all occurring within the *PML* gene: intron 6, exon 6, and intron 3. Because the breakpoints in the two genes are recurrent, most of the reported *PML-RARA* fusions can be detected by targeting these three transcript isoforms.

RT-PCR is the method of choice when high sensitivity is required to detect gene translocations. For example, *PML-RARA*

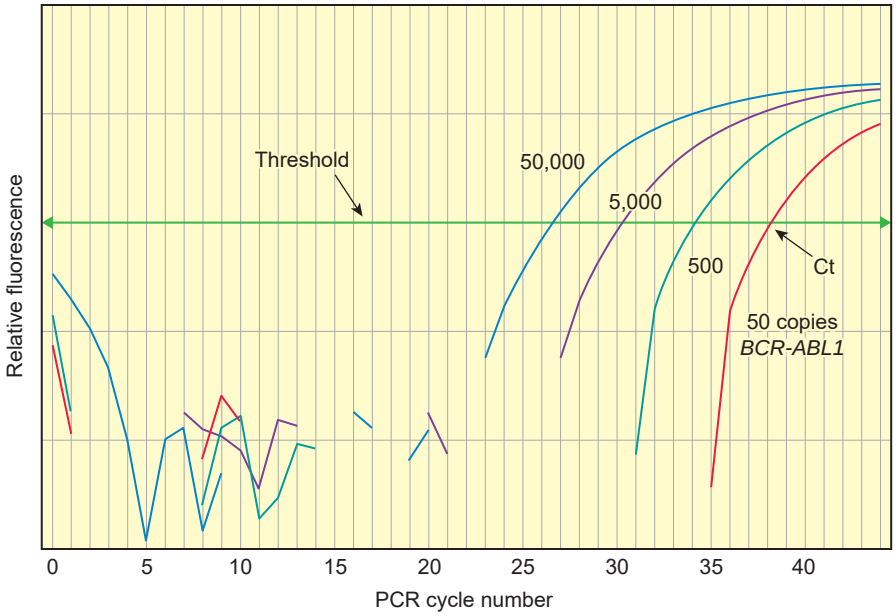


Figure 3.4 Reverse transcriptase PCR (RT-PCR) is a sensitive means to detect *BCR-ABL1* fusion transcripts in CML. RT-PCR can be combined with real-time PCR (q-PCR) to quantitate *BCR-ABL1* transcripts across four to six log range levels. Amplification products are detected during each PCR cycle using a fluorescent probe specific to the PCR product. The accumulated fluorescence in log(10) value is plotted against the number of PCR cycles. For a given specimen, the PCR cycle number is measured when the increase in fluorescence is exponential and exceeds a threshold. This point is called the Ct, which is inversely proportional to the amount of PCR target in the specimen (i.e., lower Ct values indicate a greater amount of target). Calibration standards of known quantity are used in standard curves to calculate the amount of target in a tested specimen. These are shown in the chart as different colored plots. Note that PCR increases the amount of amplification product by a factor of two with each PCR cycle. Therefore, specimens that produce a Ct value that is one cycle lower are expected to have a twofold higher concentration of target. Specimens that differ in target concentration by a factor of 10 (as shown) are expected to have a Ct value 3.3 cycles apart ($2^{3.3} = 10$).

transcript detection by RT-PCR can detect this fusion transcript down to 1 tumor cell in the background of 100,000 normal cells. Detecting low levels of fusion transcript can reveal relapse after consolidation and guide further treatment.[31] RT-PCR can also be used to quantitate the amount of expression of a gene. One major application of RT-PCR in this setting includes quantitative detection of *BCR-ABL1* fusion transcript for prognostication and minimal residual disease testing in CML (Fig. 3.4). In this setting, a three log decrease in *BCR-ABL1* levels is associated with an improved outcome.[32,33]

Fragment Analysis

A fragment analysis is a PCR amplicon-sizing technique that is relevant for the detection of small- to medium-length–affecting mutations (deletions, insertions, and duplications). This is typically performed by capillary electrophoresis, which is capable of resolving length mutations from approximately 1 to 500 base pairs in size.

Fragment analysis represents a practical strategy because it enables comprehensive detection of a wide variety of possible length mutations and has high analytic sensitivity. Further, it can provide semiquantitative information regarding the relative amount of mutated alleles. Limitations of this approach include the inability to objectively quantitate mutant allele burdens, the inability to determine the exact change in nucleotide sequence, and the inability to detect non–length-affecting mutations such as substitution mutations.

Examples of fragment analysis applications in oncology include the detection of *NPM1* insertion mutations (Fig. 3.5),[34] *EGFR* exon 19 deletions, *FLT3* internal tandem duplications, and *JAK2* exon 12 mutations.[35]

High-Resolution Melting Curve Analysis

A high-resolution melting (HRM) curve analysis is a mutation screening method that allows for the detection of DNA sequence variations based on specific sequence-related melting profiles of PCR products.[36] Because the melting property of DNA duplexes is dependent on the biophysical and chemical properties of the nucleotide sequences, mutant and wild-type DNA sequences can be differentiated from one another based on their melting characteristics.

An HRM analysis is preceded by a PCR. The reaction employs a pair of gene-specific forward and reverse primers, template DNA, and a reporter that can either be a double-stranded DNA binding dye or a fluorescent reporter probe. Following the last cycle of the PCR, the amplification products undergo a cooling step that generates homoduplexes (double-stranded molecules with perfect complementarity between alleles) and heteroduplexes (double-stranded molecules with sequence mismatch between alleles) followed by a heating step that denatures (i.e., melts) the double-stranded products. Heteroduplexes (mutant DNA) produce a melting profile different from that of wild-type samples (homoduplexes). In most cases, the reaction is performed in a q-PCR system that allows for an analysis of amplification and

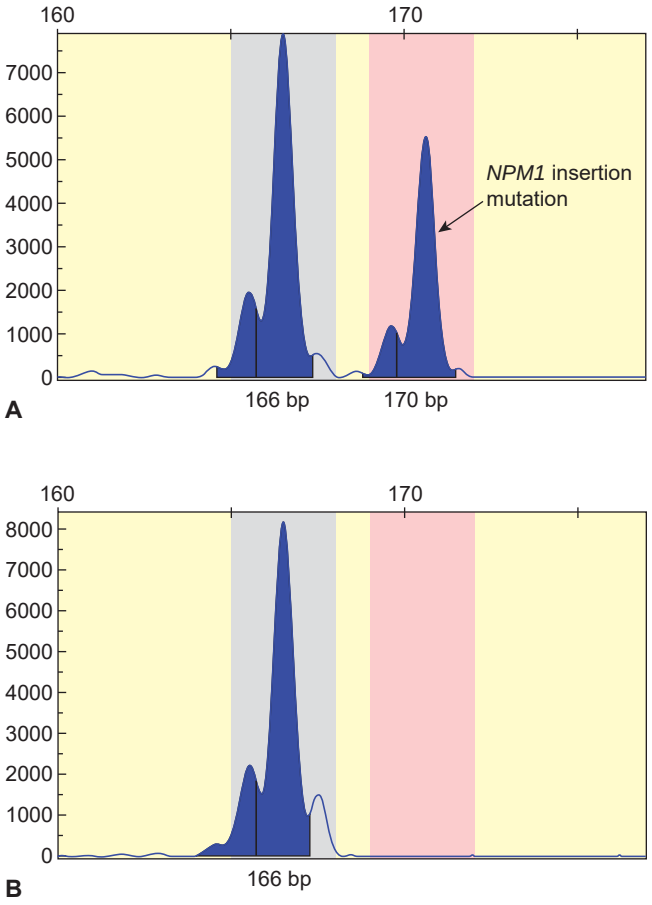


Figure 3.5 Fragment analysis. *NPM1* mutations are important prognostic markers in acute myeloid leukemia. Virtually all *NPM1* mutations result in a four nucleotide insertion within exon 12. Detection of these mutations can be accomplished by PCR utilizing primers that flank the mutation region. The amplification products are sized using capillary electrophoresis. A mutation is indicated by a PCR fragment that is 4 bp larger than the wild-type fragment. Mutation positive **(A)** and negative **(B)** cases are shown.

melting data in a close-tube format, thereby minimizing the risk of amplicon contamination.

An HRM analysis is useful for the qualitative detection of variable point mutations and small length-affecting mutations that occur within mutational hotspot regions. This method has high analytical sensitivity and can detect mutations even in a small fraction of alleles in a background of wild-type DNA. However, this assay does not characterize the specific sequence alteration in the mutant allele and may be challenging to interpret, especially for cases with mutation levels that approach the detection limit of the assay. Samples with a lower abundance of mutant alleles, and consequently a decreased fraction of heteroduplexes that produced fluorescence decay during the melting analysis, usually produce a melting curve that may not differ significantly from that of wild-type samples. Likewise, the detection of duplication mutations may be hampered by the similarity between the mutant and the duplicated wild-type genome sequences, which may produce only subtle differences in the melting behavior of the DNA duplexes, especially for samples with low mutant allele burden. Therefore, both the mutant sequence and the allelic burden play in the ability of an HRM analysis to detect mutations.[37] Poor quality and impurity of genomic DNA may also lower the sensitivity of an HRM analysis.[38] In instances of patients with a low mutant allelic burden, equivocal mutations identified by this approach may not be confirmable by an alternate method such as Sanger sequencing.

Examples of HRM applications in oncology include a mutational analysis of *KRAS* codons 12, 13, and 61[39]; a mutation screening of *BRAF* codon 600[39]; and the detection of *JAK2* exon 12 mutations (Fig. 3.6).[40]

Sanger Sequencing

Mutations in single gene assays are commonly analyzed by targeted nucleic acid sequencing, most commonly by Sanger sequencing.[41] This method, also known as dideoxy sequencing, is based on random incorporation of modified nucleotides (dideoxynucleotides [ddNTP]) into a DNA sequence during rounds of template extension that result in termination of the chain reaction at various fragment lengths. Because dideoxynucleotides lack a 3′ hydroxyl group on the DNA pentose ring, which is required for the addition of further nucleotides during extension of the new DNA strand, the chain reaction is terminated at different lengths with the random incorporation of ddNTPs to the sequence. In addition to the dideoxy modification, each ddNTP (ddATP, ddTTP, ddCTP, ddGTP) is labeled with fluorescent tags of different fluorescence wavelengths.

In this method, repetitive cycles of primer extension are performed using denatured PCR products (amplicons) as templates. Unlike PCR, in which both forward and reverse primers are added to the same reaction, in Sanger sequencing, the forward and reverse reactions are performed separately. Bidirectional sequencing is performed to ensure that the entire region of interest

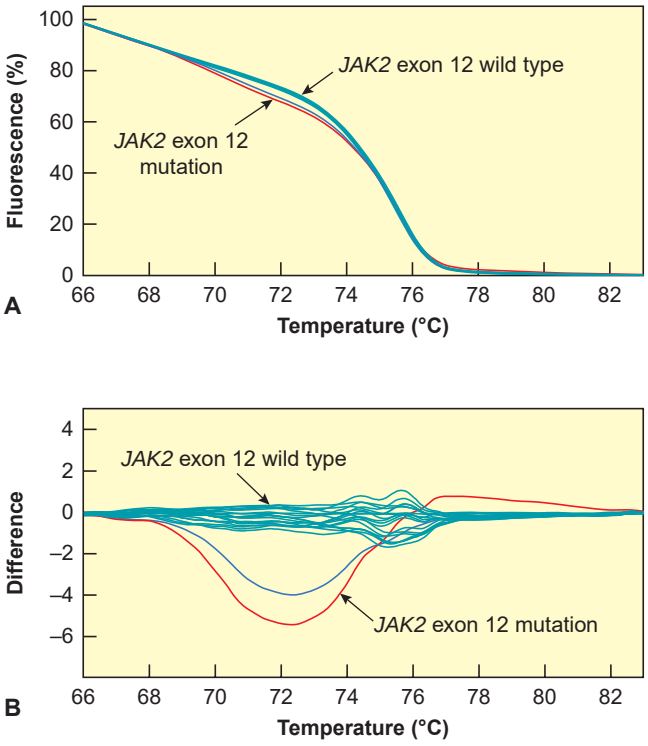
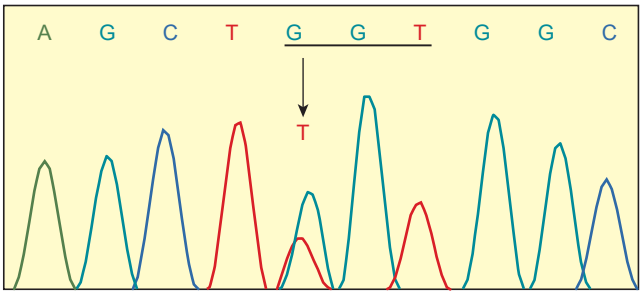


Figure 3.6 High resolution melting (HRM) curve analysis. An HRM analysis can be an efficient screening method for detecting a variety of mutations that may cluster in one or more hotspot regions, such as occurs with *JAK2* exon 12 mutations in polycythemia vera. PCR is utilized to amplify the target region in the presence of a fluorescent double-stranded DNA-binding dye. Following PCR, the product is gradually melted, and the emitted fluorescence is measured. **(A)** Plotting fluorescence versus temperature generates a melt curve characteristic of each amplicon. The presence of a mutation alters the melt profile due to mismatched double-stranded heteroduplexes of mutant and wild-type fragments. **(B)** A difference plot in which sample curves are subtracted from a wild-type control can accentuate the different melt profiles.

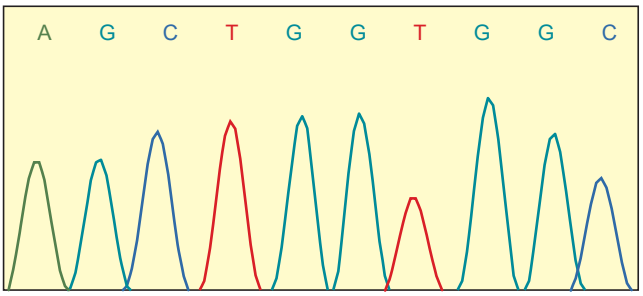
for each analysis is visualized adequately to produce unequivocal sequence readout. The sequencing products of increasing size are resolved by capillary electrophoresis, and the DNA sequence is determined by detection of the fluorescently labeled nucleotide sequences.

Sanger sequencing has the ability to detect a wide variety of nucleotide alterations in the DNA, including point mutations, deletions, insertions, and duplications. This technique is especially useful when mutations are scattered across the entire gene, when genes have not been sufficiently studied to determine mutational hot spots, or when it is relevant to determine the exact change in DNA sequence. Sanger sequencing can also provide semiquantitative information about mutation levels in a sample based on the evaluation of average peak drop values from forward and reverse mutant peaks on sequence chromatograms. Limitations of this approach include low throughput and limited diagnostic sensitivity. In general, heterozygous mutations at allelic levels lower than 20% may be difficult to detect by Sanger sequencing. This may be particularly problematic when testing for somatic mutations in oncogenes, such as *JAK2* exon 12 in polycythemia vera, which may occur at low levels.[35]

Examples of Sanger sequencing applications in oncology include the detection of *KIT* mutations for gastrointestinal stromal tumors (GIST) and melanomas that arise from mucosal membranes and acral skin, *EGFR* mutations for non–small-cell lung cancers, and *KRAS* mutations for colorectal and lung carcinomas (Fig. 3.7).



A *KRAS* G12C mutation



B *KRAS* wild type

Figure 3.7 Sanger sequencing. *KRAS* mutation testing requires a technology like Sanger sequencing, which can detect the diverse variety of mutations that span multiple nucleotide sites. Overlapping peaks in the DNA sequence chromatogram indicate the presence of a mutation. The top panel **(A)** displays a G to T nucleotide substitution in codon 12. This results in a GGT to TGT codon change, leading to a glycine to cysteine (G12C) amino acid substitution. Activating mutations in *KRAS* such as G12C are associated with resistance to epidermal growth factor receptor (EGFR) targeted therapies in colon cancer. The bottom panel **(B)** displays a wild-type *KRAS* sequence.

Pyrosequencing

Pyrosequencing, also known as *sequencing by synthesis*, is based on the real-time detection of pyrophosphate release by nucleotide incorporation during DNA synthesis.[42] In the pyrosequencing reaction, as nucleotides are added to the nucleic acid chain by polymerase, pyrophosphate molecules are released and subsequently converted to ATP by ATP sulfurylase. Light is produced by an ATP-driven luciferase reaction via oxidation of a luciferin molecule. The amount of light produced is proportional to the number of incorporated nucleotides in the sequence. When a nucleotide is not incorporated into the reaction, no pyrophosphate is released and the unused nucleotide is degraded by apyrase. Light is converted into peaks in a charge-coupled device (CCD) camera. Individual dNTP nucleotides are sequentially added to the reaction, and the sequence of nucleotides that produce chemiluminescent signals allow the template sequence to be determined. Mutations appear as new peaks in the pyrogram sequence or variations of the expected peak heights.[43]

Pyrosequencing is particularly useful for the detection of point mutations and insertion/deletion mutations that occur at short stretches in mutational hotspots. This method has higher analytical sensitivity than Sanger sequencing and can provide quantitative information about mutation levels in a sample. Pyrosequencing can also be used for the detection and quantification of gene-specific DNA methylation and gene copy number assessments. A microfluidic pyrosequencing platform is available for massive parallel sequencing. However, this method is not well suited for detecting mutations that are scattered across the entire gene because pyrosequencing read lengths are limited to ~100 to 250 base pairs.[43]

Examples of pyrosequencing applications in oncology include the mutational analysis of *BRAF* (codon 600),[44,45] *KRAS* (codons 12, 13, 61),[45] *NRAS* (codon 61),[45] and the methylation analysis of *MGMT* in glioblastoma multiforme.[46,47]

Single Nucleotide Extension Assay (SNaPshot®)

The single nucleotide extension assay is a variant of dideoxy sequencing. This method consists of a single base extension of an unlabeled primer that anneals one base upstream to the relevant mutation with fluorophore-labeled dideoxynucleotides (ddNTP). Multiplexed reactions can be designed with multiple primers of differing lengths for simultaneous amplification of multiple genomic targets.[48] Mutations are identified based on amplicon size and fluorophore color via capillary electrophoresis. When a mutation is present, an alternative dideoxynucleotide triphosphate is incorporated, resulting in a different colored peak with a different amplicon length than the expected wild-type one.

The single nucleotide extension assay is particularly useful for the simultaneous detection of recurrent point mutations. Clinically, it has been employed for analyses of mutational hotspots in multiple genes involved in melanomas, non–small-cell lung cancers, breast cancers, and metastatic colorectal cancers.[49] The assay has higher analytical sensitivity than Sanger sequencing and can detect low-level mutations in FFPE-derived DNA, making it advantageous for biopsy specimens with limited tumor involvement. This assay, however, can only detect mutations that are immediately adjacent to the 3′ to the end of the primer.

Fluorescence In Situ Hybridization

FISH allows for the visualization of specific chromosome nucleic acid sequences within a cellular preparation. This method involves the annealing of a large single-stranded fluorophore-labeled oligonucleotide probe to complementary DNA target sequences within a tissue or cell preparation. The hybridization of the probe at the specific DNA region within a nucleus is visible by direct detection using fluorescence microscopy.

FISH can be used for the quantitative assessment of gene amplification or deletion and for the qualitative evaluation of gene rearrangements. Many oncologic FISH assays employ two probe types: *locus specific probes*, which are complementary to the gene of interest, and *centromeric probes*, which hybridize to the alpha-satellite regions near the centromere of a specific chromosome and help in the enumeration of the number of copies of that chromosome.

For the quantitative assessment of gene amplification, a locus-specific probe and a centromeric probe are labeled with two different fluorophores. The signals generated by each of these probes are counted and a ratio of the targeted gene to the chromosome copy number is calculated. The amount of signal produced by the locus-specific probe is proportional to the number of copies of the targeted gene in a cell. This type of gene amplification assay can be used for the detection of *HER2* gene amplification as an adjunct to existing clinical and pathologic information as an aid in the assessment of stage II, node-positive breast cancer patients for whom Herceptin treatment is being considered. It can also be used for an assessment of *MYCN* amplification in neuroblastoma.

For the detection of deletion mutations, dual-probe hybridization is usually performed using locus-specific probes. For instance, for the detection of 1p/19q codeletion in oligodendrogliomas, locus-specific probe sets for 1p36 and 19q13, and 1q25 and 19p13 (control) are used. The frequencies of signal patterns for each of these loci are evaluated. A signal pattern with 1p and 19q signals that are less than control signals is consistent with deletion of these loci.

Gene rearrangements/chromosomal translocations in hematologic or solid malignancies can be tested using locus-specific dual-fusion or break-apart probes. Dual-color, dual-fusion translocation assays employ two probes that are located in two separate genes involved in a specific rearrangement. Each gene probe is labeled in a different color. This design detects translocations by the juxtaposition of both probe signals. Dual-color, dual-fusion translocation assays are very specific for detecting a selected translocation. But, it can only be used for detecting translocations that involve consistent partners, where both partners are known. Alternate translocations with different fusion partners are not detected by this approach. Examples of application of dual-fusion probes in oncology include for the detection of the *IGH-BCL2* translocation that occurs in most follicular lymphomas and a subset of diffuse large B-cell lymphomas (Fig. 3.8) and for the detection of *IGH-CCND1* rearrangements in mantle cell lymphomas.

In break-apart FISH assays, both dual-colored probes flank the breakpoint region in a single gene that represents the constant partner in the translocation. By this approach, rearranged alleles show two split signals, whereas normal alleles show fusion signals. This design is particularly useful for genes that fuse with multiple translocation partners (e.g., *EWSR1* gene, which may undergo rearrangement with multiple partner genes, including *FLI1*, *ERG*, *ETV1*, *FEV*, and *E1AF* in Ewing sarcoma/primitive neuroectodermal tumor [PNET]; *WT1* in desmoplastic small round cell tumors; *CHN* in extraskeletal myxoid chondrosarcoma; and *ATF1* in clear cell sarcoma and angiomatoid fibrous histiocytoma).[50] The disadvantage of this approach is that break-apart FISH does not allow for the identification of the "unknown" partner in the translocation.

FISH has the advantage of being applicable to a variety of specimen types, including FFPE tissue. Because probes are hybridized to tissue in situ, the tumor morphology is preserved, which allows for an interpretation of the assay even in the context of heterogeneous samples. However, FISH is a targeted approach that will only detect specific alterations. Because most probes are large (e.g., >100 kb), small deletions or insertions will not be detected. In addition, poor tissue fixation, fixation artifacts, nuclear truncation on tissue slides, and nuclear overlaps are potential pitfalls of this technique that may hamper interpretation. Some intrachromosomal rearrangements (e.g., *RET-PTC* and *EML4-ALK*) may be challenging to interpret by FISH due to subtle rearrangements of the probe signals on the same chromosome arm.

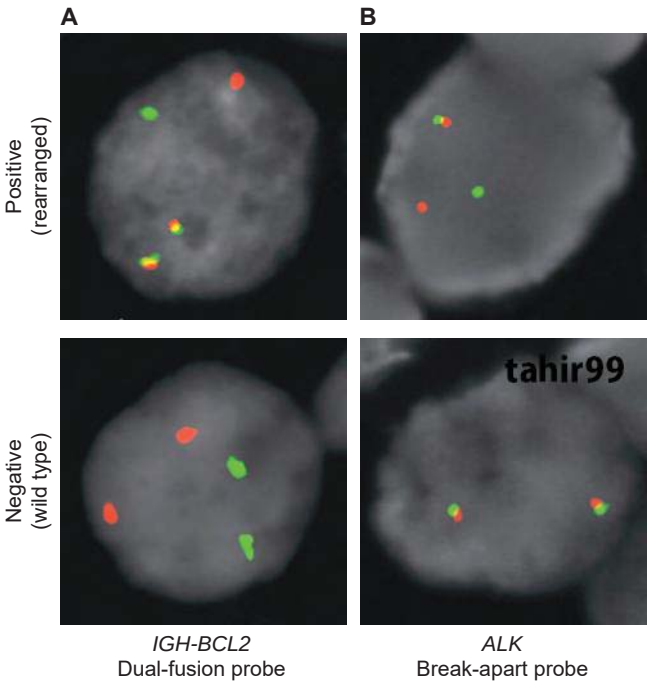


Figure 3.8 Fluorescence in situ hybridization (FISH). **(A)** Recurrent chromosomal translocations such as *IGH-BCL2* (occurring in B-cell lymphomas) can be effectively detected with a dual-fusion probe strategy. This design utilizes a green probe specific to the *IGH* locus and a red probe specific to the *BCL2* gene, with each probe spanning their respective breakpoint region. Individual green and red probe signals indicate a lack of translocation. Colocalization of green and red probes is observed when an *IGH-BCL2* translocation is present. **(B)** *ALK* rearrangements in non–small-cell lung cancers may involve a variety of translocation partners, including *EML4*, *TFG*, and *KIF5B*. Therefore, a break-apart FISH probe strategy is utilized that will detect any *ALK* rearrangement, regardless of the partner gene. Fluorescently labeled red and green probes are designed on opposite sides of the *ALK* gene breakpoint region. With this design, a normal *ALK* gene is observed as overlapping or adjacent red and green fluorescent signals, whereas a rearranged *ALK* gene is indicated by split red and green signals. *ALK* testing in lung cancer has become widespread in use because of the significant therapeutic implications.

Methylation Analysis

Changes in the methylation status of cytosine in DNA regions enriched for the sequence CpG (also known as CpG islands) are early events in many cancers and permanent changes found in many tumors. The detection of aberrant methylation of cancer-related genes may aid in the diagnosis, prognosis, and/or determination of the metastatic potential of tumors.

The most common approaches for the detection of methylation are based on the conversion of unmethylated cytosine bases into uracil after sodium bisulfite treatment, which is then converted to thymidine during PCR. By this approach, bisulfite-treated methylated alleles have different DNA sequences as compared with their corresponding unmethylated alleles. The differences between methylated and unmethylated DNA sequences can be evaluated by several methods, including methylation-sensitive restriction enzyme analysis, methylation-specific PCR, semiquantitative q-PCR, Sanger sequencing, pyrosequencing, and next-generation sequencing.

The methylation status of oncogenic genes can also be assessed by methylation-sensitive multiplex ligation-dependent probe amplification (MS-MLPA) assay.[51,52] MS-MLPA is a variant of multiplex PCR in which oligonucleotide probes hybridized to the targeted DNA samples are directly amplified using one pair of universal primers. This method is not based on bisulfite conver-

sion of unmethylated cytosine bases into uracil. Instead, the target sequences detected by MS-MLPA probes contain a restriction site recognized by methylation-sensitive endonucleases. A probe amplification product will only be obtained if the CpG site is methylated because digested probes cannot be amplified during PCR. The level of methylation is determined by resolving PCR products by capillary electrophoresis and calculating the normalized ratio of each target probe peak area in both digested and undigested specimens. The ratio corresponds to the percentage of methylation present in the specimen.

Examples of applications of methylation analysis in oncology include an analysis of *MLH1* promoter hypermethylation in microsatellite unstable sporadic colorectal carcinomas, an analysis of *MGMT* promoter methylation status in glioblastoma multiforme patients treated with alkylating chemotherapy, and *SEPT9* promoter methylation in DNA derived from blood plasma in colorectal cancer patients.[53]

Microsatellite Instability Analysis

Microsatellites are short, tandem-repeated DNA sequences with repeating units of one to six base pairs in length. Microsatellites are distributed throughout the human genome, and individual repeat loci often vary in length from one individual to another. Microsatellite instability (MSI) is the change in length of a microsatellite allele due to either insertion or deletion of repeating units and a failure of the DNA mismatch repair (MMR) system to repair these replication errors. This genomic instability arises in a variety of human neoplasms where tumor cells have a decreased ability to faithfully replicate DNA. MSI is particularly associated with colorectal cancer, where 15% to 20% of sporadic tumors show MSI, in contrast to the more common chromosomal instability (CIN) phenotype, with MSI status being an independent prognostic indicator. MSI analysis is also clinically useful in identifying patients at increased risk of hereditary nonpolyposis colorectal cancer (HNPCC)/Lynch syndrome, where a germline mutation of an MMR gene causes a familial predisposition to colorectal cancer. MSI analysis alone is not sufficient to make a diagnosis of a germline MMR mutation given the high rate of sporadic MSI-positive colorectal tumors, but a positive result is an indication for follow-up genetic testing and counseling.

In an MSI analysis, DNA is extracted from tumor tissue and the corresponding adjacent normal mucosa. The DNA is subjected to multiplex PCR using fluorescent-labeled primers for coamplification of five mononucleotide repeat markers for MSI determination and two pentanucleotide markers for confirming tumor/normal sample identity. The resulting PCR fragments are separated and detected using capillary electrophoresis. Allelic profiles of normal versus tumor tissues are compared, and MSI is scored as the presence of novel microsatellite lengths in tumor DNA compared to normal DNA. Instability in two or more out of five mononucleotide microsatellite markers in tumor DNA compared to normal DNA is defined as MSI-H (high). MSI-L (low) is defined as instability in one out of five mononucleotide markers in tumor DNA compared to normal DNA. Tumors with no instability (zero out of five altered mononucleotide markers) are defined as microsatellite stable (MSS).[54,55]

Loss of Heterozygosity Analysis

Loss of heterozygosity (LOH) is a common event in cancer that usually occurs due to deletion of a chromosome segment and results in a loss of one copy of an allele. LOH is a common occurrence in tumor suppressor genes and may contribute to tumorigenesis when the second allele is subsequently inactivated by a second "hit" due to mutation or deletion.

LOH studies are used to identify genomic imbalance in tumors, indicating possible sites of tumor suppressor gene (TSG) deletion. LOH studies can be done by multiplex PCR analysis of microsatellites (short tandem repeats [STRs]), FISH, and genomic microarrays). By PCR, microsatellites located in the vicinity of a tumor suppressor gene are used as surrogate markers for the presence of the gene of interest. DNA is extracted from tumor tissue and corresponding adjacent normal mucosa. The DNA is subjected to multiplex PCR using fluorescent-labeled STR primers. Peak height ratio of informative (nonhomozygous) alleles at each locus is calculated from both normal and tumor tissues. LOH is defined as the decrease in peak height of one of the two alleles, relative to the allele peak heights of the normal sample.

An example of applications of LOH studies in oncology include an analysis of 1p/19q loss in oligodendrogliomas, and an analysis of 1p loss in parathyroid carcinomas.

Whole Genome Analysis Methods

Next-Generation Sequencing

Next-generation sequencing (NGS), also known as massive parallel sequencing or deep sequencing, is an emerging technology that has revolutionized the speed, throughput, and cost of sequencing and has facilitated the discovery of clinically relevant genetic biomarkers for diagnosis, prognosis, and personalized therapeutics. By way of this technology, multiple genes or the entire exome or genome can be interrogated simultaneously in multiple parallel reactions instead of a single-gene basis as in Sanger sequencing or pyrosequencing. Currently, the most common NGS approach for cancer testing in the clinical setting employs targeted sequencing of specific genes and mutation hotspot regions. This targeted approach increases sensitivity for the detection of low-level mutations by increasing the depth of sequence coverage.

Presently, there are numerous NGS platforms that employ different sequencing technologies. A comprehensive review and comparison of NGS platforms is beyond the scope of this chapter and has been reviewed elsewhere.[56,57] A generalized clinical workflow is shown (Fig. 3.9). Frequently, multiple DNA samples are individually barcoded and pooled together to leverage platform throughput. Pooled libraries are prepared and enriched, and single DNA molecules are arrayed in solid surfaces, glass slides, or beads and sequenced in situ using reversible DNA chain terminators or iterative cycles of oligonucleotide ligation. NGS signal outputs are based on luminescence, fluorescence, or changes in ion concentration. Robust bioinformatics pipelines are required for an alignment of reads to a reference genome sequence, variant calling, variant annotation, and to assist with result reporting.[58]

NGS can be used for the detection of single nucleotide variants, small insertions and deletions, translocations, inversions, alternative splicing, and copy number variations given sufficient depth of genomic DNA sequence (Fig. 3.10). Technical limitations of this technique include difficulty in sequencing guanine-cytosine (GC)–rich genomic regions, and erroneous sequencing of homologous DNA regions (e.g., pseudogenes) that may confound interpretation.

Examples of applications of NGS in oncology include small targeted panels (3 to 50 genes) for non–small-cell lung cancers, melanomas, colon cancers, and acute myeloid leukemias.[57,59–62] Larger panels (50 to 500 genes) are increasingly being utilized, particularly in both clinical trials and research.

Massively parallel sequencing of RNA (RNA-Seq) can be used for determining sequence variants, alternative splicing, gene rearrangements, and allelic expression of mutant transcripts. To date, this technique has been used primarily for discovery rather than clinical applications, but it is likely to play an increasing role in clinical diagnostics as the technology improves. For transcriptome sequencing, the RNA must first be converted to cDNA, which is then fragmented and entered into library construction. After sequencing, reads are aligned to a reference genome, compared with known transcript sequences, or assembled de novo

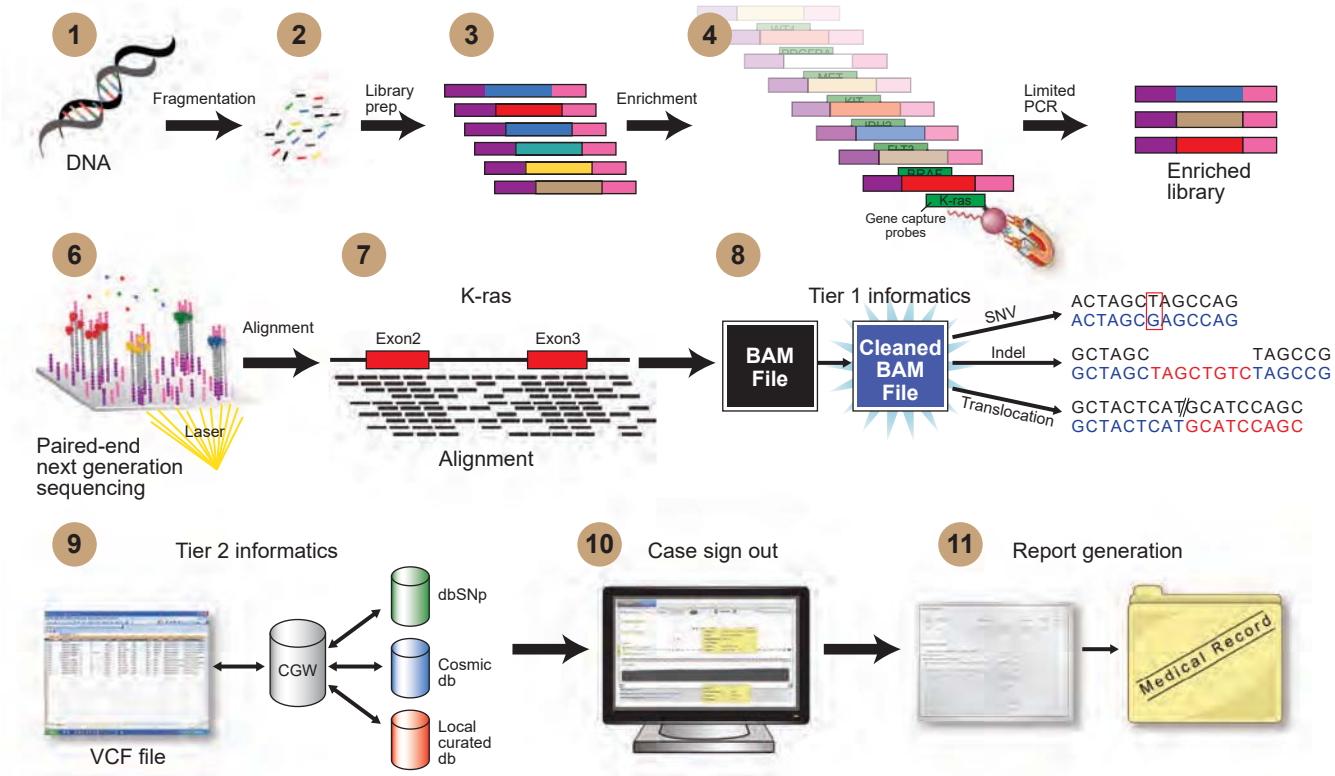


Figure 3.9 Next-generation sequencing (NGS) workflow in a clinical laboratory. Targeted-panel sequencing offers tremendous promise for cancer diagnostics due to the massive improvement in throughput, speed, and cost. NGS is a complex, multiday process that requires significant infrastructure and expertise to deploy in a clinical setting. The process begins with genomic DNA extraction, which is fragmented and to which linkers are ligated. In this targeted gene panel–based example, the sequencing libraries are enriched for the target genes, which are subjected to a limited PCR prior to sequencing. Sequence reads are mapped to a reference genome and subjected to several bioinformatics tools to provide variant calling results and variant annotation. Clinical interpretation and case sign out is performed by a physician with expertise in molecular pathology. BAM, Binary Sequence Alignment/Map; SNV, Single Nucleotide Variant; VCF, Variant Call Format; CGW, Clinical Genomicist Workstation; dbSNP, The Single Nucleotide Polymorphism Database. (Used with permission from Shashikant Kulkarni PhD and Eric Duncavage MD.)

to construct a genome-scale transcription map. Expression levels are determined from the total number of sequence reads that map to the exons of a particular gene, normalized by the length of exons that can be uniquely mapped.[56] Compared with genomic microarrays, RNA-Seq has a greater ability to distinguish RNA isoforms, determine allelic expression, and reveal sequence variants.

Chromatin immunoprecipitation with sequencing (ChIP-Seq) can be used to determine the genome-wide location of chromatin-binding transcription factors or specific epigenetic modifications of histones. This has proved to be a very powerful research tool, which to date has not been used for clinical diagnostics. Proteins in contact with genomic DNA are chemically cross-linked (usually with formaldehyde treatment) to their binding sites, the DNA is fragmented, and the proteins cross-linked with DNA are then immunoprecipitated with antibodies specific for the proteins (or specific epigenetic histone modification) of interest. The DNA harvested from the immunoprecipitate is converted into a library for NGS. The obtained reads are mapped to the reference genome of interest to generate a genome-wide protein binding map.[63,64] ChIP-Seq is rapidly replacing chromatin immunoprecipitation and microarray hybridization (ChIP-on-chip) technology[65] because of its higher sensitivity and resolution.[66]

Genomic Microarrays

High-density genomic microarrays are widely used for whole genome assessment of copy number changes, LOH, and genotyping. In array comparative genomic hybridization (aCGH), cloned genomic probes are arrayed onto glass slides and serves as targets for the competitive hybridization of normal and tumor DNA. In the aCGH reaction, tumor DNA and DNA from a normal control sample are labeled with different fluorophores. These samples are denatured and hybridized together to the arrayed single-strand probes. Digital imaging systems are used to quantify the relative fluorescence intensities of the labeled DNA probes that have hybridized to each target probe. The fluorescence ratio of the tumor and control hybridization signals is determined at different positions along the genome, which provides information on the relative copy number of sequences in the tumor genome as compared to the normal genome.[67] This method is able to detect copy number variation, such as deletions, duplications, and gene amplification, but it cannot detect polymorphic allele changes.

An SNP array has the ability to detect LOH profiles in addition to high-resolution detection of copy number aberrations, such as amplifications and deletions. This method employs thousands of unique fluorescent-labeled nucleotide probe sequences arrayed on a chip to which a fragmented single-stranded specimen DNA binds to their complementary partners. Each SNP site is interrogated by complementary sets of probes containing perfect matches and mismatches to each SNP site. Each probe is associated with one of the two alleles of an SNP (also known as A and B). Relative fluorescence intensity depends on both the amount of target DNA in the sample, as well as the affinity between target and probe. An analysis of the raw fluorescence intensity is done by computational

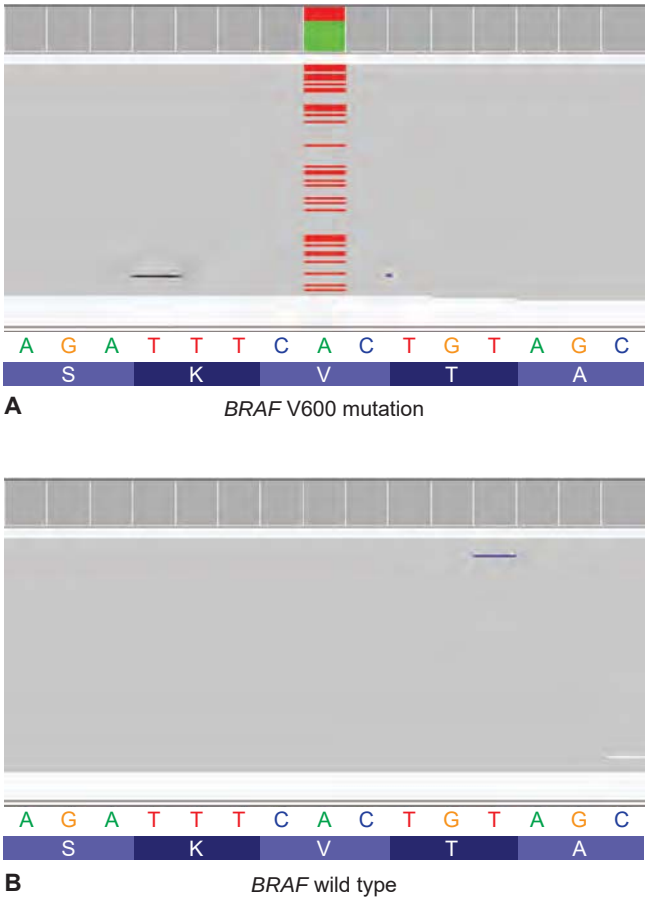


Figure 3.10 Next-generation sequencing (NGS). Hundreds to thousands of sequence reads are mapped and horizontally aligned to specific targeted regions in the reference genome (sequence shown on *bottom* of each panel). A software-assisted analysis assists in the detection of mutations, displayed as colored bars in each read above the mutation site. A wild-type sequence within each read is displayed in *gray*. Mutation frequency correlates to the number of times the mutant sequence is detected compared to the total number of reads at that nucleotide position. Shown are sequencing results from *BRAF* V600E mutation positive **(A)** and negative **(B)** melanomas. The A to T base substitution that leads to the V600E mutation is displayed in red. Patients with metastatic melanoma that harbors the *BRAF*V600E mutation are candidates for targeted therapy.

algorithms that convert the set of probe intensities into genotypes. Deleted genomic regions are identified as having an LOH associated with copy number reduction. A copy-neutral LOH is detected when SNPs expected to be heterozygous in the normal sample are detected as homozygous in the tumor sample without copy number variation. A copy neutral LOH may arise from somatic homologous recombination of a mutated tumor suppressor allele and its surrounding DNA that replaces the other allele (uniparental disomy [UPD]). SNP microarrays are the only genomic microarrays that are able to identify UPD. Array technologies cannot detect true balanced chromosome abnormalities and low-level mosaicism.

Examples of genomic microarrays applications in oncology include the detection of copy number variations and LOH in chronic lymphocytic leukemia[68] and recurrent cytogenetic abnormalities in MDS (e.g., 5q-, -7 or 7q-, +8, 20q-).[69]

Expression Panels

Gene expression signatures of multiple cancer biomarkers are starting to be incorporated into clinical practice as an adjunct to clinical and pathologic information in diverse cancer management settings. An example of a multigene expression–based test in current use includes Oncotype DX, which is a quantitative RT-PCR–based assay that measures the expression of 21 genes in FFPE breast tumors. The test is designed to predict the potential benefit of chemotherapy and the likelihood of distant breast cancer recurrence in women with node negative or node positive, estrogen receptor (ER)-positive, and *HER2*-negative invasive breast cancer. This test has been in-corporated into current American Society of Clinical Oncology (ASCO) and National Comprehensive Cancer Network (NCCN) for breast cancer management.[70] Prospective trials are in progress to evaluate other multigene tests for early stage breast cancer.

With the rapid advances in molecular diagnostic technologies, it is likely that many mutation- and expression-based panels analyzing hundreds if not thousands of genes, or even the complete genome or transcriptome, will enter widespread use. Some of the many challenges to address will be to provide evidence-based, actionable reports that guide the oncologist to more effective therapies, to learn from the results of such testing to improve the algorithms guiding therapy, to handle the incidental findings in such testing in an ethically responsible way, and ultimately, with the drugs available, to provide sufficient improvements in outcomes so that society will be willing to bear the costs.

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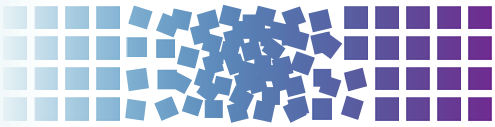
PART II

Etiology and Epidemiology of Cancer

4 Tobacco



Richard J. O'Connor

INTRODUCTION

Regrettably, tobacco use remains one of the leading causes of death worldwide. It is projected to leave over 1 billion dead in the 21st century, after killing nearly 100 million during the course of the 20th century.[1] Data from the Global Adult Tobacco Survey (GATS), which conducted representative household surveys in 14 low- and middle-income countries (Bangladesh, Brazil, China, Egypt, India, Mexico, Philippines, Poland, Russia, Thailand, Turkey, Ukraine, Uruguay, and Vietnam), suggest 41% of men and 5% of women across these countries currently smoke.[2] Compare this to approximately 24% of men and 16% of women in the United States.[3] A preponderance of the death and disease associated with tobacco use is associated with its combusted forms, particularly the cigarette. However, all forms of tobacco use have negative health consequences, the severity of which can vary among products. From the introduction of the mass-manufactured, mass-marketed cigarette (e.g., Camel in 1913), smoking rates grew, first among men then among women, and peaked in Western countries in the 1960s to 1970s, before beginning a steady decline.[4] The smoking rate among US adults has dropped from its peak in 1965 of 42% to 19% in 2011.[3] Per capita consumption has been dropping almost continuously since the 1960s, although the rate of decline has slowed since the early 2000s.[5] Among youth, smoking rates have been in decline since the 1990s,[6,7] although there is some evidence of growth in use of other forms of tobacco (e.g., cigars, water pipes, electronic cigarettes) in 2011 to 2012 that may be displacing cigarette use.[8]

Tobacco control policy interventions can impact both smoking prevalence and lung cancer incidence.[9] For example, a recent analysis suggests that implementation of graphic health warnings in Canada in 1999 resulted in a significant reduction (up to 4.5 percentage points) in smoking prevalence over a decade.[10] Increases in tobacco taxes have long been shown to reduce youth smoking initiation and to prompt more attempts to quit smoking.[11] Evidence from state comparisons in the United States suggests that comprehensive tobacco control measures effectively implemented (such as in California and Massachusetts) can reduce lung cancer incidence.[12] Indeed, Holford and colleagues[13] have shown that since the seminal 1964 Report of the Surgeon General, an estimated 157 million years of life (approximately 20 years per person) have been saved by tobacco control activities in the United States over 50 years. That is, tobacco control activities are estimated to have averted 8 million premature deaths and extended mean life span by 19 to 20 years.[13] However, the marketing of cigarettes has since shifted focus to the developing world, where smoking rates are on the increase. In an attempt to head off an epidemic of smoking and associated diseases, the World Health Organization initiated a public health treaty, the Framework Convention on Tobacco Control (FCTC), to coordinate international efforts to reduce tobacco use.[14] The FCTC binds parties to enact measures to control the labeling and marketing of tobacco products, create a framework for testing and regulating product contents and emissions, combat smuggling and counterfeiting, and protect nonsmokers from secondhand smoke.[15] To date, the FCTC has been ratified by more than 150 countries. The FCTC provides governments the opportunity to regulate the marketing, labeling, and contents/emissions of tobacco products, as well as control the global trade in tobacco products. In the United States, which is currently not a party to FCTC, the U.S. Food and Drug Administration (FDA) has, since 2009, had authority to regulate tobacco products and their marketing along similar lines.[16]

EPIDEMIOLOGY OF TOBACCO AND CANCER

Linkages between tobacco use and cancers at various sites had been noted for several decades. In the late 1800s, it was believed that excessive cigar use created irritation that led to oral cancers.[17] In the 1930s, German scientists began to establish links between cigarette smoking and lung cancers.[18] However, it was not until the Doll and Hill[19] and Wynder and Graham[20] studies were published that the association was demonstrated in large samples and well-designed studies. Table 4.1 lists the cancers currently recognized by the U.S. Surgeon General as caused by smoking, along with their corresponding estimated mortality statistics.[21–23] Of these, the most well-publicized link is between smoking and lung cancer. In a recent examination of National Health and Nutrition Examination Survey (NHANES) data, Jha[24] showed a hazard ratio for lung cancer in smokers versus nonsmokers of 17.8 in women and 14.6 in men. However, smoking contributes substantially to overall cancer burden across multiple sites, including the oropharynx, cervix, and pancreas. Hazard ratios of 1.7 for women and 2.2 for men are seen for cancers other than in the lung in smokers versus nonsmokers.[24] Emerging evidence also links smoking with breast cancer, although the data are as yet insufficient to make causal conclusions.[23,25] Cancer risks associated with smoking, as well as outcomes and survival, depend on a number of factors. A common index of cancer risk is pack-years, or the number of packs of cigarettes smoked per day multiplied by the number of years smoked in the lifetime. In general, the higher the number of pack-years, the greater the cancer risk. Risks for lung cancer decline with smoking cessation, and the longer a former smoker remains off of cigarettes, the more the risk declines.[26] However, excepting those smokers who quit with relatively few pack years accumulated (typically before age 40), cancer risk rarely approaches that of a never smoker.[24,27]

A recent study using several large cohort studies examined death rates and the relative risks associated with smoking and smoking cessation for 3 epochs (1959 to 1965, 1982 to 1988, and 2000 to 2010).[27] Of most interest here is death from lung cancer. For men, the age-adjusted death rate from lung cancer increased from 1959 through 1965 to 1982 through1988, but then fell for 2000

TABLE 4.1

Level of Evidence for Smoking-Attributable Cancers According to the United States Office of the Surgeon General by Cancer Site and Yearly Smoking-Attributable Mortality at Sites with Available Estimates, United States, 2004

	Cancer Site	Yearly Smoking-Attributable Mortality
Evidence Sufficient to Infer Causal Relationship	Bladder	4,983
	Cervix	447
	Colon and rectum	N/A
	Esophagus	8,592
	Kidney	3,043
	Larynx	3,009
	Leukemia (AML)	1,192
	Liver	N/A
	Lung	125,522
	Oral cavity and pharynx	4,893
	Pancreas	6,683
	Stomach	2,484
Evidence Suggestive but Not Sufficient to Infer Causal Relationship	Breast	
Inadequate to Infer Presence or Absence of Causal Relationship	Ovary	
Evidence Sufficient to Infer No Causal Relationship	Prostate	

N/A, not available; AML, acute myeloid leukemia.

through 2010; for women, the age-adjusted death rate continued to rise over time, with the biggest increase between 1982 through 1988 to 2000 through 2010.[27] In relative risk terms, the likelihood of dying from lung cancer given current smoking has increased from 2.73 to 12.65 to 25.66 among women, and 12.22 to 23.81 to 24.97 for men. Equivalent risks for former smokers increased from 1.3 to 3.85 to 6.7 among women, versus 3.48 to 7.41 to 6.75 for men. These and other analyses suggest that the cancer risks from smoking may have increased with time.[27,28] The histologic subtypes of lung cancer seen in the US population have also shifted with time. Into the early 1980s, squamous cell carcinomas (SCC) were the most common manifestations of lung cancer. However, a rapid rise in adenocarcinomas has been noted, and by the 1990s, had overtaken SCC as the leading type of lung cancer.[23]

Tobacco Use Behaviors

The level of tobacco exposure is ultimately driven by use behaviors, including the number of cigarettes smoked, the patterns of smoking on individual cigarettes, and the number of years smoked. The primary driver of smoking behavior is nicotine—the major addictive substance and primary reinforcer of continued smoking.[29–31] Over time, smokers learn an *acceptable* level of nicotine intake that attains the beneficial effects they seek while avoiding negative withdrawal symptoms. Smokers can affect the amount of nicotine (and accompanying toxicants) they draw from a cigarette by altering the number of puffs taken, puff size, frequency, duration, and velocity (collectively referred to as smoking topography).[32] Smokers tend to consume a relatively stable number of cigarettes per day and to smoke those cigarettes in a relatively consistent manner in order to maintain an acceptable level of nicotine in their system across the day.[33] The number of cigarettes smoked per day and the smoking pattern of an individual may be influenced by the rate of nicotine metabolism.[30] Nicotine is metabolized primarily to cotinine, which is further metabolized to trans-3′-hydroxycotinine (3HC), catalyzed by the liver cytochrome P450 2A6 enzyme.[34] Functional polymorphisms in the genes coding for these enzymes allow for the identification of *fast* metabolizers, who have more rapid nicotine clearance and show greater cigarette intake and more intensive smoking topography profiles relative to *normal* or *slow* metabolizers.[35–37] The ratio of 3HC to cotinine in plasma or saliva can be used as a reliable noninvasive phenotypic marker for CYP2A6 activity.[38,39] CYP2A6 activity is known to vary across racial/ethnic groups, with those of African or Asian descent showing slower metabolism than those of Caucasian descent.[40–42] Clinical trial data clearly show that the metabolite ratio can be used to predict success in quitting, and that the likelihood of quitting decreases as the ratio increases, such that slower metabolizers are more successful at achieving abstinence.[37,41,43] Despite their addiction to nicotine, most smokers in Western countries report that they regret ever starting to smoke and want to quit smoking, and there is evidence for similar regret in developing countries as well.[44–46] However, most smokers are unsuccessful in their attempts to quit smoking; the most effective evidence-based treatments increase the odds of quitting by 3 times, with 12-month cessation rates of approximately 40% relative to placebo.[47]

Evolution of Tobacco Products

Historically, tar was believed to be the main contributor to smoking-caused disease.[48] It is important to note that *tar* is not a specific substance, but simply the collected particulate matter from cigarette smoke, less water and nicotine (in technical reports, it is often referred to as nicotine-free dry particulate matter). Soon after the first studies were done showing that painting mice with cigarette tar caused cancerous tumors, it was theorized that reducing tar yields of cigarettes might also reduce the disease burden of smoking.[48] Concurrently, cigarette manufacturers were seeking to reassure their customers that their products were safe, that if hazardous compounds were identified they would be removed, and that product modifications could help to reduce risks.[4,49–51] Indeed, in the United States and United Kingdom, average tar levels of cigarettes dropped dramatically from the 1960s through the 1990s, and have since leveled off.[52,53] The European Union took the tar reduction mentality to heart in crafting maximum levels of tar in cigarettes that could be sold in member countries, beginning at 15 mg in 1992, then dropping to 12 mg in 1998, and 10 mg in 2005.[54] Unfortunately, these reductions in tar yields have not translated into changes in disease risks among smokers.[55] Despite initial optimism about these products, both laboratory-based and epidemiologic studies indicate neither an individual, nor a public health benefit from *low-tar* cigarettes as compared to *full-flavor* varieties.[56–58] The health consequences of mistakenly accepting the purported benefits of lower tar and nicotine products have been significant. The increases in adenocarcinoma of the lung observed in the United States over recent decades may reflect changes made to the cigarette, such as filters, filter ventilation, and tobacco-specific nitrosamines (TSNA) in smoke produced by the relatively high amount of burley tobacco used in the typical US cigarette blend.[23,59] Tobacco manufacturers engineered cigarettes be *elastic*; that is, cigarettes allow smokers to adjust their puffing patterns to regulate their intake of nicotine, regardless of how the cigarette might perform under the standard

testing conditions that drove the labeling and advertising of the products.[55] Researchers have since come to determine that filter vents are the main design feature the industry relied on in creating elastic products.[54,55,60,61] Vents facilitate taking larger puffs and also contribute to sensory perceptions, because they dilute the smoke with air. [62] So, even with a larger puff, the same mass of toxins can seem less harsh and irritating because it is diluted by a proportionate amount of air, which may in turn underscore smokers' beliefs that they are smoking safer cigarettes.[62–64] Other smoke components (e.g., acetaldehyde, ammonia, minor tobacco alkaloids) and aspects of cigarette engineering (e.g., menthol, flavor additives) may further contribute to the addictiveness of cigarettes.[65]

Since the 1980s, manufacturers have introduced products that make more explicit claims about reduced health risks. Examples of modified cigarettelike products include Premier (RJ Reynolds), Eclipse (RJ Reynolds), Accord/Heatbar (Philip Morris), Omni (Vector Tobacco), and Advance (Brown and Williamson).[66] In the 2000s, as evidence of reduced lung cancer incidence and coincident increases in snus use in Sweden appeared,[67,68] manufacturers began to promote smokeless tobacco products as reduced harm alternatives. Most recently, electronic cigarettes, which vaporize a nicotine solution, have gained increasing popularity and generated concern among public health practitioners, particularly with regard to effects on youth.[8,69,70] In the United States, the FDA has authority to authorize marketing claims about reduced risk, which an Institute of Medicine panel concluded should be based on extensive testing of abuse liability, likely health effects, and effects on the whole population.[71]

CARCINOGENS IN TOBACCO PRODUCTS AND PROCESSES OF CANCER DEVELOPMENT

Cigarette smoke has been identified as carcinogenic since the 1950s, and efforts have continued to identify specific carcinogens in smoke and smokeless tobacco products. The International Agency for Research on Cancer (IARC) has classified both cigarette smoke and smokeless tobacco as Group 1 carcinogens.[72,73] IARC has also identified 72 measurable carcinogens in cigarette smoke where evidence is sufficient to classify them as Group 1 (carcinogenic to humans), 2A (probably carcinogenic to humans), or 2B (possibly carcinogenic to humans).[72] The IARC list, in addition to data from the U.S. Environmental Protection Agency (EPA), the National Toxicology Program, and the National Institute for Occupational Safety and Health (NIOSH), informed the FDA's development of a list of Harmful and Potentially Harmful Constituents (HPHC) in tobacco and tobacco smoke, which manufacturers will be required to report.[74] Table 4.2 illustrates the carcinogens listed as HPHC alongside their carcinogenicity classifications by IARC or the EPA.

Compounds of Particular Concern

Research groups have listed components of cigarette smoke theorized to impact health risk, often relying on carcinogenic potency indices and relative concentrations in smoke.[75,76] In these analyses, the N-nitrosamines, benzene, 1,3-butadiene, aromatic amines, and cadmium often rank highly. Polycyclic aromatic hydrocarbons (PAH), many of which are carcinogenic, consist of three or more fused aromatic rings resulting from incomplete combustion of organic (carbonaceous) materials, and are often found in coal tar, soot, broiled foods, and automobile engine exhaust.[77] A compound of particular concern in cigarette smoke historically has been benzo(a)pyrene (BaP), which has substantial carcinogenic activity and is considered carcinogenic to humans by the IARC.[77] In addition to PAH, other hydrocarbons found in significant quantities in cigarette smoke include benzene (a long-established cause of leukemia), 1,3-butadiene (a potent multiorgan carcinogen), naphthalene, and styrene. Carbonyl compounds, such as formaldehyde and acetaldehyde, are found in copious amounts in cigarette smoke, primarily coming from the combustion of sugars and cellulose.[78] However, there are numerous other noncigarette exposures to these compounds, including endogenous formation during metabolism. Smoke contains a number of aromatic amines, such as known bladder carcinogens 2-aminonaphthalene and 4-aminobiphenyl, heterocyclic amines, and furans. Toxic metals, including beryllium, cadmium, lead, and polonium-210, are also present in cigarette smoke in measurable quantities,[79,80] levels of which may depend in part on the region of the world where the tobacco was grown.[81] Much attention has been focused on the *N*-nitrosamines, primarily because they are well-established carcinogens.[82–85] Nitrosamines form through reactions of nitrite with amino groups. In tobacco, two compounds of concern are 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), which is derived from nitrosation of nicotine, and *N′*-nitrosonornicotine (NNN), which is derived from nitrosation of nornicotine. Both of these compounds are tobacco specific. NNN and NNK primarily form during the curing process for tobacco, where the leaves are dried through contact with combustion gases from heat (flue) curing or microbial activity in air curing.[78] NNK is known to be a potent lung carcinogen, but also shows tumor induction activity in the nasal cavity, the pancreas, and the liver, whereas NNN has been shown to induce tumors along the respiratory tract and esophagus in various animal models. Because they are produced in the curing process and transfer into smoke, rather than being formed by combustion, it is possible to reduce nitrosamines by changing curing and storage practices.[78,86,87]

Smokeless tobacco products, although they are not burned, nonetheless contain substantial levels of carcinogens, most prominently the *N*-nitrosamines.[73] Here, product type and composition has an enormous effect on nitrosamine levels. For example, US moist snuff has substantially higher levels than that sold in Sweden (snus), whereas smokeless products available in India are often far higher in nitrosamines.[88] US smokeless products also can contain PAH and carbonyl compounds, likely derived from fire curing the constituent tobacco.[89] Similar to cigarettes, smokeless products would also contain toxic metals.[79,80]

Although tobacco is an exceedingly complex mixture, it is possible to use animal model and epidemiologic evidence to postulate relationships between specific components and known tobacco-induced cancers.[90–92] There is strong evidence from multiple studies to suggest that PAH and *N*-nitrosamines are involved in lung carcinogenesis. For example, PAH–DNA adducts are observed in lung tissues, and p53 tumor suppressor mutations in lung tumors resemble the damage created by PAH diol epoxide metabolites in vitro.[93–96] NNK appears to preferentially induce lung tumors in the rat, regardless of the route of administration, and DNA–nitrosamine adducts are detectable in lung tissues.[97,98] Most importantly, nitrosamine metabolite levels measured in smokers were prospectively related to the risk of lung cancer in cohort studies, even adjusting for other indices of smoking exposure (e.g., cotinine, pack-years).[98–102] PAH and nitrosamines are also likely to be implicated in cancers along the respiratory tract and the cervix.[103,104] Considerable evidence exist that aromatic amines such as 4-aminobiphenyl and 2-naphthylamine are potent bladder carcinogens, and smokers are known to be at an elevated risk of bladder cancer, so these are presumed to be the primary causative agents.[105–107] Similarly, as benzene is a known cause of leukemia, it is presumed that this is the link to leukemia observed in smokers.

Important to examining the role of various smoke components in cancer is the ability to measure the exposure of smokers to these components. Biomarkers of exposure may also be crucial for examining products for their potential to reduce health risks associated with tobacco use.[71,108,109] Validation of tobacco exposure biomarkers is threefold: method validation, validation with respect to product use, and validation with respect to disease risk.[71]

TABLE 4.2

Carcinogens in Tobacco and Tobacco Smoke Identified as Harmful and Potentially Harmful by the U.S. Food and Drug Administration, with International Agency for Research on Cancer Carcinogenecity (IARC) Classifications as of 2013

Compound	CAS No.	IARC Group	IARC Volume	Year
1,3-Butadiene	106-99-0	1	100F	2012
2-Aminonaphthalene	91-59-8	1	100F	2012
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	64091-91-4	1	100E	2012
4-Aminobiphenyl	92-67-1	1	100F	2012
Aflatoxin B1	1162-65-8	1	100F	2012
Arsenic	7440-38-2	1	100C	2012
Benzene	71-43-2	1	100F	2012
Benzo[a]pyrene	50-32-8	1	100F	2012
Beryllium	7440-41-7	1	100C	2012
Cadmium	7440-43-9	1	100C	2012
Chromium (Hexavalent compounds)	18540-29-9	1	100C	2012
Ethylene oxide	75-21-8	1	100F	2012
Formaldehyde	50-00-0	1	100F	2012
N-Nitrosonornicotine (NNN)	16543-55-8	1	100E	2012
Nickel (compounds)		1	100C	2012
o-Toluidine	95-53-4	1	100F	2012
Polonium-210	7440-08-6	1	100D	2012
Uranium (235, 238 Isotopes)	7440-61-1	1	100D	2012
Vinyl chloride	75-01-4	1	100F	2012
Acrylamide	79-06-1	2A	60	1994
Cyclopenta[c,d]pyrene	27208-37-3	2A	92	2010
Dibenz[a,h]anthracene	53-70-3	2A	92	2010
Dibenzo[a,l]pyrene	191-30-0	2A	92	2010
Ethyl carbamate (urethane)	51-79-6	2A	96	2010
IQ (2-Amino-3-methylimidazo[4,5-f]quinoline)	76180-96-6	2A	56	1993
N-Nitrosodiethylamine	55-18-5	2A	SUP 7	1987
N-Nitrosodimethylamine (NDMA)	62-75-9	2A	SUP 7	1987
2-Nitropropane	79-46-9	2B	71	1999
2,6-Dimethylaniline	87-62-7	2B	57	1993
5-Methylchrysene	3697-24-3	2B	92	2010
A-α-C (2-Amino-9H-pyrido[2,3-b]indole)	26148-68-5	2B	SUP 7	1987
Acetaldehyde	75-07-0	2B	71	1999
Acetamide	60-35-5	2B	71	1999
Acrylonitrile	107-13-1	2B	71	1999
Benz[a]anthracene	56-55-3	2B	92	2010
Benz[j]aceanthrylene	202-33-5	2B	92	2012
Benzo[b]fluoranthene	205-99-2	2B	92	2010
Benzo[b]furan	271-89-6	2B	63	1995
Benzo[c]phenanthrene	195-19-7	2B	92	2010
Benzo[k]fluoranthene	207-08-9	2B	92	2010
Caffeic acid	331-39-5	2B	56	1993
Catechol	120-80-9	2B	71	1999
Chrysene	218-01-9	2B	92	2010
Cobalt	7440-48-4	2B	52	1991

(continued)

TABLE 4.2

Carcinogens in Tobacco and Tobacco Smoke Identified as Harmful and Potentially Harmful by the U.S. Food and Drug Administration, with International Agency for Research on Cancer Carcinogenecity (IARC) Classifications as of 2013 *(continued)*

Compound	CAS No.	IARC Group	IARC Volume	Year
Dibenzo[a,h]pyrene	189-64-0	2B	92	2010
Dibenzo[a,i]pyrene	189-55-9	2B	92	2010
Ethylbenzene	100-41-4	2B	77	2000
Furan	110-00-9	2B	63	1995
Glu-P-1 (2-Amino-6-methyldipyrido[1,2-a:3′,2′-d]imidazole)	67730-11-4	2B	SUP 7	1987
Glu-P-2 (2-Aminodipyrido[1,2-a:3′,2′-d]imidazole)	67730-10-3	2B	SUP 7	1987
Hydrazine	302-01-2	2B	71	1999
Indeno[1,2,3-cd]pyrene	193-39-5	2B	92	2010
Isoprene	78-79-5	2B	71	1999
Lead	7439-92-1	2B	SUP 7	1987
MeA-α-C (2-Amino-3-methyl)-9H-pyrido[2,3-b]indole)	68006-83-7	2B	SUP 7	1987
N-Nitrosodiethanolamine (NDELA)	1116-54-7	2B	77	2000
N-Nitrosomethylethylamine	10595-95-6	2B	SUP 7	1987
N-Nitrosomorpholine (NMOR)	59-89-2	2B	SUP 7	1987
N-Nitrosopiperidine (NPIP)	100-75-4	2B	SUP 7	1987
N-Nitrosopyrrolidine (NPYR)	930-55-2	2B	SUP 7	1987
N-Nitrososarcosine (NSAR)	13256-22-9	2B	SUP 7	1987
Naphthalene	91-20-3	2B	82	2002
Nickel	7440-02-0	2B	49	1990
Nitrobenzene	98-95-3	2B	65	1996
Nitromethane	75-52-5	2B	77	2000
o-Anisidine	90-04-0	2B	73	1999
PhIP (2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine)	105650-23-5	2B	56	1993
Propylene oxide	75-56-9	2B	60	1994
Styrene	100-42-5	2B	82	2002
Trp-P-1 (3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]indole)	62450-06-0	2B	SUP 7	1987
Trp-P-2 (3-Amino-1-Methyl-5H-pyrido[4,3-b]indole)	62450-07-1	2B	SUP 7	1987
Vinyl acetate	108-05-4	2B	63	1995
1-Aminonaphthalene	134-32-7	3	SUP 7	1987
Chromium	7440-47-3	3	49	1990
Crotonaldehyde	4170-30-3	3	63	1995
Dibenzo[a,e]pyrene	192-65-4	3	92	2010
Mercury	7439-97-6	3	58	1993
Quinoline	91-22-5	EPA Group B2		
Cresols (o-, m-, and p-cresol)	1319-77-3	EPA Group C		

Notes: Most recently published IARC monograph for each compound is listed.
Quinoline and cresols have not been evaluated by IARC, but have been evaluated by U.S. Environmental Protection Agency.
IARC Groups: 1, Carcinogenic to humans; 2A, Probably carcinogenic to humans; 2B, Possibly carcinogenic to humans; 3, Not classifiable as to its carcinogenicity to humans; http://monographs.iarc.fr/ENG/Classification/ClassificationsAlphaOrder.pdf
EPA Groups: B2, Likely to be carcinogenic in humans; C, Possible human carcinogen.
CAS No., Chemical Abstracts Service registry number. *CAS Registry Number is a Registered Trademark of the American Chemical Society.* EPA, Environmental Protection Agency.
Quinoline: http://www.epa.gov/iris/subst/1004.htm
Cresols: http://www.epa.gov/iris/subst/0300.htm; http://www.epa.gov/iris/subst/0301.htm; http://www.epa.gov/iris/subst/0302.htm

TABLE 4.3

Commonly Used Biomarkers of Exposure to Carcinogens in Tobacco Smoke

Biomarker	Tobacco Smoke Source	Matrices
Monohydroxy-30butenyl mercapturic acid (MHBMA)	1,3-butadiene	Urine
4-Aminobiphenyl-globin	4-aminobiphenyl	Blood
N-(2-hydroxypropyl)methacrylamide (HPMA)	Acrolein	Urine
Carbamoylethylvaline	Acrylamide	Blood
Cyanoethylvaline	Acrylonitrile	Blood
S-phenylmercapturic acid (SPMA)	Benzene	Urine
Cd	Cadmium	Urine
3-hydroxypropyl mercapturic acid (HBMA)	Crotonaldehyde	Urine
2-hydroxyethyl mercapturic acid (HEMA)	Ethylene oxide	Urine
Nicotine equivalents (nicotine, cotinine, trans-3′-hydroxycotinine, and their respective glucuronides)	Nicotine	Urine
Total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) (NNAL + NNAL glucuronide)	NNK	Urine
Total NNN (NNN + NNN glucuronide)	NNN	Urine
1-Hydroxypyrene	Pyrene (representative of other PAH)	Urine

Adapted from Hecht SS, Yuan JM, Hatsukami D. Applying tobacco carcinogen and toxicant biomarkers in product regulation and cancer prevention. *Chem Res Toxicol* 2010;23:1001–1008.

Validation with respect to product use means that levels of a given biomarker differ substantially between users and nonusers, and that biomarker levels decrease substantially when product use is stopped. Validation with respect to disease risk implies that variation in biomarker levels in product users are predictive of variations in disease outcomes. Over the last decade, the development of modern high-throughput, high-resolution mass spectrometry has allowed for the measurement of multiple metabolites of tobacco carcinogens.[110–113] Commonly used biomarkers of tobacco exposure are listed in Table 4.3.

How Tobacco Use Leads to Cancer

A recent U.S. Surgeon General's report provides extensive detail on the current state of knowledge of how smoking causes cancer.[65] Therefore, only a brief overview is provided here. Hecht[101,113–116] has argued for a major pathway by which tobacco use leads to cancer: carcinogen exposure leads to the formation of carcinogen–DNA adducts, which then cause mutations that, if not repaired or removed by apoptosis, will eventually give rise to cancer. It is important to keep perspective that, whereas each cigarette may contain seemingly low levels of a given carcinogen, smoking is, for most people, a long-term addiction. Thus, a mixture of numerous carcinogens is administered multiple times per day over the course of decades. Further, compounds taken in during smokers can be metabolically activated, thus increasing their activity. Cigarette smoke compounds appear to induce the cytochrome P450 system, which facilitates the metabolic activation of carcinogens to electrophilic entities that are able to covalently bind DNA.[117,118] DNA adducts appear to be crucial to the cancer process, and numerous studies show that smoker tissues contain higher levels of DNA adducts than nonsmokers, and that DNA adduct levels are associated with cancer risk.[119,120] At the same time, other systems are involved in the detoxification and deactivation of smoke constituents, typically catalyzed by UDP-glucuronosyltransferases and glutathione-S-transferases, resulting in excretion of inactive compounds.[121,122] An individual's balance of activation and deactivation of toxicants may be an important predictor of cancer risk, although evidence for this is mixed in the literature.[123,124] Similarly, DNA repair capacity is an important consideration, because, even if adducts are formed, processes exist to remove such perturbations to normalize DNA structure. Enzymatic processes of DNA repair include alkytransferases, nucleotide excision, and mismatch repair. Polymorphisms in genes coding for these enzymes may relate to individual cancer susceptibility. Table 4.4 outlines the metabolic activation/detoxification, DNA-adduct formation, and repair processes believed to be involved for four tobacco carcinogens (nitrosamines, PAH, benzene, 4-aminobiphenyl).[65,120]

Those DNA adducts that persist can cause miscoding during DNA replication. Smoke carcinogens are known to cause G:A and G:T mutations, and mutations in the *KRAS* oncogene and the *P53* tumor suppressor gene are strongly associated with tobacco-caused cancers.[93,114,125–127] Inactivation of *P53*, together with the activation of *KRAS*, appear to reduce survival in non–small-cell lung cancer.[65] Gene mutations that do not result in apoptosis may go on to influence a number of downstream processes, which may lead to genomic instability, proliferation, and eventually, malignancy.[128–130] Some smoke constituents may also act in ways that indirectly support the development of cancer. Nicotine, although not a carcinogen in itself, is known to reduce apoptosis and increase angiogenesis and transformation processes via nuclear factor kappa B (NF-κB).[65,131] Activation of nicotinic acetylcholine receptors (nAChR) in lung epithelium by nicotine or NNK is associated with survival and proliferation of malignant cells.[65] Nitrosamines also appear to have similar activities via the activation of protein kinases A and B.[132] NNK may bind β-adrenergic receptors to stimulate the release of arachidonic acid, which is converted to prostaglandin E2 by cyclooxygenase (COX)-2. Smoke compounds appear to activate epidermal growth factor receptor (EGFR) and COX-2, both of which are found to be elevated in many cancers.[133] Ciliatoxic, inflammatory, and oxidizing compounds, such as acrolein and ethylene oxide in smoke, may also impact the likelihood of cancer development. Epigenetic changes such as hypermethylation, particularly at P16, may also play a role in lung cancer development.[65]

TABLE 4.4

Key Pathways and Processes Where Selected Smoke Constituents Are Activated and Detoxified

	NNN, NNK	PAH	Benzene	4-ABP
Metabolic Activation	Alpha hydroxylation	Diol epoxide formation	Epoxide/oxepin formation	N-oxidation
Cytochrome P450 Enzymes Involved	2A6, 2A13, 2E1	1A1, 1B1	2E1	1A2
Enzymes Involved in Detoxification/ Activation	UGT	MEH, GST, UGT	MEH, GST	UGT, NAT
DNA Adduct Formation Sites				
Lung	*O6*-POB-deoxyguanosine	BPDE-N2-deoxyguanosine		
Bladder				C-8 deoxyguanosine
DNA Repair Pathways	AGT, BER	NER, MMR	BER, NER, NIR	NER

UGT, uridine-5′-diphosphate-glucuronosyltransferases; MEH, microsomal epoxide hydrolases; NAT, *N*-Acetyltransferases; GST, glutathione-S-transferases; AGT, *O6*-alkylguanine–DNA alkyltransferase; BER, base excision repair; NER, nucleotide excision repair; MMR, mismatch repair; NIR, nucleotide incision repair.

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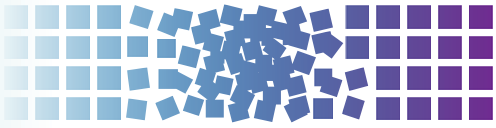
5 Oncogenic Viruses



Christopher B. Buck and Lee Ratner

PRINCIPLES OF TUMOR VIROLOGY

Viral infections are estimated to play a causal role in at least 11% of all new cancer diagnoses worldwide.[1] A vast majority of cases (>85%) occur in developing countries, where poor sanitation, high rates of cocarcinogenic factors such as HIV/AIDS, and lack of access to vaccines and cancer screening all contribute to increased rates of virally induced cancers. Even in developed countries, where effective countermeasures are widely available, cancers attributable to viral infection account for at least 4% of new cases.[2,3]

Viruses thought to cause various forms of human cancer come from six distinct viral families with a range of physical characteristics (Table 5.1). All known human cancer viruses are capable of establishing durable, long-term infections and cause cancer only in a minority of persistently infected individuals. The low penetrance of cancer induction is consistent with the idea that a virus capable of establishing a durable productive infection would not benefit from inducing a disease that kills the host.[4] The slow course of cancer induction (typically over a course of many years after the initial infection) suggests that viral infection alone is rarely sufficient to cause human malignancy and that virally induced cancers arise only after additional oncogenic "hits" have had time to accumulate stochastically.

In broad terms, viruses can cause cancer through either (or both) of two broad mechanisms: direct or indirect. Direct mechanisms, in which the virus-infected cell ultimately becomes malignant, are typically driven by the effects of viral oncogene expression or through direct genotoxic effects of viral gene products. In most established examples of direct viral oncogenesis, the cancerous cell remains "addicted" to viral oncogene expression for ongoing growth and viability.

A common feature of DNA viruses that depend on host cell DNA polymerases for replication (e.g., papillomaviruses, herpesviruses, and polyomaviruses) is the expression of viral gene products that promote progression into the cell cycle. A typical mechanism of direct oncogenic effects is through the inactivation of tumor suppressor proteins, such as the guardian of the genome, p53, and retinoblastoma protein (pRB). This effectively primes the cell to express the host machinery necessary for replicating the viral DNA. The study of tumor viruses has been instrumental in uncovering the existence and function of key tumor suppressor proteins, as well as key cellular proto-oncogenes, such as Src and Myc.

In theory, viruses could cause cancer via direct hit-and-run effects. In this model, viral gene products may serve to preserve cellular viability and promote cell growth in the face of otherwise proapoptotic genetic damage during the early phases of tumor development. In principle, the precancerous cell might eventually accumulate enough additional genetic hits to allow for cell growth and survival independent of viral oncogene expression. This would allow for stochastic loss of viral nucleic acids from the nascent tumor, perhaps giving a growth advantage due to the loss of "foreign" viral antigens that might otherwise serve as targets for immune-mediated clearance of the nascent tumor. Although hit-and-run effects have been observed in animal models of virally induced cancer,[5] these effects are extremely difficult to address in humans. Currently, there are no clearly established examples of hit-and-run effects in human cancer.

In indirect oncogenic mechanisms, the cells that give rise to the malignant tumor have never been infected by the virus. Instead, the viral infection is thought to lead to cancer by attracting inflammatory immune responses that, in turn, lead to accelerated cycles of tissue damage and regeneration of noninfected cells. In some instances, virally infected cells may secrete paracrine signals that drive the proliferation of uninfected cells. At a theoretical level, it may be difficult to distinguish between indirect carcinogenesis and hit-and-run direct carcinogenesis, because, in both cases, the metastatic tumor may not contain any viral nucleic acids.

A variety of hunting approaches have been used to uncover etiologic roles for viruses in human cancer. The first clues that high-risk human papillomaviruses (HPVs), Epstein-Barr virus (EBV), Kaposi's sarcoma–associated herpesvirus (KSHV), and Merkel cell polyomavirus (MCPyV) might be carcinogenic were based on the detection of virions, viral DNA, or viral RNA in the tumors these viruses cause. A common feature of known virally induced cancers is that they are more prevalent in immunosuppressed individuals, such as individuals suffering from HIV/AIDS or patients on immunosuppressive therapy after organ transplantation. This is thought to reflect the lack of immunologic control over the cancer-causing virus. Studies focused on AIDS-associated cancers provided the first evidence for the carcinogenic potential of KSHV and MCPyV. A theoretical limitation of this approach is that some virally induced cancers may not occur at dramatically elevated rates in all types of immunosuppressed subjects, particularly if the virus causes only a fraction of cases (e.g., HPV-induced head and neck cancers). Fortunately, the unbiased analysis of nucleic acid sequences found in tumors has become substantially more tractable as deep-sequencing methods have continued to fall in price. In the coming years, it should be increasingly possible to search for viral sequences without making the starting assumption that all virally induced tumors are associated with immunosuppression.[6]

One limitation of tumor sequencing approaches is that they might miss undiscovered divergent viral species within viral families known to have extensive sequence diversity[7] and could miss viral families that have not yet been discovered.[8] Tumor-sequencing approaches might also miss viruses that cause cancer by hit-and-run or indirect mechanisms. It is conceivable that this caveat could be addressed by focusing on sequencing early precancerous lesions thought to ultimately give rise to metastatic cancer.

An additional successful approach to hunting cancer viruses involves showing that individuals who are infected with a particular virus have an increased long-term risk of developing particular forms of cancer. This approach was successful for identifying and validating the carcinogenic roles of high-risk HPV types, hepatitis B virus (HBV), hepatitis C virus (HCV), KSHV, and human T-lymphotropic virus 1 (HTLV-1). Although viruses that are extremely prevalent, such as EBV and MCPyV, are not amenable to this approach per se, it may still be possible to draw connections

TABLE 5.1

Oncogenic Viruses

Virus	Taxon	Viral Genome	Virion	Infection Rate	Site of Persistance	Diseases in Normal Hosts	Diseases in Immunocompromised Hosts	Associated Cancers
High-risk human papillomavirus types (e.g., HPV16)	*Alphapapillomavirus*	8 kb circular dsDNA	Nonenveloped	>70%	Anogential mucosa, oral mucosa	Carcinomas of the cervix, penis, anus, vagina, vulva, tonsils, base of tongue	Increased incidence of same diseases	610,000
Hepatitis B virus (HBV)	*Hepadnaviridae*	3 kb ss/ dsDNA	Enveloped	2%–8%	Hepatocytes	Cirrhosis, hepatocellular carcinoma	Same diseases, increased incidence with AIDS	380,000
Hepatitis C virus (HCV)	*Flaviviridae*	10 kb +RNA	Enveloped	~3%	Hepatocytes	Cirrhosis, hepatocellular carcinoma, splenic marginal zone lymphoma	Same diseases, increased incidence with AIDS	220,000
Epstein-Barr virus (EBV, HHV-4)	*Gammaherpesvirinae*	170 kb linear DNA	Enveloped	90%	B cells, pharyngeal mucosa	Mononucleosis, Burkitt lymphoma, other non-Hodgkin lymphoma, nasopharyngeal carcinoma	Increased incidence of same diseases, lymphoproliferative disease, other lymphomas, oral hairy leukoplakia, leiomyosarcoma	110,000
Kaposi's sarcoma herpesvirus (KSHV, HHV-8)	*Gammaherpesvirinae*	170 kb linear DNA	Enveloped	2%–60%	Oral mucosa, endothelium, B cells	Kaposi's sarcoma (KS), multicentric Castleman disease (MCD)	Increased KS, MCD incidence, primary effusion lymphoma	43,000
Merkel cell polyomavirus (MCPyV, MCV)	*Orthopolyomavirus*	5 kb circular dsDNA	Nonenveloped	75%	Skin (lymphocytes?)	Merkel cell carcinoma (MCC)	Increased MCC incidence	1,500 (US)
Human T-cell leukemia virus (HTLV-1)	*Deltaretrovirus*	9 kb +RNA (RT)	Enveloped	0.01%–6%	T and B cells	Adult T-cell leukemia/ lymphoma, tropical spastic paraparesis, myelopathy, uveitis, dermatitis	Unknown	2,100

Note: Ranges for infection rates imply major variations in prevalence among populations in different world regions. *Associated cancers* indicates the annual number of new cases clearly attributable to viral infection. An estimate for the worldwide incidence of Merkel cell carcinoma is not currently available and an estimate of the annual new cases in the United States alone is given instead.
ds, double-stranded; ss, single-stranded; HHV, human herpesvirus; RT, reverse transcriptase.
Adapted from de Martel C, Ferlay J, Franceschi S, et al. Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. *Lancet Oncol* 2012;13(6):607–615; Schiller JT, Lowy DR. Virus infection and human cancer: an overview. *Recent Results Cancer Res* 2014;193:1–10; Chen CJ, Hsu WL, Yang HI, et al. Epidemiology of virus infection and human cancer. *Recent Results Cancer Res* 2014;193:11–32; and Virgin HW, Wherry EJ, Ahmed R. Redefining chronic viral infection. *Cell* 2009;138(1):30–50.

between cancer risk and either unusually high serum antibody titers against viral antigens or unusually high viral load. Relatively high serologic titers reflect either comparatively poor control of the viral infection in at-risk individuals or expression of viral antigens in tumors or tumor precursor cells.[9,10]

The finding that a virus causes cancer is good news, in the sense that it can suggest possible paths to clinical intervention. These can include the development of vaccines or antiviral agents that prevent, attenuate, or eradicate the viral infection and thereby prevent cancer; the development of methods for early detection or diagnosis of cancer based on assays for viral nucleic acids or gene products; or the development of drugs or immunotherapeutics that treat cancer by targeting viral gene products. Unfortunately, establishing the carcinogenicity of a given viral species is an arduous process that must inevitably integrate multiple lines of evidence.[11] The demonstration that the virus can transform cells in culture and/or cause cancer in animal models provides circumstantial evidence of the oncogenic potential of a virus. All known human cancer viruses meet this criterion. However, it is important to recognize that viruses can theoretically coevolve to be noncarcinogenic in their native host (e.g., humans) and cause cancer only in the dysregulated environment of a nonnative host animal. This caveat may apply to human adenoviruses.

Finding that viral DNA is clonally integrated in a primary tumor and its metastatic lesions helps address the caveat that the virus might merely be a hitchhiker that finds the tumor cell a conducive environment in which to replicate (as opposed to playing a causal carcinogenic role). This caveat is also addressed by the observation that, in most instances, viruses found in tumors have lost the ability to exit viral latency and are functionally unable to produce new progeny virions. An unfortunate consequence of this is that vaccines or antiviral agents that target virion proteins (e.g., vaccines against high-risk HPVs or HBV) or gene products expressed late in the viral life cycle (e.g., herpesvirus thymidine kinase, which is the target of drugs such as ganciclovir) are rarely effective for treating existing virally induced tumors.

Demonstrating that a vaccine or antiviral agent targeting the virus either prevents or treats human cancer is by far the strongest form of evidence that a given virus causes human cancer. This type of proof has fully validated the causal role of HBV in human liver cancer. Compelling clinical trial data also show that antiherpesvirus therapeutics can prevent KSHV- or EBV-associated lymphoproliferative disorders, and that vaccination against HPV can prevent the development of precancerous lesions on the uterine cervix.

PAPILLOMAVIRUSES

History

The idea that cancer of the uterine cervix might be linked to sexual behavior was first proposed in the mid 19th century by Dominico Rigoni-Stern, who observed that nuns rarely contracted cervical cancer, whereas prostitutes suffered from cervical cancer more often than the general populace.[12] Another major milestone in cervical cancer research was Georgios Papanikolaou's development of the so-called Pap smear for early cytologic diagnosis of precancerous cervical lesions.[13] This form of screening, which allows for surgical intervention to remove precancerous lesions, has saved many millions of lives in developed countries, where public health campaigns have made testing widely available.

Although observations in the early 1980s suggested the possibility of a hit-and-run carcinogenic role for herpes simplex viruses in cervical cancer,[14] this hypothesis was abandoned in light of studies led by Harald zur Hausen. Low-stringency hybridization approaches revealed the presence of two previously unknown papillomavirus types, HPV16 and HPV18, in various cervical cancer cell lines, including the famous HeLa cell line.[15,16] There is now overwhelming evidence that a group of more than a dozen sexually transmitted HPV types, including HPV16 and HPV18, play a causal role in essentially all cases of cervical cancer. HPVs associated with a high risk of cancer also cause about half of all penile cancers, 88% of anal cancers, 43% of vulvar cancers, 70% of vaginal cancers,[2] and an increasing fraction of head and neck cancers (see the following). In 2008, zur Hausen was awarded the Nobel Prize for his groundbreaking work establishing the link between HPVs and human cancer.

The viral family *Papillomaviridae* is named for the benign skin warts (papillomas) that some members of the family cause. In the early 1930s, Richard Edwin Shope and colleagues demonstrated viral transmission of papillomas in a rabbit model system.[17] Using this system, Peyton Rous and others showed that cottontail rabbit papillomavirus-induced lesions can progress to malignant skin cancer.[18,19] This was the first demonstration of a cancer-causing virus in mammals, building on Rous' prior work demonstrating a virus capable of causing cancer in chickens (the Rous sarcoma retrovirus).

Tissue Tropism and Gene Functions

Although papillomaviruses can achieve infectious entry into a wide variety of cell types in vitro and in vivo, the late phase of the viral life cycle, during which the viral genome undergoes vegetative replication and the L1 and L2 capsid proteins are expressed, is strictly dependent on host cell factors found only in differentiating keratinocytes near the surface of the skin or mucosa. Interestingly, a majority of HPV-induced cancers appear to arise primarily at zones of transition between stratified squamous epithelia and the single-layer (columnar) epithelia of the endocervix, the inner surface of the anus, and tonsillar crypts. It is thought that the mixed phenotypic milieu in cells at squamocolumnar transition zones may cause dysregulation of the normal coupling of the HPV life cycle to keratinocyte differentiation.

There are nearly 200 known HPV types.[20] In general, each papillomavirus type is a functionally distinct serotype, meaning that serum antibodies that neutralize one HPV type do not robustly neutralize other HPV types. Various HPV types preferentially infect different skin or mucosal surfaces. Different types tend to establish either transient infections that may be cleared over the course of months, or stable infections where virions are chronically shed from the infected skin surface for the lifetime of the host. HPV infections may or may not be associated with the formation of visible warts or other lesions. High-risk HPV types, with clearly established causal links to human cancer, are preferentially tropic for the anogenital mucosa and the oral mucosa, are usually transmitted by sexual contact, rarely cause visible warts, and usually establish only transient infections in a great majority of exposed individuals. The lifetime risk of sexual exposure to a high-risk HPV type has been estimated to be >70%. Individuals who fail to clear their infection with a high-risk HPV type and remain persistently infected are at much greater risk of developing cancer. Polymerase chain reaction (PCR)-based screening for the presence of high-risk HPV types thus serves as a useful adjunct to, or even a replacement for, the traditional Pap test.[21]

A consequence of the strict tissue-differentiation specificity of the papillomavirus life cycle is that HPVs do not replicate in standard monolayer cell cultures. Papillomaviruses also seem to be highly species restricted, and there are no known examples of an HPV type capable of infecting animals.[22] Thus, the investigation of key details of papillomavirus biology has relied almost entirely on modern recombinant DNA and molecular biologic analyses.

Papillomavirus genomes are roughly 8 kb, double-stranded, closed-circular DNA molecules (essentially reminiscent of a plasmid). During the normal viral life cycle, the genome does not adopt a linear form, does not integrate into the host cell chromosome, and remains as an extrachromosomal episome or minichromosome.

All the viral protein-coding sequences are arranged on one strand of the genome. The expression of various proteins is regulated by differential transcription and polyadenylation, as well as effects at the level of RNA splicing, export from the nucleus, and translation. In addition to the late half of the viral genome, which encodes the L1 and L2 capsid proteins, all papillomaviruses encode six key early region genes: E1, E2, E4, E5, E6, and E7.

The master transcriptional regulator E2 serves as a transcriptional repressor, and loss of E2 expression (typically through integration of the viral episome into the host cell DNA) results in the upregulation of early gene expression. The most extensively studied early region proteins are the E6 and E7 oncogenes of HPV16 and HPV18. The E6 protein of high-risk HPV types triggers the destruction of p53 by recruiting a host cell ubiquitin–protein ligase, E6AP.[23–25] Another important oncogenic function of E6 is the activation of cellular telomerase.[26] A wide variety of additional high-risk E6 activities that do not involve p53 have been identified.[27]

Most E7 proteins, including those of many low-risk HPV types, contain a conserved LXCXE motif that mediates interaction with pRB and the related "pocket" proteins p107 and p130.[28] Interestingly, the LXCXE motif is present in a wide variety of other oncogenes, most notably the T antigens of polyomaviruses and the E1A oncogenes of adenoviruses. The interaction of E7 with pRB disrupts the formation of a complex between pRB and E2F transcription factors, thereby blocking the ability of pRB to trigger cell cycle arrest.[29] The E7 proteins of high-risk HPVs can also contribute to chromosomal mis-segregation and aneuploidy, which may in turn contribute to malignant progression.[30] Like E6, E7 interacts with a wide variety of additional cellular targets, the spectrum of which seems to vary with different HPV types.[27]

Some papillomavirus types express an E5 oncogene, which functions as an agonist for cell surface growth factor receptors such as platelet-derived growth factor beta (PDGF-β) and epidermal growth factor (EGF) receptor.[31] Because E5 expression is uncommon in cervical tumors, it is uncertain whether the protein plays a key role in human cancer.

Human Papilloma Virus Vaccines

Two preventive vaccines against cancer-causing HPVs, trade named Gardasil (Merck) and Cervarix (GSK), are currently marketed worldwide for the prevention of cervical cancer. Both vaccines contain recombinant L1 capsid proteins based on HPV16 and HPV18 that are assembled in vitro into virus-like particles (VLPs). Together, HPV16 and HPV18 cause about 70% of all cases of cervical cancer worldwide. Gardasil also includes VLPs based on HPV types 6 and 11, which rarely cause cervical cancer but together cause about 90% of all genital warts. The VLPs contained in the vaccines are highly immunogenic in humans, eliciting high-titer serum antibody responses against L1 that are capable of neutralizing the infectivity of the cognate HPV types represented in the vaccine. It appears that the current HPV vaccines may confer lifelong immunity against new infection with the HPV types represented in the vaccine.[32] The vaccines elicit lower titer cross-neutralizing responses against a subset of cancer-causing HPV types that are closely related to HPV16 and HPV18.[33] Although these cross-neutralizing responses can at least partially protect vaccinees against a new infection with additional high-risk types, such as HPV31 and HPV45, it remains unclear how durable the lower level cross-protection will be.[33]

Because L1 is not expressed in latently infected keratinocyte stem cells residing on the epithelial basement membrane, current HPV vaccines are very unlikely to eradicate existing infections.[34,35] Like keratinocyte stem cells, cervical cancers and precursor lesions rarely or never express L1. Thus, the existing L1-based vaccines seem unlikely to serve as therapeutic agents for treating cervical cancer.

Three types of next-generation HPV vaccines are currently in human clinical trials. Merck has recently announced that a newer version of Gardasil, which contains VLPs based on a total of nine different HPV types, remained highly effective against HPV16 and HPV18 and also prevented 97% of precancerous cervical lesions caused by a wider variety of high-risk HPV types.[36] Another class of second-generation vaccines targets the papillomavirus minor capsid protein L2. An N-terminal portion of L2 appears to represent a highly conserved "Achilles' heel", which contains conserved protein motifs required for key steps of the infectious entry process.[37] Anti-L2 antibodies can neutralize a broad range of different human and animal HPV types, and thus, L2 vaccines are hoped to offer protection against all HPVs that cause cervical cancer, all low-risk HPV types that cause abnormal Pap smear results, as well as the full range of HPV types that cause skin warts. Finally, a wide variety of vaccines that seek to elicit cell-mediated immune responses against the E6 and E7 oncoproteins are aimed at a therapeutic intervention for the treatment of cervical cancer.[38]

Oropharyngeal Cancer

It is well established that tobacco products and alcohol cause head and neck cancer. In the late 1990s, Maura Gillison and colleagues noted a surprising number of new cases of tonsillar cancer in nonsmokers.[39] Many of the tumors found in nonsmokers were found to have wild-type p53 genes, raising the possibility that the tumor might be dependent on a p53-suppressing viral oncogene (as seen in cervical cancer). Gillison and colleagues went on to show that nearly half of all tonsillar cancers contain HPV DNA, most commonly HPV16. Interestingly, HPV-positive oropharyngeal cancers tend to be less lethal than tobacco-associated HPV-negative tumors. This finding has important implications for treatment of HPV-positive head and neck cancers.[40]

Although the incidence of tobacco-associated head and neck cancer has been declining in recent decades due to decreased tobacco use, recent studies suggest an ongoing increase in the incidence of HPV-associated cancers of the tonsils and the base of the tongue. By 2025, the number of new HPV-induced head and neck cancer cases in the United States is expected to roughly equal the number of new cervical cancer cases.[39] Based in part on these observations, the U.S. Centers for Disease Control and Prevention recommends that boys, in addition to girls, should be vaccinated against high-risk HPVs.

Nonmelanoma Skin Cancer

Epidermodysplasia verruciformis (EV) is a rare immunodeficiency that is characterized by the appearance of numerous flat, wartlike lesions across wide areas of skin. The lesions typically contain genus betapapillomaviruses, such as HPV5 or HPV8. EV patients frequently develop squamous cell carcinomas (SCC) in sun-exposed skin areas (suggesting that ultraviolet [UV] light exposure is a cofactor). It is also well established that other immunosuppressed individuals, such as organ transplant recipients and HIV-infected individuals, are at increased risk of developing SCC.[41,42] Although the E6 and E7 proteins of betapapillomaviruses appear to exert a different spectrum of effects than the E6 and E7 proteins of HPV types associated with cervical cancer,[43–45] Betapapillomavirus oncogenes can transform cells in vitro.[46] Although these circumstantial lines of evidence suggest that infectious agents, such as Betapapillomaviruses, might play a causal role in SCC, recent deep sequencing studies have observed few or no viral sequences in SCC tumors.[47] Although the results argue against durable direct oncogenic effects of any known viral species in SCC, an animal model system using bovine papillomavirus type 4 strongly suggests that papillomaviruses can cause cancer by hit and run mechanisms.[5] Thus, the question of whether hit-and-run or indirect oncogenic effects of HPVs may be at play in human SCC remains open.

POLYOMAVIRUSES

History

In the early 1950s, Ludwik Gross showed that a filterable infectious agent could cause salivary gland cancer in laboratory mice.[48] Later work by Bernice Eddy and Sarah Stewart showed that the murine polyoma (Greek for "many tumors") virus caused many different types of cancer in experimentally infected mice.[49] The discovery that murine polyomavirus could be grown in cell culture helped rekindle research interest in tumor virology and interest in the question of whether viruses might cause human cancer.

Like papillomaviruses, polyomaviruses have a nonenveloped capsid assembled from 72 pentamers of a single major capsid protein (VP1). Both viral families also carry circular dsDNA genomes. These physical similarities initially led to the classification of both groups into a single family, *Papovaviridae*. When sequencing studies ultimately revealed that polyomaviruses have a unique genome organization (with early and late genes being arranged on opposing strands of the genome) and almost no sequence homology to papillomaviruses, the two groups of viruses were divided into separate families.

In the early 1960s, Bernice Eddy, Maurice Hilleman, and Benjamin Sweet reported the discovery of simian vacuolating virus 40 (SV40), a previously unknown polyomavirus that was found as a contaminant in vaccines against poliovirus.[50,51] SV40 was derived from the rhesus monkey kidney cells used to amplify poliovirus virions in culture.[52] SV40 rapidly became an important model polyomavirus, and studies of its major and minor tumor antigens (large T [LT]and small t [ST], respectively) have played an important role in understanding various aspects of carcinogenesis. Despite significant alarm about the possible risk SV40 might pose to exposed individuals, a comprehensive, decades long series of studies have failed to uncover compelling evidence that SV40 exposure is causally associated with human cancer.[53]

Two naturally human-tropic polyomaviruses, BK virus (BKV) and John Cunningham virus (JCV), were first reported in back-to-back publications in 1971.[54,55] BKV and JCV are known to cause kidney disease and a lethal brain disease called progressive multifocal leukoencephalopathy, respectively, in immunosuppressed individuals. Although both viruses can cause cancer in experimentally exposed animals, it remains unclear whether either virus plays a causal role in human cancer. Although BKV LT expression can frequently be observed in the inflammatory precursor lesions that are thought to give rise to prostate cancer,[56] there is no evidence for the persistence of BKV DNA in malignant prostate tumors.[57] There have been case studies finding BKV T-antigen expression in bladder cancer,[58] and some reports have indicated the presence of JCV DNA in colorectal tumors. The long history of conflicting evidence concerning possible roles for BKV or JCV in human cancer is reviewed elsewhere.[59,60]

Merkel Cell Polyomavirus

In 2008, Yuan Chang and Patrick Moore reported their lab's discovery of the fifth known human polyomavirus species, which they named Merkel cell polyomavirus (MCV or MCPyV) based on its presence in Merkel cell carcinoma (MCC).[61] The discovery used an RNA deep sequencing approach called digital transcriptome subtraction. Using classic Southern blotting, this report demonstrated the clonal integration of MCPyV in an MCC tumor and its distant metastases. Many other labs worldwide have independently confirmed the presence of MCPyV DNA in about 80% of MCC tumors.[11]

MCC is a rare but highly lethal form of cancer that typically presents as a fast-growing lesion on sun-exposed skin surfaces (Fig. 5.1).[62] The risk of MCC is dramatically higher in HIV/AIDS patients, offering an initial clue that MCC might be a virally induced cancer.[63] Although MCC tumors express neuroendocrine markers associated with sensory Merkel cells of the epidermis, one recent report has shown that some MCC tumors also express B-cell markers, including rearranged antibody loci.[64] Currently, there is no clear evidence for the involvement of MCPyV in other tumors with neuroendocrine features.

In 2012, the International Agency for Research on Cancer (IARC) concluded that MCPyV is a class 2A carcinogen (probably carcinogenic to humans).[10,53] It should be noted that IARC evaluations rely heavily on animal carcinogenicity studies, and the 2A designation was assigned prior to a recent report showing that MCV-positive MCC lines are tumorigenic in a mouse model system.[65]

A great majority of healthy adults have serum antibodies specific for the MCPyV major capsid protein VP1. A majority also shed MCPyV virions from apparently healthy skin surfaces, and there is a strong correlation between individual subjects' serologic titer against VP1 and the amount of MCPyV DNA they shed.[66–68] Interestingly, MCC patients tend to have exceptionally strong serologic titers against VP1.[69] MCC tumors do not express detectable amounts of VP1, so this is unlikely to reflect direct exposure to

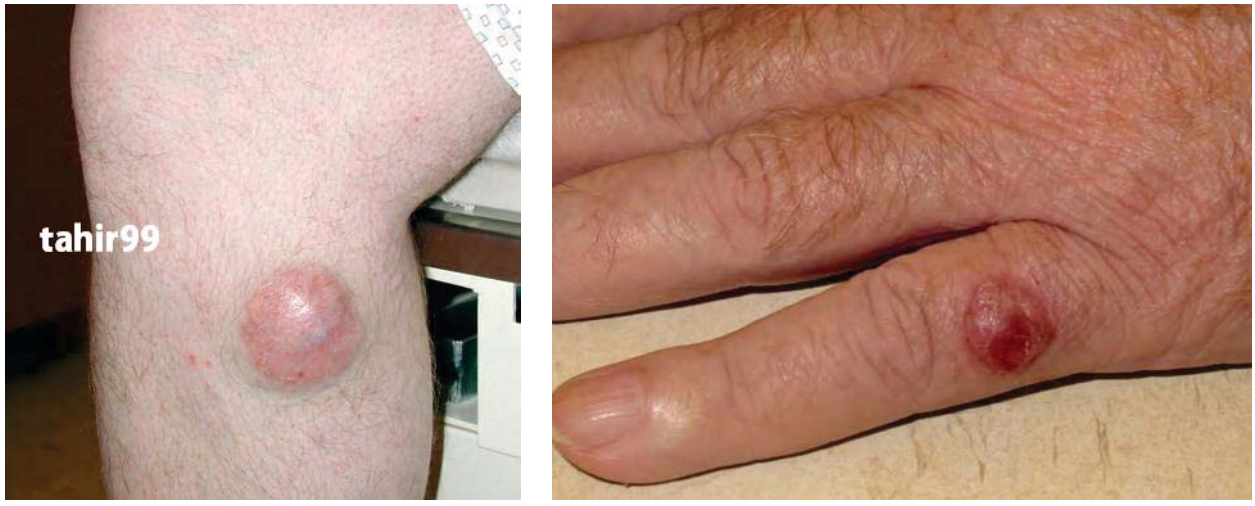


Figure 5.1 Merkel cell carcinoma (MCC). The *left panel* shows an MCC tumor on the calf. The *right panel* shows an MCC tumor on the finger. Photographs provided with permission by Dr. Paul Nghiem (University of Washington, www.merkelcell.org).

the tumor and instead likely represents a history of a high MCPyV load in MCC patients. A recent study of archived serum samples shows that unusually high serologic titers against MCPyV VP1 often precede the development of MCC by many years.[70]

Like the LT protein of SV40 (and the E7 proteins of high-risk HPVs), an N-terminal portion of the MCPyV LT protein contains an LXCXE motif that mediates inactivation of pRB function. In contrast to SV40 LT, which carries a p53-inactivation domain that overlaps the C-terminal helicase domain, MCPyV LT does not appear to inactivate p53 function.[71] Instead, the MCPyV LT helicase domain activates DNA damage responses and induces cell cycle arrest in cultured cell lines.[72] This may explain why the LT genes found in MCC tumors essentially always carry mutations that truncate LT upstream of the helicase domain. siRNA experiments indicate that most (although possibly not all) MCC tumors are "addicted" to the expression of MCPyV T antigens.[73–75] Interestingly, patients with higher levels of MCPyV DNA in their tumors, stronger T-antigen expression, and tumors that have been infiltrated by CD8+ T cells appear to have better prognoses.[76] This is consistent with the idea that cell-mediated immunity can help clear MCC tumors that express MCPyV antigens.

Recent work has shown that the pRB interacting domain of LT mediates increased expression of the cellular gene survivin. The knockdown of survivin using siRNAs results in MCC tumor cell death and YM155, a small molecule inhibitor of survivin expression, protects mice from MCC tumors in a xenograft challenge system.[77,78]

In contrast to SV40, where LT appears to be the dominant oncogene, the MCPyV ST protein appears to play a key role in cell transformation. In addition to modifying the signaling functions of the cellular proto-oncogene PP2A, ST triggers the phosphorylation of eukaryotic translation initiation factor 4E binding protein 1.[79] This results in dysregulation of cap-dependent translation and cellular transformation.

Although there is an intriguing epidemiologic correlation between MCC and chronic lymphocytic leukemia (CLL),[80] there are conflicting reports concerning the presence of MCPyV in CLL and other lymphocytic cancers.[81–83]

Other Human Polyomaviruses

In recent years, the number of known human polyomaviruses has expanded dramatically. Of the 12 currently known HPyV species, only MCPyV has been clearly linked to human cancer. One new HPyV, trichodysplasia spinulosa polyomavirus (TSV or TSPyV) has been found in association with abnormal spiny growths on the facial skin of a small number of immunocompromised individuals.

EPSTEIN-BARR VIRUS

History

In 1958, Denis Burkitt provided the first clear clinical description of an unusual B-cell–derived tumor that frequently affects the jawbones of children in equatorial Africa.[84] After hearing Burkitt give a 1961 lecture entitled "The Commonest Children's Cancer in Tropical Africa – A Hitherto Unrecognized Syndrome," Michael Epstein became interested in the idea that an insect vector-borne infection might account for the high incidence of Burkitt lymphoma in tropical Africa. Epstein, together with then PhD candidate Yvonne Barr, began examining tumor samples sent to them by Burkitt. Electron micrographs of lymphoid cells that grew out of the tumors in culture revealed viral particles with a morphology strikingly similar to herpes simplex viruses.[85] It was soon shown that Epstein-Barr herpesvirus (EBV, later designated human herpesvirus 4 [HHV-4]) can transform cultured B cells and is the agent responsible for infectious mononucleosis.[86–88]

Although the initial conjecture that tropically endemic Burkitt lymphoma depends on a geographically restricted infectious agent ultimately proved correct, it was quickly established that the EBV infection is not restricted to the tropics. It instead appears likely that the malaria parasite *Plasmodium falciparum* is a key geographically restricted cocarcinogen responsible for endemic Burkitt lymphoma.[53] In areas where children suffer repeated malaria infections, it appears that the parasite triggers abnormal B-cell responses, as well as weakened cell-mediated immune function, and these effects of recurring malaria infection in turn promote or allow the development of EBV-induced Burkitt tumors.[11]

Epstein-Barr Virus Life Cycle

EBV chronically infects nearly all humans. In a great majority of individuals, the infection is initially established in early childhood and is never associated with any noticeable symptoms. The infection is typically transmitted when virions, shed in the saliva of a chronically infected individual, come in contact with the oropharyngeal epithelium of a naïve individual. Although infected epithelial cells, such as keratinocytes, might serve to amplify the virus in some circumstances,[89] the establishment of chronic infection is ultimately dependent on mature B cells, as subjects with X-linked agammaglobulinemia (who lack mature B cells) appear to be immune to stable EBV infection.[90] Individuals who escape infection during childhood and instead first become infected during adolescence or adulthood often develop mononucleosis, which is associated with fevers and extreme fatigue lasting for weeks or sometimes months. Interestingly, late-infected individuals who experience mononucleosis and high EBV viral load are at increased risk of developing EBV-positive Hodgkin lymphoma.[91]

EBV-infected B cells can either go on to produce new virions, which are typically associated with cell lysis, or the virus can enter a nonproductive state known as latency. Viral latency is defined as a condition in which the virus expresses few (or possibly no) gene products but can, under some conditions, "reawaken" to express the full range of viral gene products and produce new progeny virions. Latently infected cells are highly resistant to immune clearance.

There are three recognized forms of EBV latency. In latency I, EBV nuclear antigen-1 (EBNA1), which is required for the stable maintenance of the circularized viral DNA minichromosome, is the only viral protein expressed. EBV-derived microRNAs (miRs) may also be expressed. At the other end of the spectrum, latency III is characterized by the expression of EBNA1–6, several latent membrane proteins (LMP1, 2A, and 2B), two noncoding RNAs (EBER1 and 2), the BCL-2 homolog BHRF1, BARF0, and multiple miRs. Although the initial discovery of EBV involved the visualization of virions, indicating that the virus had exited latency and entered the productive lytic phase of the life cycle, viral gene expression in EBV-induced cancers generally follows one of the three latent patterns. The oncogenic activities of various EBV gene products have recently been reviewed.[87,88]

In a great majority of healthy individuals, EBV exists almost exclusively in a latent state, with the occasional asymptomatic shedding of virions in the saliva. The infection is controlled, at least in part, by CD8+ T cells specific for various latency proteins. EBV, like other herpesviruses, expresses a variety of proteins that interfere with cell-mediated immune responses. Intriguingly, results from mouse model systems suggest that the chronic immunostimulatory effects of persistent gammaherpesvirus emergence (or abortive emergence) from latency in healthy hosts can nonspecifically boost immunity to other infections.[92]

Lymphomas

In addition to endemic Burkitt lymphoma, EBV is often present in sporadic cases of Burkitt lymphoma in individuals who have not been exposed to malaria. Although nearly all cases of endemic

Burkitt's lymphoma contain EBV DNA in the tumor (typically in a latency I–like state), only about 20% of sporadic cases arising in immunocompetent individuals contain EBV. Rates of Burkitt lymphoma are elevated in HIV-infected individuals, and HIV-associated Burkitt lymphomas contain EBV in about 30% of cases.

A common hallmark of all types of Burkitt's lymphomas is deregulation of the cellular Myc proto-oncogene. A classic mutation involves chromosomal translocation of the Myc gene to the antibody heavy chain locus. Burkitt's lymphoma tumors that lack detectable EBV DNA tend to carry multiple additional mutations in host cell genes, raising the possibility that an originally EBV-positive precursor cell ultimately accumulated mutations that rendered it independent of viral genes.[88,93]

In addition to Burkitt lymphoma, EBV is associated, to varying extents, with a histologically diverse range of other lymphoid cancers, including Hodgkin lymphoma, natural killer (NK)/T-cell lymphoma, primary central nervous system (CNS) lymphoma, and diffuse large B-cell lymphoma. The incidence of these various forms of lymphoma is significantly increased both in AIDS patients as well as in iatrogenically and congenitally immunosuppressed individuals.[88] In particular, the essentially universal presence of EBV in CNS lymphomas in AIDS patients makes it possible to diagnose the disease with a PCR test for EBV that, together with radiologic findings, can obviate the need for a brain biopsy.

EBV is almost invariably associated with lymphoproliferative disorders, such as plasmacytic hyperplasia and polymorphic B cell hyperplasia, which are often observed in organ transplant recipients. These polyclonal lymphoproliferative responses can, in some instances, progress to oligoclonal or monoclonal lymphomas of various types. The occurrence of EBV-associated lymphoproliferative disease in immunosuppressed patients is generally heralded by the increased detection of EBV DNA in the peripheral blood and the oral cavity. This presumably reflects the failure of cellular immune responses to drive the virus into full latency and perhaps also a failure of cell-mediated immune responses targeting latency-associated EBV gene products present in the nascent tumor.

Carcinomas

In Southern China, NPC affects 25 out of 100,000 people, accounting for 18% of all cancers in China as a whole.[94] Most other world regions have a 25- to 100-fold lower rate of NPC. EBV is present in nearly all cases of NPC, both in endemic and nonendemic regions. Although there is support for the idea that dietary intake of salted fish and other preserved foods is a factor in endemic NPC, it remains possible that genetic traits or as yet unidentified environmental cocarcinogenic factors may play a role as well. Individuals with rising or relatively high IgA antibody responses to EBNA1, DNase, and/or EBV capsid antigens have a dramatically increased risk of developing NPC, offering an early detection method for at-risk individuals.[87]

EBV is also present in a small percentage (5% to 15%) of gastric adenocarcinomas and over 90% of gastric lymphoepithelioma-like carcinomas. In contrast to NPC, the prevalence of EBV-associated gastric cancer is similar in all world regions. As with NPC, elevated antibody responsiveness to EBV antigens may offer a method for identifying individuals at greater risk of gastric cancer.

Prevention and Treatment

The reduction of immunosuppression in response to increasing EBV loads is a standard approach to preventing EBV diseases in T-cell immunosuppressed individuals. Another approach to the prevention of EBV disease relies on ganciclovir (or related antiherpesvirus drugs), which can trigger the death of cells that express the EBV thymidine kinase gene. Pretreating at-risk individuals, such as organ transplant recipients, with ganciclovir has been shown to effectively prevent the development of EBV-induced lymphoproliferative disorders.[95] However, it is important to note that thymidine kinase is only expressed in the lytic phase of the viral life cycle, and drugs of this class are not generally effective for treating existing tumors, presumably due to the fact that EBV gene expression in tumors is typically of a latent type.

Although a recently developed vaccine targeting the EBV gp350 virion surface antigen did not provide sterilizing immunity to EBV infection, vaccinees did experience lower peak EBV viral loads upon infection.[96] Given the strong correlation between high EBV loads and the development of EBV diseases, it is hoped that the vaccine's ability to merely blunt the acute infection may offer significant protection against disease.

Most forms of EBV-associated lymphoid cancers express the B-cell marker CD20, making rituximab (an anti-CD20 mAb) a potentially effective adjunct therapy.[97,98] An emerging treatment approach that has recently entered clinical trials involves stimulating T cells ex vivo against peptides based on EBV antigens or against autologous EBV-transformed B cells.

KAPOSI'S SARCOMA HERPESVIRUS

History and Epidemiology

In the late 19th century, Hungarian dermatologist Moritz Kaposi's described a relatively rare type of indolent pigmented skin sarcoma affecting older men.[99] Kaposi's sarcoma (KS) was later found to be more prevalent in the Mediterranean region and in eastern portions of sub-Saharan Africa.[100] An early clue to the emergence of the HIV/AIDS pandemic in the early 1980s was a dramatic increase in the incidence of highly aggressive forms of KS, particularly in gay men who were much younger than typical KS patients. After the discovery of HIV, it was briefly hypothesized that HIV might be a direct cause of KS. However, this hypothesis failed to explain the existence of KS long prior to the HIV pandemic and the low incidence of KS in individuals who became infected with HIV via blood products. This latter observation was more easily explained by the existence of a sexually transmitted cofactor other than HIV.[101]

Using a subtractive DNA hybridization approach known as representational difference analysis, Yuan Chang, Patrick Moore, and colleagues discovered the presence of a previously unknown herpesvirus in KS tumors.[102] The newly founded field of research rapidly established key lines of evidence supporting the conclusion that KSHV (later designated human herpesvirus-8 [HHV-8]) is a causal factor in KS.[11]

It is now clear that the rate of KSHV infection varies greatly in different world regions.[11,103] In North America and Western Europe, KSHV seroprevalence in the general population ranges from 1% to 7%. Seroprevalence among gay men in these regions is substantially higher (25% to 60%), suggesting a possible link to sexual transmission. KSHV infection is much more prevalent in the general population in central and eastern Africa, where seroprevalence ranges from 23% to 70%. In endemic areas, up to 15% of children are seropositive, suggesting either vertical transmission or transmission via nonsexual casual contact (presumably via saliva). In endemic regions, KS is estimated to be the third most common cancer among adults.[104]

Kaposi's Sarcoma-Associated Herpesvirus in Kaposi's Sarcoma

KS tumors are complex on a number of levels. In contrast to most other forms of cancer, where it is often clear that a single cell type has proliferated out of control, KS tumors are composed of cells from multiple lineages (Fig. 5.2). KSHV-infected cells in the tumor often have a spindle-shaped morphology. Interestingly,

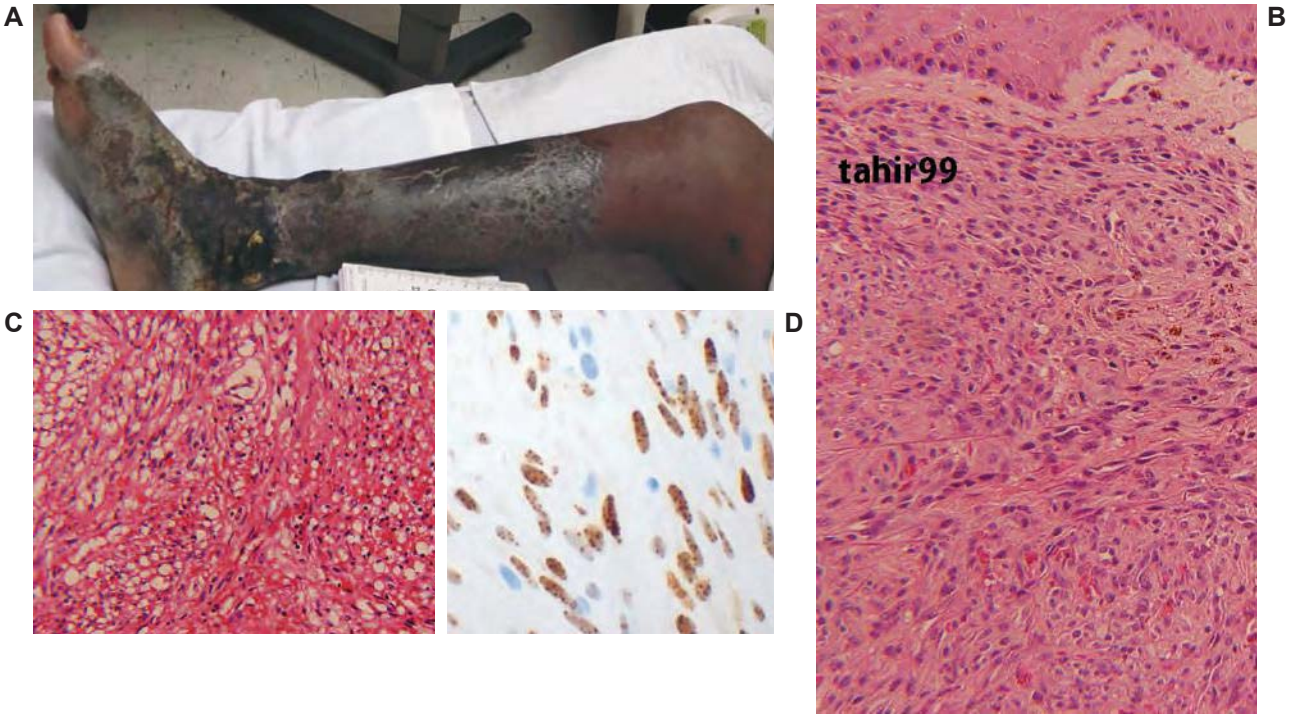


Figure 5.2 Kaposi's sarcoma (KS). **(A)** Photograph of the lower leg of an individual with severe, diffuse KS involving the lower leg. **(B)** Histology of the skin. **(C)** Lung shows a mixture of spindle to epithelioid cells, with slitlike vascular spaces intermixed with red blood cells and red blood cell fragments. **(D)** Immunohistochemical detection of KSHV LANA in the cutaneous tumor. Photographs provided with permission by Drs. Odey Ukpo and Ethel Cesarman.

spindle cells do not exhibit a highly transformed phenotype and tend to show relatively little chromosomal instability. In a culture, the cells are highly dependent on exogenous cytokines and other factors present in the tumor microenvironment in vivo. Although spindle cells express a number of markers of the endothelial lineage, it is uncertain whether they are derived from mature endothelial cells, the early precursor cells that give rise to smooth muscle and vascular endothelial cells, or cells of the lymphatic endothelial lineage. KS tumors also contain infiltrating lymphocytes and monocytes, as well as aberrant neovascular spaces lined with infected and uninfected endothelial cells. The aberrant blood vessels in KS lesion vessels rupture easily and leak red blood cells, giving KS tumors their classic dark red, brown, or purple color.

The latency status of KSHV in KS tumors is also complex, with the expression of gene products typical of latency (e.g., LANA) as well as lytic-phase genes (e.g., RTA/ORF50). Some of these gene products, such as the viral interleukin (IL)-6 homolog (vIL-6), trigger proliferation and secondary cytokine signaling in noninfected cells within the tumor. The tumorigenic effects of individual KSHV gene products have recently been reviewed.[88,103] In contrast to EBV, where tumorigenesis is driven by latency gene expression, it appears that KS pathogenesis is often dependent on lytic phase gene expression. This may explain why ganciclovir, which is not a particularly effective treatment for EBV tumors, was found to prevent the formation of new KS lesions in HIV-positive patients.[105] However, it should be noted that this outcome has more recently proven difficult to reproduce.[106] At present, there are no recommended preventive therapies for individuals at risk of KS, but this is an area of active investigation.

There are a variety of possible explanations for the need for lytic-phase KSHV gene expression during tumor development. For example, infected spindle cells may lose the viral DNA during cell division and require reinfection for ongoing tumorigenicity. Alternatively, factors secreted by a small fraction of tumor cells that enter the lytic phase may be required for tumorigenesis. An important area of current research focus is the role of KSHV gene products in the regulation of angiogenesis in KS lesions[107] and several current trials are investigating inhibitors of angiogenic pathways for the treatment of KS.

Lymphoproliferative Disorders

KSHV causes two forms of B-cell proliferative disorder: multicentric Castleman disease (MCD) and primary effusion lymphoma (PEL). Both diseases are most commonly found in association with HIV infection. In HIV-infected individuals, MCD tumors contain KSHV in nearly all cases, whereas in HIV-negative individuals, the tumor contains KSHV in only about 50% of cases.[108] KSHV in MCD tumors exhibits periodic activation of lytic replication and the expression of lytic phase genes.[109] The expression of vIL-6 during disease flare-ups appears to play a role in MCD pathogenesis, raising the possibility that tocilizumab (a mAb therapeutic that targets the IL-6 receptor) may be of therapeutic benefit.

PEL comprises about 4% of all HIV-associated non-Hodgkin lymphomas.[110] Typically, PEL tumors express markers of both plasma cells (akin to multiple myeloma tumors) and immunoblasts (similar to some EBV-induced tumors). In AIDS patients, essentially all PEL tumors are infected with KSHV and a great majority are also coinfected with EBV.[88] Although PEL is rare in HIV-negative individuals, PEL tumors in such individuals contain KSHV in about 50% of cases.

A common approach to the treatment of all KSHV-associated diseases is the restoration of immune function, either through antiretroviral therapy of HIV/AIDS or through a reduction of immunosuppressive therapy. The general success of immune reconstitution in many KSHV-associated diseases presumably involves an immune-mediated attack of cells expressing KSHV gene products, particularly the many lytic-phase gene products the virus can produce in various disease states.

ANIMAL AND HUMAN RETROVIRUSES

The first oncogenic retroviruses were discovered by Ellerman and Bang in 1908 and by Rous in 1911, but it was many years before the significance of these findings was appreciated.[111] One reason the field was stymied was the failure to identify RNA forms of the viral genome in infected cells. This led to the discovery of the reverse transcriptase independently by Baltimore and Temin in 1970. Another major development was the finding in 1976 of viral oncogenes derived from cellular genes, with the identification by Varmus and Bishop of the first dominant oncogene, *src*. With the discovery of IL-2 by Gallo in 1976, it became possible to culture the first human retrovirus, HTLV-1, from a form of adult T-cell leukemia/lymphoma (ATLL) that was first recognized by Takatsuki and coworkers.[112] These advances opened the door for Montagnier and colleagues' isolation of HIV-1 in 1983, a discovery confirmed independently by Gallo and Levy. This breakthrough led to the first licensed HIV test in 1985.

Retroviruses are positive single-strand RNA viruses that utilize transcription of their RNA genome into a DNA intermediate during virus replication.[111] This accounts for their name, retroviruses, because this is opposite to the normal flow of eukaryotic genetic information. They infect a wide range of vertebrate animal species and are distantly related to repetitive elements in the human genome, known as retrotransposons. Retroviruses are also related to hepadnaviruses, double-stranded DNA viruses, such as hepatitis B virus, which also undergo a reverse transcription step in their replication.

Retroviruses may be classified as *endogenous* or *exogenous* depending on whether they appear in the genome of the host species. There are approximately 100,000 endogenous retroviral elements in the human genome, making up nearly 8% of the genetic information, but their potential roles in disease are unclear.[113] Retroviruses may also be classified as *ecotropic*, *xenotropic*, or *polytropic* depending on whether they infect cells of the same animal species from which they are derived, infect cells of a different species, or both. *Amphotropic* retroviruses infect cells of the species of origin without producing disease, but infect cells of other species and may produce disease.

Retroviruses that produce disease after a long incubation period are termed *lentiviruses* and include human, simian, feline, ovine, caprine, and bovine immunodeficiency viruses. Another group of retroviruses that are not clearly associated with disease are known as *spumaviruses* and include human and simian foamy viruses. HTLV-1, which is classified in the genus Delta, is the only retrovirus known to be oncogenic in humans. A member of the retroviral genus Gamma identified in 2008, designated xenotropic murine leukemia virus-related virus (XMRV), was thought to be associated with human prostate cancer; however, more recent studies showed XMRV to be a lab-derived artifact.[114] A genus betaretrovirus related to the mouse mammary tumor virus has been suggested to be associated with biliary cirrhosis, but this finding requires independent validation.[115]

Retroviruses producing tumors in animals or birds are designated transforming viruses and may be classified as acute or chronic transforming retroviruses. Acute transforming retroviruses have acquired a mutated cellular gene, termed *oncogene*, and induce cancer in an animal within a few weeks. Many dominant acting proto-oncogenes in humans (e.g., *ras*, *myc*, and *erbB*), were first identified as retroviral oncogenes.

Chronic transforming retroviruses integrate almost randomly in the genome, but when integrated in the vicinity of specific genes disrupt their regulation and induce cell proliferation or resistance to apoptosis. Chronic transforming retroviruses induce malignancy only after many weeks to months of infection. The use of a murine leukemia virus vector for gene therapy in children with a form of severe combined immune deficiency syndrome characterized by defective expression of the common gamma chain of the IL-2 receptor resulted in T-cell acute lymphoblastic leukemia. This was found to be the result of persistent expression of the LIM domain only 2 (LMO2) gene triggered by the nearby integration of the retroviral vector.[116]

In addition to acute or chronic transformation mechanisms, retroviruses can transform cells through direct effects on cell physiology mediated by structural or nonstructural viral proteins. Transforming genes of HTLV-1 are nonstructural viral proteins that activate host cell signaling pathways.[117] Because the oncogenic effects of HTLV-1 transforming genes generally take many years to cause cancer, the virus does not fit the precise definition of having either an acute or a chronic oncogenic mechanism.

HIV-1 infection is also associated with a variety of malignancies, but only by indirect effects of suppressing immunity to oncogenic virus infections, such as gammaherpesviruses, high-risk human papillomaviruses, and hepatitis viruses.

Human T-Cell Leukemia Virus Epidemiology

Four species of human T-cell leukemia virus have been identified. HTLV-1 was identified in 1980 as the first human retrovirus associated with cancer, and it is the focus of the remainder of this section.[118] HTLV-2 was discovered in 1982 and shares 70% genomic homology with HTLV-1.[119] HTLV-3 and -4 were sporadically isolated from individuals who had contact with monkeys.[120] HTLV-2, -3, and -4 do not appear to be associated with disease in humans.

HTLV-1 is present in 15 to 20 million individuals worldwide, most commonly in the Caribbean Islands, South America, southern Japan, and parts of Australia, Melanesia, Africa, and Iran.[121] In the United States, Canada, and Europe, 0.01% to 0.03% of blood donors are infected with HTLV-1. It is most commonly found in individuals who emigrated from endemic regions or among African Americans. HTLV-1 is transmitted sexually, by contaminated cell-associated blood products, or by breast-feeding.[122] Only 2% to 5% of HTLV-1–infected individuals develop disease, and ATLL only occurs in individuals who acquired HTLV-1 by breast-feeding.

Human T-Cell Leukemia Virus Molecular Biology

HTLV-1, like other retroviruses, encodes Gag, Protease, Pol, and Envelope proteins.[123] Gag proteins compose the inner nucleocapsid core of the virus. The Pol proteins include the reverse transcriptase and integrase. The reverse transcriptase copies the single-stranded viral RNA into double-stranded DNA, and it is inhibited by several nucleoside analogs, but not by the nonnucleoside reverse transcriptase inhibitors approved for HIV-1.[124] The integrase is responsible for inserting the linear double-stranded DNA product of reverse transcription into the host chromosomal DNA. At least one integrase inhibitor, raltegravir, now approved for HIV-1, is active against HTLV-1.[125] Integration occurs throughout the human genome, but there is preference for integration into transcriptionally active genomic regions.[126] The viral protease proteolytically processes Gag, Protease, and Pol precursor proteins to the mature individual proteins, but it is not affected by inhibitors of HIV-1 protease. The envelope proteins include the transmembrane protein, which anchors the surface envelope protein on the virion, which mediates binding to the viral receptor.[127]

The viral genome also encodes regulatory proteins, including Tax and HTLV-1 bZIP factor (HBZ).[117] Tax is a transcriptional transactivator protein that functions as a coactivator to induce members of the cAMP response element-binding protein/activating transcription factor (CREB/ATF) family, nuclear factor kappa B (NF-κB), and serum response factor (SRF) pathways. Tax activation of the CREB/ATF pathway is responsible for upregulation of the viral promoter. Tax induction of NF-κB promotes cell proliferation and resistance to apoptosis. Tax also binds and activates cyclin-dependent kinases and inhibits cell cycle checkpoint proteins. Tax

is important for tumor initiation, whereas HBZ may be important in tumor maintenance.[128]

HTLV-1 preferentially immortalizes CD4+ T lymphocytes and induces tumors in mice.[129] Tax also promotes the leukemia-initiating activity of ATLL cells in mouse models.[130] In immunodeficient mice reconstituted with human hematopoietic cells, HTLV-1 causes CD4+ lymphomas.[131]

Clinical Characteristics and Treatment of HTLV-Associated Malignancies

The diagnosis of HTLV-1 is based on serologic assays.[132] HTLV-1 is associated with various inflammatory disorders, including uveitis, polymyositis, pneumonitis, Sjögren syndrome, and myelopathy. Infected patients are susceptible to certain infectious disorders (e.g. staphylococcal dermatitis) and opportunistic infections such as pneumocystis pneumonia, disseminated cryptococcosis, strongyloidiasis, or toxoplasmosis.[133] Vaccines have not been developed for HTLV infections.

T-lymphocyte proliferative disorders develop in 1% to 5% of infected individuals and are generally CD2+, CD3+, CD4+, CD5+, CD25+, CD29+, CD45RO+, CD52+, HLA-DR+, T-cell receptor αβ+, and variably CD30+, and lack CD7, CD8, and CD26 expression. The virus is clonally integrated in the malignant cells. Complex karyotypes are often found, and cytogenetic analysis is rarely useful. The histologic features of lymph nodes in ATLL may be indistinguishable from those of other peripheral T-cell lymphomas.[134] Circulating tumor "flower cells" are helpful in the diagnosis (Fig. 5.3).

ATLL is categorized in four subtypes.[135] (1) Smoldering ATLL is defined as 5% or more abnormal T lymphocytes and lactate dehydrogenase (LDH) levels up to 1.5× the upper limit of normal, with normal lymphocyte count, calcium, and no lymph node or visceral disease other than skin or pulmonary disease. (2) Chronic ATLL is characterized by lymphocytosis, LDH up to 2× the upper limit of normal, no hypercalcemia, and no CNS, bone, pleural, peritoneal, or gastrointestinal involvement, although the lymph nodes, liver, spleen, skin, or lungs may be involved. The mean survival of these forms of ATLL is 2 to 5 years.[136] No intervention in these subtypes of ATLL has been defined that prevents progression to the more aggressive forms of ATLL. Although chronic or smoldering ATLL may respond to zidovudine and interferon, randomized studies have not been conducted.[137] (3) Lymphoma-type ATLL is characterized by ≤1% abnormal T lymphocytes and features of non-Hodgkin lymphoma. (4) Acute-type ATLL includes the remaining patients. Even with optimal therapy, the median survival of lymphoma and acute-type ATLL is less than 1 year.[138] Lymphoma and acute types of ATLL are the most common presenting subtypes. Other major prognostic factors include performance status, age, the presence of more than three involved lesions, and hypercalcemia.[139]

Combination chemotherapy for lymphoma or acute-type ATLL with the infusional etoposide, prednisone, vincristine, and doxorubicin (EPOCH) regimen or the LSG-15 regimen results in complete remission rates of 15% to 40%.[140,141] However, responses are short lived, with <10% of patients free of disease at 4 years. The addition of anti-CCR4 antibody, mogamulizumab, may improve response rates, but studies are still underway.[142]

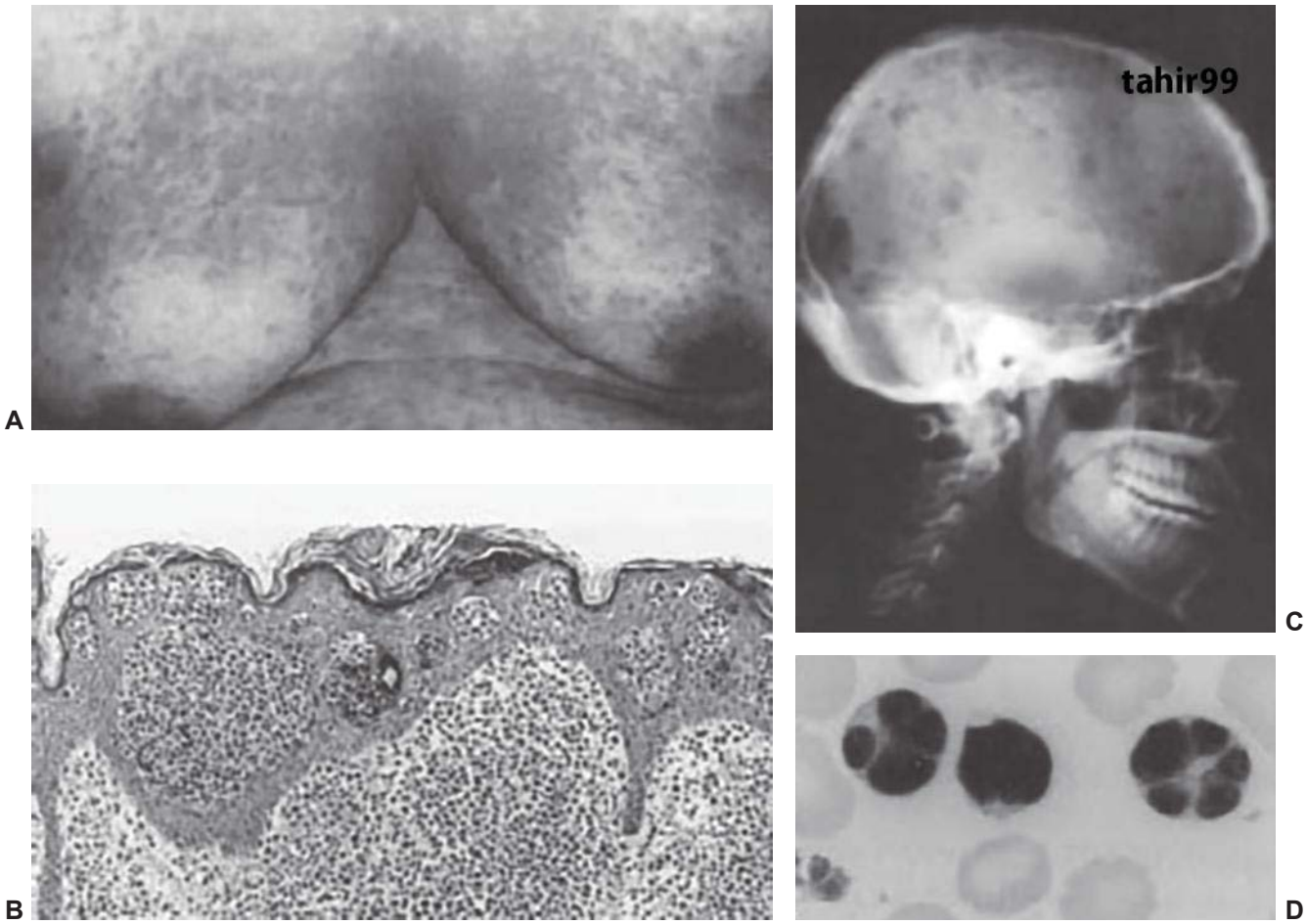


Figure 5.3 Clinical manifestation of adult T-cell leukemia/lymphoma. **(A–B)** Infiltration of malignant T lymphocytes into the skin. **(C)** Lytic bone lesions seen on lateral skull x-ray. **(D)** "Flower cells" in the blood.

The combination of interferon and zidovudine with or without arsenic may result in the remission of acute, but not lymphoma subtypes.[143] Allogenic transplantation may result in long-term, disease-free survival for patients with complete or near complete remission of disease, although infectious complications have been notable in these studies.[144]

HEPATITIS VIRUSES

The earliest record of an epidemic caused by a hepatitis virus was in 1885, occurring in individuals vaccinated for smallpox with lymph from other people.[145] The cause of the epidemic, HBV, was not identified until 1966, when Blumberg discovered the *Australian antigen* now known to be the hepatitis B surface antigen (HBsAg). This was followed by the discovery of the virus particle by Dane in 1970. In the early 1980s, the HBV genome was sequenced and the first vaccines were tested. In the mid 1970s, Alter described cases of hepatitis not due to hepatitis A or B viruses, and the suspected agent was designated non-A, non-B hepatitis virus, now known as HCV.[146] In 1987, Houghton used molecular cloning to identify the HCV genome and develop a diagnostic test, which was licensed in 1990.

Approximately 240 million people are chronically infected with HBV and 150 to 200 million people are infected with HCV worldwide, according to the World Health Organization (WHO). About 1 million deaths per year are attributed to the chronic diseases such as liver cirrhosis and hepatocellular carcinoma (HCC) that result from viral hepatitis infections. HBV and HCV are the leading cause of liver cancer in the world, accounting for almost 80% of the cases. In the United States, Europe, Egypt, and Japan, more than 60% of HCC cases are associated with HCV, and 20% are related to HBV and chronic alcoholism.[147] In Africa and Asia, 60% of HCC is associated with HBV, 20% related to HCV, and the remainder related to other risk factors, such as alcohol and aflatoxin. HCC is the sixth most common cancer worldwide and is the third most common cause of cancer death.[148]

In Asia and Africa, up to 70% of individuals have serologic evidence of current or prior HBV infection, and 8% to 15% of these subjects have a chronic active infection. Rates of HCV infection of >3.5% occur in Central and East Asia, North Africa, and the Middle East. In the United States, 0.8 to 1.4 million individuals are infected with HBV, and 3.2 million with HCV. The incidence of HCC in the United States tripled between 1975 and 2005, particularly in African American and Hispanic males.[149]

HBV is transmitted primarily through exposure to infected blood, semen, and other body fluids, whereas HCV is transmitted primarily by contact with contaminated blood. Acute HCV infection causes mild and vague symptoms in about 15% of individuals and resolves spontaneously in 10% to 50% of cases.[150] Liver enzymes are normal in 5% to 50% of individuals with chronic HCV infection.[151] After 20 years of an HCV infection, the likelihood of cirrhosis is 10% to 15% for men, and 1.5% for women.[152] Cofactors that increase the likelihood of cirrhosis are coinfection with both hepatitis viruses, persistently high levels of HBV or HCV viremia, HBeAg, certain viral genotypes, schistosoma, HIV, alcoholism, male gender, advanced age at the time of infection, diabetes, and obesity.[153,154]

Hepatitis B Virus

HBV is an enveloped DNA virus that is a member of the *Hepadnaviridae* family.[155] HBV has a strong preference for infecting hepatocytes, but small amounts of viral DNA can also be found in kidney, pancreas, and mononuclear cells, although it is not linked to extrahepatic disease. The viral genome is a relaxed circular, partially double-stranded (ds) DNA of 3.2 kb. The genome exists as an episomal covalently closed circular dsDNA (cccDNA) molecule in the nucleus of infected cells, although chromosomal integration of viral genomic sequences can occur during cycles of hepatocyte regeneration and proliferation. In addition to 40 to 42 nm virions, HBV-infected cells also produce noninfectious 20-nm spherical and filamentous particles. The viral genome encodes four open reading frames. The presurface–surface (preS-S) region encodes three proteins from different translational initiation sites; these include the S (HBsAg), M (or pre-S2), and L (or pre-S1) proteins. The L protein is responsible for receptor binding and virion assembly. The precore–core (preC-C) region encodes the HBcAg and HBeAg. The P region encodes the viral polymerase, and the X (HBx) protein modulates host-signal transduction.

After infection, the viral genome is transcribed by host RNA polymerase II, and viral proteins are translated. Nucleocapsids assemble in the cytosol, incorporating a molecule of pregenomic RNA into the viral core, where reverse transcription occurs to produce the dsDNA viral genome. Viral cores are enveloped with intracellular membranes and viral L, M, and S surface antigens, which are exported from the cells.

HBV replication is not cytotoxic. Instead, liver injury is due to the host immune response, primarily T-cell and proinflammatory cytokine responses. Chronic HBV carriers exhibit an attenuated virus-specific T-cells response, although a vigorous humoral response is still evident. About 5% of infections in adults and up to 90% of infections in neonates result in a persistent infection, which may or may not be associated with symptoms and elevated serum aminotransferase levels. About 20% of such individuals develop cirrhosis. Immunosuppressed individuals also have a higher likelihood of a persistent infection.

With acute infection, viral titers of 10^9 to 10^{10} virions per mililiter are present, whereas levels of 10^7 to 10^9 virions per mililiter and HBsAg, and in some cases, HBeAg are present in the blood of individuals with a persistent infection. The resolution of infection, which is associated with declining viral DNA titers, is observed at a rate of 5% to 10% per year in persistently infected individuals. However, even subjects who have resolved the infection continue to have very low levels of viral DNA (10^3 to 10^5 copies per mililiter) for most of their lives.

HBV infection can be managed with alpha interferon or nucleos(t)ide analogs that inhibit the viral polymerase, such as lamivudine, telbivudine, entecavir, adefovir, and tenofovir.[156] Entecavir and tenofovir are both effective at inducing viral suppression, and may be used in combination in patients with high HBV DNA load or multidrug resistance. Because these agents are all associated with some toxicity, current guidelines recommend therapy only when liver disease is clinically apparent, with continued treatment for 6 to 12 months after clearance of HBeAg or HBsAg. Although these drugs effectively control HBV, they typically fail to cure the infection due to the long-term persistence of the cccDNA form of the viral genome. Other nucleos(t)ide analogs are currently in clinical trials, as well as a novel form of interferon (IFN-λ) and an inhibitor of virus release.[157]

Hepatitis D virus (HDV) occurs only in individuals coinfected with HBV. HDV is composed a single-stranded circular viral RNA genome of 1,679 nucleotides, a central core of HDAg, and an outer coat with all three HBV envelope proteins. HDV infection results in more severe complications than infection with HBV alone, with a higher likelihood and more rapid progression to cirrhosis and HCC.

Hepatits C Virus

HCV is an enveloped RNA virus associated with cancer, primarily HCC and, rarely, splenic marginal zone lymphoma.[158] HCV is a positive-sense, single-stranded RNA virus of the *Flaviviridae* family.[159] There are seven genotypes of HCV; in the United States, about 70% of infections are caused by genotype 1.[160] HCV replicates in the cytoplasm and does not integrate into the host cell

genome. The viral RNA is 9.6 kb and encodes a single polyprotein of 3,010 amino acids that is proteolytically processed into structural and nonstructural proteins. In addition to the structural roles of the core (C) protein, it has also been reported to affect various host cell functions. The envelope glycoproteins E1 and E2 mediate infectious entry through tetraspanin CD81 and other receptors on hepatocytes and B lymphocytes.

HCV non structural proteins NS2, NS3, NS4A, NS4B, NS5A, NS5B, and p7 are required for virus replication and assembly. NS2 is a membrane-associated cysteine protease. NS3 is a helicase and NTPase that unwinds RNA and DNA substrates. The complex of NS3 with NS4A forms a serine protease. NS4B induces the formation of a membranous web associated with the viral RNA replicase. NS5A is an RNA-binding phosphoprotein, whereas NS5B is the RNA-dependent RNA polymerase. The p7 protein forms a cation channel in infected cells that has a role in particle maturation and release.

Treating an HCV infection typically utilizes 24 to 48 weeks of pegylated IFN-α and ribavirin.[161] Treatment with IFN and ribavirin alone produces sustained virologic responses in 70% to 80% of subjects with genotype 2 or 3 infections. Recently approved inhibitors of the NS3-4A protease (e.g., telaprevir, boceprevir, or simeprevir) may be included in IFN-based regimens, particularly if the patient has failed prior therapy. Protease inhibitors are currently approved for use in IFN/ribavirin combination therapy for HCV genotype 1 or 4 infection. Sofosbuvir, a nucleoside analog inhibitor of the viral NS5B polymerase, has recently been approved for use in combination with ribavirin alone for genotypes 2 or 3, or in triple therapy for genotypes 1 and 4. Recently, IFN-free regimens have also been approved. Additional protease and polymerase inhibitors are currently in development. A recent meta-analysis of eight randomized controlled trials comparing antiviral therapy with placebo suggested that antiviral therapy resulted in a 50% reduced risk of HCC.[162]

Hepatitis Virus Pathogenesis

HBV and HCV depress innate immune responses by inhibiting Toll-like receptor signaling through effects of HBx and NS3-4A.[147] In addition, HCV C inhibits the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling, and NS5A and E2 inhibit IFN signaling. Through an undefined mechanism, HBV can inhibit JAK-STAT signaling as well.

HBV and HCV induce HCC by direct and indirect mechanisms.[147] Both HBV and HCV encode proteins that have pro- and antiapoptotic properties. High levels of HBx block activation of the NF-κB pathway, whereas HCV C and NS5A block apoptosis by the activation of AKT and NF-κB, respectively. The C and NS5A proteins may also induce epithelial–mesenchymal transition (EMT), which is important for liver fibrosis, through effects on transforming growth factor β and Src signaling. Mice transgenic for NS5A develop steatosis and HCC.

HBx and HCV C are associated with mitochondria, where they trigger oxidative stress that induces apoptosis. In addition, HBs and HBx and NS3-4A alter calcium signaling and increase reactive oxygen species, which trigger endoplasmic reticulum (ER) stress, an unfolded protein response, and the production of proinflammatory cytokines that induce collagen synthesis, which drives the development of fibrosis. Autophagy is triggered by both viruses to restore ER integrity, which promotes cell survival and viral persistence.

HBV and HCV also disrupt tumor suppressor proteins. HCV NS5B recruits an ubiquitin ligase protein to modify pRB and induce its degradation, whereas HBx and HCV C proteins both inhibit p16INK4a and p21 cell cycle inhibitors, which leads to the inactivating phosphorylation of pRB. The HBx and HCV C, NS3, and NS5A proteins deregulate p53 tumor suppressor activity, by compromising p53-mediated DNA repair. HBV and HCV also induce alterations in micro-RNAs that are partially responsible for cell cycle effects.

Although not part of the normal virus replication cycle, the tendency of HBV genomic DNA sequences to integrate within the host cell chromosomes also contributes to the pathogenesis of HBV-associated HCC. In most hepatoma cells, HBV replication is extinguished, and integration at certain sites provides a growth or survival advantage, leading to tumors that are clonal with respect to viral integration. Whole-genome sequencing studies have identified a number of cellular loci, including *TERT* and *MLL*, where HBV integration is associated with HCC.[163,164]

Both HBV and HCV promote characteristics of cancer stem cells. HBx promotes the expression of Nanog, Kruppel-like factor 4, octamer-binding transcription factor 4, and Myc. These markers are also induced by HBV and HCV-induced hypoxia and hypoxia-induced factors.

Clinical Characteristics and Treatment of Hepatitis Virus-Associated Malignancies

HBV and HCV infections are diagnosed by serologic assays, and/or antigen assays in the case of HBV.[153] Quantitative HBV DNA and HCV RNA polymerase chain reactions are utilized to measure virus load. No vaccine has been identified that protects against HCV because infections consist of a genetically heterogenous "swarm" of virus particles, some of which escape neutralization. However, a vaccine, which now utilizes a recombinant HBsAg produced in yeast cells, has been available for HBV prevention for more than 30 years. The HBV vaccine reduces the risk of infection by more than 70%.[157] Factors associated with HBV vaccination failure in adults include increased age, obesity, smoking, diabetes, end-stage renal disease, HIV infection, alcoholism, or recipients of liver or kidney transplantation. There have been recent suggestions that emerging HBV strains may be evolving to escape neutralizing antibodies elicited by the current vaccine.[165] Novel vaccine adjuvants are currently in clinical trials, as well as studies of a therapeutic HBV vaccine.

Because an early diagnosis of HCC is key to a successful treatment, there has been extensive research on surveillance techniques in HBV- and HCV-infected individuals.[166] The U.S. Centers for Disease Control and Prevention has recently recommended that all individuals born between 1945 and 1965 be tested for HCV infection. The American Association for the Study of Liver Diseases, as well as the European and Asian Pacific Associations for the Study of the Liver, endorse surveillance in HCV-infected individuals with cirrhosis using ultrasound every 6 months. Viral eradication does not fully eliminate the risk of HCC, and thus, continued surveillance is still recommended in cirrhotic patients.

Therapeutic options for HCC are determined not only by the number and size of HCC nodules as well as the presence or absence of vascular invasion and metastases, but also by liver function and the presence or absence of portal hypertension.[167] HCC amenable to liver transplantation is usually defined as either one tumor measuring ≤50 mm in diameter or two to three tumors measuring ≤30 mm in diameter without vascular extension or metastasis (Milan criteria).[168] Up to 30% of all cases of HCC present with multiple nodules of HCC, suggesting a field carcinogenesis effect of HBV and HCV.[169] HBV- and HCV-infected patients may have a lower survival than noninfected patients after liver transplantation.[170] Hepatitis B immune globulin and nucleos(t)ide analogs are recommended for reinfection prophylaxis in the posttransplant period for HBV-infected individuals.[171] Studies are underway to examine the appropriate use of antiviral therapy for HCV-infected patients undergoing liver transplantation.

Reactivation of HCV can occur with chemotherapy or monoclonal antibody-based immunosuppressive therapies, but is less frequent as compared to HBV infection.[172] Individuals who appear to have cleared an HBV infection and who have an undetectable viral load can experience HBV reactivation on rituximab therapy. Monitoring hepatic function and virus load is indicated during

chemoimmunotherapy of HBV- or HCV-positive patients.[173] Although there is controversy regarding the role of virus screening for patients undergoing chemotherapy, antiviral therapy is recommended for high-risk HBV-infected patients undergoing chemoimmunotherapy, such as rituximab-based chemotherapy regimens.[174]

An association between HCV and B-cell non-Hodgkin lymphoma (NHL) has also been demonstrated in highly endemic geographic areas.[175] Lymphoproliferation has been linked to type II mixed cryoglobulinemia in many of these individuals. In addition to diffuse large B-cell lymphoma, marginal zone lymphomas and lymphoplasmacytic lymphomas are the histologic subtypes most frequently associated with HCV infection. Antiviral treatment with IFNα with or without ribavirin has been effective in the treatment of HCV-infected patients with indolent lymphoma, but rarely in individuals with aggressive lymphomas.

CONCLUSION

Oncogenic viruses are important causes of cancer, especially in less industrialized countries and in immunosuppressed individuals. They are common causes of anogenital cancers, lymphomas, oral and hepatocellular carcinomas and are associated with a variety of other malignancies. Vaccines and antiviral agents play an important role in the prevention of virus-induced cancers. Studies of virus pathogenesis will continue to establish paradigms that are critical to our understanding of cancer etiology in general.

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6 Inflammation

Sahdeo Prasad and Bharat B. Aggarwal

INTRODUCTION

Extensive research over the last half a century indicates that inflammation plays an important role in cancer. Although acute inflammation can play a therapeutic role, low-level chronic inflammation can promote cancer. Different inflammatory cells, the various cell signaling pathways that lead to inflammation, and biomarkers of inflammation have now been well defined. These inflammatory pathways, which are primarily mediated through the transcription factors nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3), have been linked to cellular transformation, tumor survival, proliferation, invasion, angiogenesis, and metastasis of cancer. These pathways have also now been linked with chemoresistance and radioresistance. This chapter considers the role of inflammation in cancer and its potential for cancer prevention and treatment.

Inflammation is the complex biologic responses of the body to irritation, injury, or infection. The recognition of inflammation dates back to antiquity. As documented by Aulus Cornelius Celsus, a Roman of the 1st century AD, inflammation is characterized by the tissue response to injury that results in *rubor* (redness, due to hyperemia), *tumor* (swelling, caused by increased permeability of the microvasculature and leakage of protein into the interstitial space), *calor* (heat, associated with increased blood flow and the metabolic activity of the cellular mediators of inflammation), and *dolor* (pain, in part due to changes in the perivasculature and associated nerve endings). Rudolf Virchow subsequently added *functio laesa* (dysfunction of the organs involved) in the 1850s. The process includes increased blood flow with an influx of white blood cells and other chemical substances that facilitate healing. Inflammation is also considered the body's self-protective attempt to remove harmful stimuli, including damaged cells, irritants, or pathogens, and to begin the healing process.

The word inflammation is derived from the Latin *inflammo* (meaning "I set alight, I ignite"). Because inflammation is a stereotyped response, it is considered a mechanism of innate immunity, as compared with adaptive immunity. On the basis of longevity, inflammation is classified as acute or chronic. When inflammation is short term, usually appearing within a few minutes or hours and ceasing upon the removal of the injurious stimulus, it is called acute. However, if it persists longer, it is called chronic inflammation, which leads to simultaneous destruction from the inflammatory process. Inflammation is beneficial when it is acute; however, chronic inflammation leads to several diseases, including cancer. Cancer is primarily a disease of lifestyle, with 30% of all cancers having been linked to smoking, 35% to diet, 14% to 20% to obesity, 18% to infection, and 7% to environmental pollution and radiation (Fig. 6.1).[1] Smoking, obesity, infections, pollution, and radiation are all known to activate proinflammatory pathways.[2] Therefore, understanding how inflammation contributes to cancer etiology is important for both cancer prevention and treatment.[3]

MOLECULAR BASIS OF INFLAMMATION

Although it is clear that inflammation and cancer are closely related, the mechanisms underlying persistent and chronic inflammation in chronic diseases remain unclear. Numerous cytokines have been linked with inflammation, including tumor necrosis factor (TNF), interleukin (IL)-1, IL-6, IL-8, IL-17, and vascular endothelial growth factor (VEGF). Among various cytokines that have been linked with inflammation, TNF is a primary mediator of inflammation linked to cancer.[4] However, it has been shown that proinflammatory transcriptional factors (activator protein [AP]-1, STAT3, NF-κB, hypoxia-inducible factor [HIF]-1, and β-catenin/Wnt) are ubiquitously expressed and control numerous physiologic processes, including development, differentiation, immunity, and metabolism in chronic diseases. Although these transcription factors are regulated by completely different signaling mechanisms, they are activated in response to various stimuli, including stresses and cytokines, and are involved in inflammation-induced tumor development and its metastasis.[5] Interestingly, inflammation plays a role at all stages of tumor development: initiation, progression, and metastasis.[2] In initiation, inflammation induces the release of a variety of cytokines and chemokines that promote the release of inflammatory cells and associated factors. This further causes oxidative damage, DNA mutations, and other changes in the tissue microenvironment, making it more conducive to cell transformation, increased survival, and proliferation. Inflammation also contributes to tissue injury, remodeling of the extracellular matrix, angiogenesis, and fibrosis in diverse target tissues. Among all the inflammatory cell signaling pathways, NF-κB has been shown to play a major role in cancer,[6,7] and TNF is one of the most potent activators of NF-κB.[8,9]

ROLE OF INFLAMMATION IN TRANSFORMATION

Transformation is the process by which the cellular and molecular makeup of a cell is altered as it becomes malignant. Numerous factors are involved in the process of cell transformation, including inflammation. A clinical study has shown that chronic inflammation due to heavy metal deposition in lymph nodes leads to malignant transformation and, finally, to patient death.[10] More recently, chronic exposure to cigarette smoke extract[11] and arsenite[12] has been shown to induce inflammation followed by epithelial–mesenchymal transition and transformation of human bronchial epithelial (HBE) cells. Furthermore, activation of NF-κB and HIF-2α increased the levels of the proinflammatory IL-6, IL-8, and IL-1β, which are essential for the malignant progression of transformed HBE cells. Sox2, another important molecular factor, cooperates with inflammation-mediated STAT3 activation, which precedes the malignant transformation of foregut basal progenitor cells.[13] A clinical study reported that the p53 mutation is a critical event for the malignant transformation of sinonasal inverted papilloma. This p53 mutation resulted in cyclooxygenase (COX)-2–mediated inflammatory signals that contribute to the proliferation

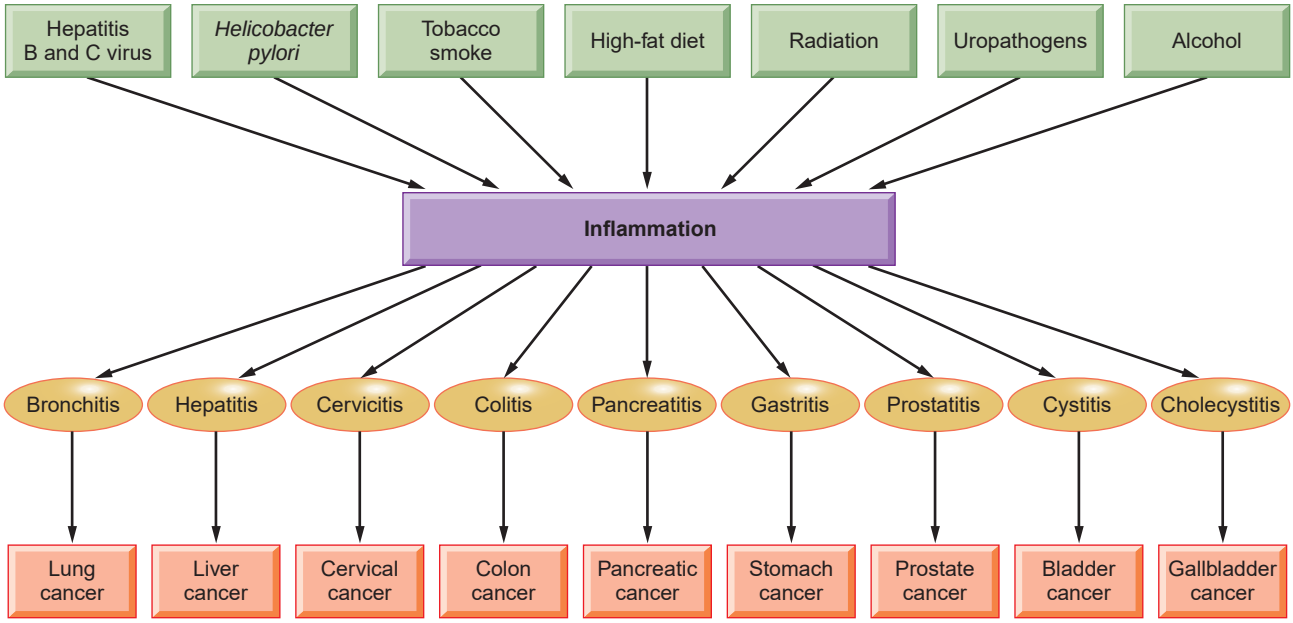

Figure 6.1 Origin of inflammation and its role in various cancers.

of advanced sinonasal inverted papilloma.[14] In another study in patients, the YKL-40 protein was found to be involved in chronic inflammation and oncogenic transformation of human breast tissues.[15] Inflammation-mediated transformation was also found to be regulated by MyD88 in a mouse model through Ras signaling.[16] In addition, inflammation contributed to the activation of the epidermal growth factor receptor (EGFR) and its subsequent interaction with PKCδ, which leads to the transformation of normal esophageal epithelia to squamous cell carcinoma.[17] Activation of Src oncoprotein triggers an inflammatory response mediated by NF-κB that directly activates Lin28 transcription and rapidly reduces let-7 microRNA levels. The inflammatory cytokine IL-6 mediates the activation of STAT3 transcription factor, which results in the transformation of cells.[18]

ROLE OF INFLAMMATION IN SURVIVAL

Numerous findings across different cancer populations have suggested that inflammation has an important role in carcinogenesis and disease progression.[19,20] The important markers of systemic inflammatory response in both in vitro findings and clinical outcomes include plasma C-reactive protein (CRP) concentration,[21,22] hypoalbuminemia,[23] and the Glasgow Prognostic Score (GPS), which combines CRP and albumin.[24,25] In addition to these, hematologic markers of systemic inflammatory response such as absolute white-cell count or its components (neutrophils, neutrophil-to-lymphocyte ratio [NLR]),[26–28] platelets, and a platelet-to-lymphocyte ratio[29,30] are also prognostic indicators for cancer clinical outcomes. Whether these inflammatory biomarkers influence the survival of cancer patients is discussed in this section.

In a study of 416 patients with renal cell carcinoma, with 362 patients included in the analysis, elevated neutrophil count, elevated platelet counts, and a high NLR were found. This inflammatory response was predictive for shorter overall patient survival.[31] Another study in unresectable malignant biliary obstruction (UMBO) found that patients with low GPS (0 and 1) had better postoperative survivals than did patients with a higher GPS. The 6-month and 1-year survival rates were 58.1% to 27.3%, respectively, for patients with low GPS and 25% to 6.2%, respectively, for patients with a higher GPS.[32] It has been also shown that prostate cancer patients with aggressive, clinically significant disease and an elevated GPS[2] had a higher risk of death overall as well as high-grade disease.[33] Other than GPS, age and gastrectomy have also been shown to independently influence the disease-specific and progression-free survival of gastric cancer patients.[34] A biomarker of systemic inflammation, the blood NLR, predicted patient survival with hepatocellular carcinoma (HCC) after transarterial chemoembolization. Patients in whom the NLR remained stable or became normalized after transarterial chemoembolization showed improved overall survival compared with patients showing a persistently abnormal index of NLR.[35]

A further study found that inflammatory transcription factors and cytokines contribute to the overall survival of patients. One study found that 97% of patients with epithelial tumors of malignant pleural mesothelioma and 95% of patients with nonepithelial tumors expressed IL-4Rα protein, and this strong IL-4Rα expression was correlated with a worse survival. In response to IL-4, human malignant pleural mesothelioma cells showed increased STAT6 phosphorylation and increased production of IL-6, IL-8, and VEGF without any effect on proliferation or apoptosis. This finding indicates that high expression of STAT6 as well as STAT3 and cytokines is inversely correlated with survival in patients.[36,37] NF-κB, along with IL-6, contributes to the survival of mammospheres in culture, because NF-κB and IL-6 were hyperactive in breast cancer–derived mammospheres.[38] In addition, elevated CRP and serum amyloid A (SAA) were associated with reduced disease-free survival of breast cancer patients.[39] In gastroesophageal cancer, proinflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α and acute phase protein concentrations (CRP) were found to be elevated, and these levels were associated with reduced survival of patients.[40] Additionally, the Bcl-2 family protein COX-2, which is regulated by inflammatory transcription factors, is also involved in the survival of cancer cells.[41,42] Thus, we conclude that inflammation in general contributes to poor survival of patients.

In contrast to these findings, an in vivo study of dogs with osteosarcoma showed that survival improvement was apparent with inflammation or lymphocyte-infiltration scores >1, as well as in dogs that had apoptosis scores in the top 50th percentile.[43] Also, in patients with epithelioid malignant pleural mesothelioma, a high degree of chronic inflammatory cell infiltration in the stromal component was associated with improved overall survival.[44]

ROLE OF INFLAMMATION IN PROLIFERATION

Several studies have shown that cell proliferation is affected by inflammation.[45] More significantly, proliferation in the setting of chronic inflammation predisposes humans to carcinoma in the esophagus, stomach, colon, liver, and urinary bladder.[46] In postgastrectomy patients, *Helicobacter pylori* induced inflammation and was associated with increased epithelial cell proliferation.[47] Even in the mouse model, chronic infection with *Helicobacter hepaticus* induced hepatic inflammation, which further led to hepatic cell proliferation.[48] Other reports found an increased expression of the cell proliferative markers PCNA and Ki-67 in the linings of inflamed odontogenic keratocysts compared with noninflamed lesions.[49,50] These findings suggest the existence of greater proliferative activity in the cells with inflammation. Wang et al.[51] showed an increased expression of cell proliferative markers PCNA and Ki-67 in a sample of 45 patients with benign prostatic hyperplasia.

The inflammatory biomarker COX-2 was also associated with the proliferation of cells. The highest proliferation index was found in COX-2–positive epithelium.[51] The association of COX-2 and proliferation was also reported in a rat model. The carcinogen dimethylhydrazine (DMH) induces an increase in epithelial cell proliferation and in the expression of COX-2 in the colon of rats.[52] Erbb2, a kinase, regulates inflammation through the induction of NF-κB, Comp1, IL-1β, COX-2, and multiple chemokines in the skin by ultraviolet (UV) exposure. This inflammation has been shown to increase the proliferation of skin tissue after UV irradiation.[53]

ROLE OF INFLAMMATION IN INVASION

A characteristic of invasive cancer cells is survival and growth under nonadhesive conditions. This invasion of cancer cells causes the disease to spread, which results in poor patient survival.[54] A strong relationship has been documented between inflammation and cancer cell invasion.[55,56] In a study of 150 patients with HCC, a high GPS score was associated with a high vascular invasion of cancer cells.[57] Another study of colorectal cancer also supports the links between inflammation and the invasion of cancer cells, with a finding that a high GPS increased the invasion of colorectal cancer cells.[58] In patients with esophageal squamous cell carcinoma, a high GPS score also showed a close relationship with lymphatic and venous invasion.[59]

At the molecular level, various proteins are known to be involved in tumor cell invasion. MMP-9, a gelatinase that degrades type IV collagen—the major structural protein component in the extracellular matrix and basement membrane—is thought to play an important role in facilitating tumor invasion, as it is highly expressed in various malignant tumors.[60,61] Additionally, the high expression of HIF-1α has been proposed as being associated with a greater incidence of vascular invasion of HCC. This expression of HIF-1α was further correlated with high expression of the inflammatory molecule COX-2.[62]

Breast cancer invasion has been linked to proteolytic activity at the tumor cell surface. In inflammatory breast cancer (IBC) cells, high expression of cathepsin B, a cell surface proteolytic enzyme, has been shown to be associated with invasiveness of IBC. In addition, a high coexpression of cathepsin B and caveolin-1 was found in IBC patient biopsies. Thus, proteolytic activity of cathepsin B and its coexpression with caveolin-1 contributes to the invasiveness of IBC.[63] In IBC, RhoC GTPase is also responsible for the invasive phenotype.[64] In addition, the PI3K/Akt signaling pathway is crucial in IBC invasion. The molecules involved in cell motility are specifically upregulated in IBC patients compared with stage-matched and cell-type-of-origin–matched non-IBCs patients. Distinctively, RhoC GTPase is a substrate for Akt1, and its phosphorylation is absolutely essential for IBC cell invasion.[65]

ROLE OF INFLAMMATION IN ANGIOGENESIS

Angiogenesis—the formation of new blood vessels from existing vessels—is tightly linked to chronic inflammation and cancer. Angiogenesis is one of the molecular events that bridges the gap between inflammation and cancer. Angiogenesis results from multiple signals acting on endothelial cells. Mature vessels control exchanges of hematopoietic cells and solutes between blood and surrounding tissues by responding to microenvironmental cues, including inflammation. Although inflammation is essential to defend the body against pathogens, it has adverse effects on the surrounding tissue, and some of these effects induce angiogenesis. Inflammation and angiogenesis are thereby linked processes, but exactly how they are related has not been well understood. Both inflammation and angiogenesis are exacerbated by an increased production of chemokines/cytokines, growth factors, proteolytic enzymes, proteoglycans, lipid mediators, and prostaglandins.

A close relationship has been reported between inflammation and angiogenesis in breast cancer. Tissue section staining showed increased vascularity with the intensity of diffuse inflammation.[66] Offersen et al.[67] found that inflammation was significantly correlated in bladder carcinoma with microvessel density, which is a marker of angiogenesis. Leukocytes have been described as mediators of inflammation-associated angiogenesis. In addition, the stable expression of TNF-α in endothelial cells increased angiogenic sprout formation independently of angiogenic growth factors. Furthermore, in work using the Matrigel plug assay in vivo, increased angiogenesis was observed in endothelial TNF-α–expressing mice. Thus, chronic inflammatory changes mediated by TNF-α can induce angiogenesis in vitro and in vivo, suggesting a direct link between inflammation and angiogenesis.[68] TNF-α–induced inhibitor of nuclear factor kappa kinase (IKK)-β activation also activates the angiogenic process. IKK-β activates the mammalian target of rapamycin (mTOR) pathway and enhances angiogenesis through VEGF production.[66] In addition to TNF-α, proinflammatory cytokines IL-1 (mainly IL-1β) and IL-8 were also found to be major proangiogenic stimuli of both physiologic and pathologic angiogenesis.[69,70] Recently, another cytokine macrophage migration inhibitory factor (MIF) was found to play a role in neoangiogenesis/vasculogenesis by endothelial cell activation along with inflammation.[71]

Benest et al.[72] found that a well-known regulator of angiogenesis, angiopoietin-2 (Ang-2), can upregulate inflammatory responses, indicating a common signaling pathway for inflammation and angiogenesis. TGF-β induction was also reported in head and neck epithelia and human head and neck squamous cell carcinomas (HNSCC), with severe inflammation that leads to angiogenesis.[73] The tumor-derived cytokine endothelial monocyte-activating polypeptide II (EMAP-II) has been shown to have profound effects on inflammation as well as on the processes involved in angiogenesis.[74] NF-κB plays an important role in inflammation as well as in angiogenesis, because the suppression of NF-κB and IkB-2A blocks basic fibroblast growth factor–induced angiogenesis in vivo. NF-κB regulates the angiogenic protein VEGF promoted by α5β1 integrin, which coordinately regulates angiogenesis and inflammation.[75] It has been also reported that a coculture of cancer cells with macrophages synergistically increased the production of various angiogenesis-related factors when stimulated by the inflammatory cytokine. This inflammatory angiogenesis was mediated by the activation of NF-κB and activator protein 1 (Jun/Fos), because the administration of either NF-κB–targeting drugs or COX-2 inhibitors or the depletion of macrophages blocked inflammatory angiogenesis.[76]

In a mouse model, cigarette smoke induced the inflammatory protein 5-lipoxygenase (5-LOX), and this induction activated matrix metalloproteinase 2 (MMP-2) and VEGF to induce the angiogenic process.[77] A cellular enzyme, Tank-binding kinase 1

(TBK-1), has been proposed as a putative mediator in tumor angiogenesis. TBK-1 mediates angiogenesis through the upregulation of VEGF and exerts proinflammatory effects via the induction of inflammatory cytokines. Thus, these pathways, including TBK-1, are an important cross-link between angiogenesis and inflammation.[78]

ROLE OF INFLAMMATION IN METASTASIS

Inflammation plays a regulatory role in cancer progression and metastasis. Chronic or tumor-derived inflammation and inflammation-related stimuli within the tumor microenvironment promote blood and lymphatic vessel formation and aid in invasion and metastasis.[79,80] The association of inflammation and metastasis has been observed in several cancer types. In an immunohistochemical analysis of lung cancer tissues, a remarkably high level of metastasis was observed with severe inflammation.[81] A mouse model of breast cancer found that mammary tumors increased the frequency of lung metastases, and this effect was associated with the recruitment of inflammatory cells to the lung as well as elevated levels of IL-6 in the lung airways.[82] In another murine model, implanting human ovarian tumor cells into the ovaries of severe combined immunodeficient mice resulted in peritoneal inflammation and tumor cell dissemination from the ovaries. In addition, enhancement of the inflammatory response with thioglycolate accelerated the development of ascites and metastases, and its suppression with acetylsalicylic acid delayed metastasis.[83] Thus, it can be concluded that inflammation facilitates ovarian tumor metastasis by a mechanism largely mediated by cytokines.

It has been shown that metastatic tumor cells entering a distant organ such as the liver trigger a proinflammatory response involving the Kupffer cell–mediated release of TNF-α and the upregulation of vascular endothelial cell adhesion receptors, such as E-selectin.[84] The physiologic expression of the selectins is tightly controlled to limit the inflammatory response, but dysregulated expression of selectins contributes to inflammatory and thrombotic disorders as well as tumor metastases.[85] Using P-selectin knockout mice, the importance of P-selectin–mediated cell adhesive interactions in the pathogenesis of inflammation and metastasis of cancers has been clearly demonstrated.[86]

Tumor-associated inflammatory monocytes and macrophages are essential promoters of tumor cell migration, invasion, and metastasis.[87] Macrophages and their mediators affect the multistep process of invasion and metastasis, from interaction with the extracellular matrix to the construction of a premetastatic niche. Monocytes are attracted by cytokines and chemokines (e.g., CSF-1, GM-CSF, and MCP-1), which are released by tumor cells or cells of the tumor microenvironment. These monocytes are then induced to express proangiogenic and metastatic factors, including VEGF, fibroblast growth factor (FGF)-2, platelet-derived growth factor (PDGF), intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, E-selectin, P- selectin, and MMP-9.[88] Versican, a large extracellular matrix proteoglycan, has been shown to activate tumor-infiltrating myeloid cells through Toll-like receptor (TLR) 2 and its coreceptors TLR6 and CD14 and to elicit the production of proinflammatory cytokines (including TNF-α), which enhance tumor metastasis. TLR2 increases the secretion of IL-8, which potentiates metastatic growth. Ligation of TLR2 by versican induces inflammatory cytokine secretion, providing a link between inflammation and cancer metastasis.[89]

IKK-α has been shown to be important in the inflammation-associated metastasis of cancer cells. Luo et al.[90] demonstrated that activation and nuclear localization of IKK-α by tumor-infiltrating immune cells in prostatic epithelial tumor cells leads to malignant prostatic epithelial cells with a metastatic fate. Src family kinases, when inappropriately activated, promote pathologic inflammatory processes and tumor metastasis, in part through their effects on the regulation of endothelial monolayer permeability.[91] Platelet-activating factor (PAF), an inflammatory biolipid, has also been shown to increase metastasis. In particular, Melnikova et al.[92] demonstrated that PAF receptor antagonists can effectively inhibit the metastatic potential of human melanoma cells in nude mice. Mesenchymal stem cells promote HCC metastasis under the influence of inflammation through TGF-β.[93]

EPIGENETIC CHANGES AND INFLAMMATION

Epigenetics considers the heritable changes in the activity of gene expression without the alteration of DNA sequences, and such changes have been linked to many human diseases, including cancer.[94] DNA methylation and histone modification are well-known epigenetic changes that can lead to gene activation or inactivation.[94–96] DNA methylation occurs primarily at cytosine-phosphate-guanine (CpG) dinucleotides as well as at transcriptional regulatory sites on the gene promoter.[96–98] Epigenetic abnormalities result in dysregulated gene expression and function, which can further lead to cancer. Inflammation and epigenetic abnormalities in cancer are highly associated. Inflammation induces aberrant epigenetic alterations in a tissue early in the process of carcinogenesis, and accumulation of such alterations forms an epigenetic field for cancer. Yara et al.[99] have shown that increased inflammation, as evidenced by the activation of NF-κB, production of IL-6 and COX-2, as well as the decrease of IκB, leads to the promoter's methylation. However, preincubation of cells with a demethylating agent prevented inflammation.

Infectious agents also contribute to inflammation-induced epigenetic changes. Infectious agents such as *H. pylori* and hepatitis C virus as well as intrinsic mediators of inflammatory responses, including proinflammatory cytokines, induce genetic and epigenetic changes, including point mutations, deletions, duplications, recombinations, and methylation of various tumor-related genes. Interestingly, disturbances in cytokine and chemokine signals and the induction of cell proliferation are important ways that inflammation induces aberrant DNA methylation. A study has shown that infection of human gastric mucosae with *H. pylori* induces chronic inflammation and further gastric cancers.[100] This inflammation is associated with high methylation levels or high incidences of methylation.[101–103]

Furthermore, numerous reports have documented the fact that inflammation is linked with epigenetic changes in carcinogenesis. Recently, Achyut[104] reported that inflammation in stromal fibroblasts caused epigenetic silencing of p21 and further tumor progression. Chronic inflammation also led to epigenetic regulation of p16 and activation of DNA damage in a lung carcinogenesis model.[105]

A transient inflammatory signal has been shown to initiate an epigenetic switch from nontransformed cells to cancer cells via a positive feedback loop involving NF-κB, Lin28, let-7, and IL-6. This IL-6 induced STAT3, directly activated miR-21 and miR-181b-1, and further induced the epigenetic switch. Thus, STAT3 underlies the epigenetic switch of mir-21 and mir-181b-1 that links inflammation to cancer.[106] Another report also showed that transient activation of Src oncoprotein mediates an epigenetic switch from immortalized breast cells to a stably transformed line that contained cancer stem cells. Thus, inflammation activates a positive feedback loop that maintains the epigenetic transformed state for many generations in the absence of the inducing signal.[18]

DNA hypermethylation at promoter CpG islands is an important mechanism by which carcinogenesis occurs through the inactivation of tumor-suppressor genes. Aberrant CpG island hypermethylation is also frequently observed in chronic inflammation and precancerous lesions, which again suggests links between inflammation and epigenetic change.[107] In addition, inflammation induced the halogenation of cytosine nucleotide. Damage products of this inflammation-mediated halogenated cytosine interfere with normal epigenetic control by altering DNA-protein interac-

tions that are critical for gene regulation and the heritable transmission of methylation patterns. These inflammation-mediated cytosine damage products also provide a mechanistic link between inflammation and cancer.[108]

ROLE OF INFLAMMATION IN CANCER DIAGNOSIS

Chronic inflammation plays an important role in the etiology and progression of chronic diseases, including cancer. Hence, chronic inflammation may have an important diagnostic role in cancer. Inflammation induced by inflammatory cells such as infiltrating cells and mesothelial cells is mediated via the release of various mediators and proteins, including PDGF, IL-8, monocyte chemotactic peptide (MCP-1), nitric oxide (NO), collagen, antioxidant enzymes, and the plasminogen activation inhibitor (PAI). Furthermore, several inflammatory mediators have been shown to be detected at increased concentrations, thereby aiding in the disease diagnosis.[109]

In one study, numerous inflammatory disorders were detected based on inflammation measured in gastric biopsies of patients by Fourier transform infrared spectroscopy (FT-IR). Using endoscopic samples, gastritis and gastric cancer were diagnosed.[110] Furthermore, the degree of prostate inflammation has been used to determine the level of incidental prostatitis.[111] An assessment of the expression of cytokines and other immune stimulatory molecules that drive B-cell activation provides insight into the etiology of cancers. It has been shown that the dysregulation of cytokine production precedes the diagnosis of non-Hodgkin lymphoma.[112]

Inflammation parameters have been used to diagnose cancer in patients. Inflammation parameters, including CRP, were found to differ in patients with cancer and in those without. In clinical practice, however, such parameters are considered to have modest diagnostic value for cancer.[113] In a study with 1,275 patients, granulomatous inflammation was identified in 154 patients (12.1%), of whom 12 out of 154 (7.8%) had a concurrent diagnosis of cancer.[114] In another study with 173 patients, 52% had lung adenocarcinoma. Patients with high systemic inflammation were more likely to have more than two sites of metastatic disease and to have poor performance status and less likely to receive any chemotherapy. Systemic inflammation at diagnosis is considered to be an independent marker of poor outcome in patients with advanced non-small cell lung cancer (NSCLC).[115]

INFLAMMATION AND GENOMICS

Recently, the genomic landscape of the most common forms of human cancer have been examined.[116] Almost 140 genes and 12 cell signaling pathways have been linked with most cancers. Several of these genes and pathways are directly or indirectly linked with inflammation. A cytokine pattern in patients with cancer has been identified.[117]

INFLAMMATION AND TARGETED THERAPIES

That inflammation can be used as a target for cancer prevention and treatment is indicated by the fact that several drugs approved by the U.S. Food and Drug Administration (FDA) actually modulate proinflammatory pathways. For instance, EGFR, HER2, VEGF, CXCR4, and proteasome have been shown to activate NF-κB–mediated proinflammatory pathways, and their inhibitors have been approved by the FDA for the treatment of various cancers. Similarly, steroids such as dexamethasone, nonsteroidal anti-inflammatory drugs (NSAIDs), and statins that are currently used for prevention or treatment have also been found to suppress the NF-κB pathway. Thus, these observations indicate that inflammatory pathways are excellent targets for cancer.

CONCLUSIONS

According to Colditz et al.,[118] almost 50% of all cancers can be prevented based on what we know today. All the studies summarized previously suggest that inflammation is closely linked to cancer, and the incidence of most cancers can be reduced by controlling inflammation. Proinflammatory conditions such as colitis, bronchitis, hepatitis, and gastritis can all eventually lead to cancer. Thus, one must find ways to treat these conditions before the appearance of cancer. All these studies indicate that an anti-inflammatory lifestyle could play an important role in both the prevention and treatment of cancer.

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The full reference list can be accessed at lwwhealthlibrary.com/oncology.

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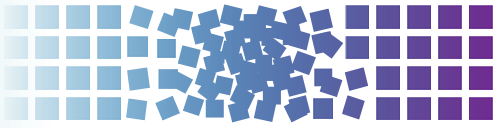
7 Chemical Factors



Stuart H. Yuspa and Peter G. Shields

INTRODUCTION

As early as the 1800s, initial observations of unusual cancer incidences in occupational groups provided the first indications that chemicals were a cause of human cancer, which was then confirmed in experimental animal studies during the early and mid 1900s. However, the extent to which chemical exposures contribute to cancer incidence was not fully appreciated until population-based studies documented differing organ-specific cancer rates in geographically distinct populations and in cohort studies such as those that linked smoking to lung cancer.[1] The most commonly occurring chemical exposures that increase cancer risk are tobacco, alcoholic beverages, diet, and reproductive factors (e.g., hormones). Today, it is recognized that cancer results not solely from chemical exposure (e.g., in the workplace or at home), but that a variety of biologic, social, and physical factors contribute to cancer pathogenesis.[2,3] For some common cancers, it also has been recognized that heritable factors also contribute to cancer risk from chemical exposure (e.g., genes involved in carcinogen metabolism, DNA repair, a variety of cancer pathways).[4] Twin studies show that for common cancers, nongenetic risk factors are dominant, and the best associations for genetic risks of sporadic cancers indicate that the risks for specific genetic traits are typically less than 1.5-fold.[5–7] The role of the tumor microenvironment, the cancer stem cells, and feedback signaling to and from the tumor also have been recently recognized as important contributors to carcinogenesis, although how chemicals affect these have yet been clearly demonstrated.[8–10]

The experimental induction of tumors in animals, the neoplastic transformation of cultured cells by chemicals, and the molecular analysis of human tumors have revealed important concepts regarding the pathogenesis of cancer and how laboratory studies can be used to better understand human cancer pathogenesis.[7,11,12] Chemical carcinogens usually affect specific organs, targeting the epithelial cells (or other susceptible cells within an organ) and causing genetic damage (genotoxic) or epigenetic effects regulating DNA transcription and translation. Chemically related DNA damage and consequent somatic mutations relevant to human cancer can occur either directly from exogenous exposures or indirectly by activation of endogenous mutagenic pathways (e.g., nitric oxide, oxyradicals).[13,14] The risk of developing a chemically induced tumor may be modified by nongenotoxic exogenous and endogenous exposures and factors (e.g., hormones, immunosuppression triggered by the tumor), and by accumulated exposure to the same or different genotoxic carcinogens.[7,15]

Analyses of how chemicals induce cancer in animal models and human populations has had a major impact on human health. Experimental studies have been instrumental in replicating hypotheses generated from human studies and identifying pathobiologic mechanisms. For example, animal experiments confirmed the carcinogenic and cocarcinogenic properties of cigarette smoke and identified bioactive chemical and gaseous components.[1] The transplacental carcinogenicity of diethylstilbestrol and the hazards of specific occupational carcinogens such as vinyl chloride, benzene, aromatic amines, and bis(chloromethyl)ether led to a reduction in allowable exposures of suspected human carcinogens from the workplace and a reduction in cancer rates. Dietary factors that enhance or inhibit cancer development and the contribution of obesity to specific organ sites have been identified in models of chemical carcinogenesis, and alterations in diet and obesity are expected to result in reduced cancer risk. Experimental animal studies are the mainstay of risk assessment as a screening tool to identify potential carcinogens in the workplace and the environment, although these studies do not prove specific chemical etiologies as a cause of human cancer because of interspecies differences and the use of maximally tolerated doses that do not replicate human exposure.

THE NATURE OF CHEMICAL CARCINOGENS: CHEMISTRY AND METABOLISM

The National Toxicology Program, based mostly on experimental animal studies and supported by epidemiology studies when available, lists 45 chemical, physical, and infectious agents as known human carcinogens and about 175 that are reasonably anticipated to be human carcinogens (http://ntp.niehs.nih.gov/?objectid=035E57E7-BDD9-2D9B-AFB9D1CADC8D09C1), whereas the International Agency for Research on Cancer (IARC) lists 113 agents as carcinogenic to humans and 66 that are probably carcinogenic to humans (http://monographs.iarc.fr/ENG/Classification/index.php). Table 7.1 provides a selected list of known human carcinogens, as indicated by the IARC, which are continuously updated.[16] Most chemical carcinogens first undergo metabolic activation by cytochrome P450s or other metabolic pathways so that they react with DNA and/or alter epigenetic mechanisms.[11,17] This process, evolutionarily presumed to have been developed to rid the body of foreign chemicals for excretion, inadvertently generates reactive carcinogenic intermediates that can bind cellular molecules, including DNA, and cause mutations or other alterations.[18] Recent data indicate that metabolizing enzymes also have the ability to cross-talk with transcription factors involved in the regulation of other metabolizing and antioxidant enzymes.[19] DNA is considered the ultimate target for most carcinogens to cause either mutations or gross chromosomal changes, but epigenetic effects, such as altered DNA methylation and gene transcription, also promote carcinogenesis.[20] The formation of DNA adducts, where chemicals bind directly to DNA to promote mutations, is likely necessary but not sufficient to cause cancer.

Genotoxic carcinogens may transfer simple alkyl or complexed (aryl) alkyl groups to specific sites on DNA bases.[18,21] These alkylating and aryl-alkylating agents include, but are not limited to, N-nitroso compounds, aliphatic epoxides, aflatoxins, mustards, polycyclic aromatic hydrocarbons, and other combustion products of fossil fuels and vegetable matter. Others transfer arylamine residues to DNA, as exemplified by aryl aromatic

TABLE 7.1

Known Chemical Carcinogens in Humans[a]

Target Organ	Agents	Industries	Tumor Type
Lung	Tobacco smoke, arsenic, asbestos, crystalline silica, benzo(a)pyrene, beryllium, bis(chloro)methyl ether, 1,3-butadiene, chromium VI compounds, coal tar and pitch, diesel exhaust, nickel compounds, soot, mustard gas, cobalt-tungsten carbide powders	Aluminum production, coal gasification, coke production, painting, hematite mining, painting, grinding in oil and gas	Squamous, large cell, and small cell cancer and adenocarcinoma
Pleura	Asbestos, erionite, painting	Insulation, mining	Mesothelioma
Oral cavity	Tobacco smoke, alcoholic beverages, nickel compounds, betel quid	–	Squamous cell cancer
Esophagus	Tobacco smoke, alcoholic beverages, betel quid	–	Squamous cell cancer
Gastric	Tobacco smoking	Rubber industry	Adenocarcinoma
Colon	Alcohol, tobacco smoking	–	Adenocarcinoma
Liver	Aflatoxin, vinyl chloride, tobacco smoke, alcoholic beverages	–	Hepatocellular carcinoma, hemangiosarcoma
Kidney	Tobacco smoke, trichloroethylene	–	Renal cell cancer
Bladder	Tobacco smoke, 4-aminobiphenyl, benzidine, 2-napthylamine, cyclophosphamide, phenacetin	Magenta manufacturing, auramine manufacturing, painting, rubber production	Transitional cell cancer
Prostate	Cadmium	–	Adenocarcinoma
Skin	Arsenic, benzo(a)pyrene, coal tar and pitch, mineral oils, soot, cyclosporin A, azathioprine, shale oils	–	Squamous cell cancer, basal cell cancer
Bone marrow	Benzene, tobacco smoke, ethylene oxide, antineoplastic agents, cyclosporin A, formaldehyde	Rubber workers	Leukemia, lymphoma

[a]The carcinogen designations are determined by the International Agency for Research on Cancer (http://monographs.iarc.fr/index.php). They do not imply proof of carcinogenicity in individuals. This table is not all inclusive. For additional information, the reader is referred to agency documents and publications.

amines, aminoazo dyes, and heterocyclic aromatic amines. For genotoxic carcinogens, the interaction with DNA is not random, and each class of agents reacts selectively with purine and pyrimidine targets.[7,18,21] Furthermore, targeting carcinogens to particular sites in DNA is determined by nucleotide sequence, by host cell, and by selective DNA repair processes (see later discussion), making some genetic material at risk over others. As expected from this chemistry, genotoxic carcinogens can be potent mutagens and particularly adept at causing nucleotide base mispairing or small deletions, leading to missense or nonsense mutations. Others may cause macrogenetic damage, such as chromosome breaks and large deletions. In some cases, such genotoxic damage may result in changes in transcription and translation that affect protein levels or function, which in turn alter the behavior of the specific host cell type. For example, there may be effects on cell proliferation, programmed cell death, or DNA repair. This is best typified by the signature mutations detected in the p53 gene caused by ingested aflatoxin in human liver cancer[22] and by polycyclic aromatic hydrocarbons human lung cancer caused by the inhalation of cigarette smoke.[15,23,24] Similarly, a distinct pattern of mutations is detected in pancreatic cancers from smokers when compared with pancreatic cancers from nonsmokers.[25]

Some chemicals that cause cancers in laboratory rodents are not demonstrably genotoxic. In general, these agents are carcinogenic in laboratory animals at high doses and require prolonged exposure. Synthetic pesticides and herbicides fall within this group, as do a number of natural products that are ingested. The mechanism of action by nongenotoxic carcinogens is not well understood, and may be related in some cases to toxic cell death and regenerative hyperplasia. They may also induce endogenous mutagenic mechanisms through the production of free radicals, increasing rates of depurination, and the deamination of 5-methylcytosine. In other cases, nongenotoxic carcinogens may have hormonal effects on hormone-dependent tissues. For example, some pesticides, herbicides, and fungicides have endocrine-disrupting properties in experimental models, although the relation to human cancer risk is unknown.

ANIMAL MODEL SYSTEMS AND CHEMICAL CARCINOGENESIS

Most human chemical carcinogens can induce tumors in experimental animals; however, the tumors may not be in the same organ, the exposure pathways may differ from human exposure, and the causative mechanisms may not exist in humans. In many cases, however, the cell of origin, morphogenesis, phenotypic markers, and genetic alterations are qualitatively identical to corresponding human cancers. Furthermore, animal models have revealed the constancy of carcinogen–host interaction among mammalian species by reproducing organ-specific cancers in animals with chemicals identified as human carcinogens, such as coal tar and squamous cell carcinomas, vinyl chloride and hepatic angiosarcomas, aflatoxin and hepatocellular carcinoma, and aromatic amines and bladder cancer. The introduction of genetically modified mice designed to reproduce specific human cancer syndromes and precancer models has accelerated both the understanding of the contributions of chemicals to cancer causation and the identification of potential exogenous carcinogens.[26,27] Furthermore, construction of mouse strains genetically altered to express human drug–metabolizing enzymes has added both to the relevance of mouse studies for understanding human carcinogen metabolism and the prediction of genotoxicity from suspected

human carcinogens and other chemical exposures.[28] Together, these studies have indicated that carcinogenic agents can directly activate oncogenes, inactivate tumor suppressor genes, and cause the genomic changes that are associated with autonomous growth, enhanced survival, and modified gene expression profiles that are required for the malignant phenotype.[29]

Genetic Susceptibility to Chemical Carcinogenesis in Experimental Animal Models

The use of inbred strains of rodents and spontaneous or genetically modified mutant strains have led to the identification and characterization of genes that modify risks for cancer development.[30–32] For a variety of tissue sites, including the lungs, the liver, the breast, and the skin, pairs of inbred mice can differ by 100-fold in the risk for tumor development after carcinogen exposure. Genetically determined differences in the affinity for the aryl hydrocarbon hydroxylase (Ah) receptor or other differences in metabolic processing of carcinogens is one modifier that has a major impact on experimental and presumed human cancer risk.[33–35] The development of mice reconstituted with components of the human carcinogen–metabolizing genome should facilitate the extrapolation of metabolic activity by human enzymes and cancer risk.[27,28,36] Such mice also show that other loci regulate the growth of premalignant foci, the response to tumor promoters, the immune response to metastatic cells, and the basal proliferation rate of target cells.[30] In mice susceptible to colon cancer due to a carcinogen-induced constitutive mutation in the APC gene, a locus on mouse chromosome 4 confers resistance to colon cancer.[31] The identification of the phospholipase A2 gene at this locus and subsequent functional testing in transgenic mice revealed an interesting paracrine protective influence on tumor development.[31] This gene, and several other genes mapped for susceptibility to chemically induced mouse tumors (PTPRJ, a receptor type tyrosine phosphatase, and STK6/STK15, an aurora kinase), have now been shown to influence susceptibility to organ-specific cancer induction in humans.[30,31]

MOLECULAR EPIDEMIOLOGY, CHEMICAL CARCINOGENESIS, AND CANCER RISK IN HUMAN POPULATIONS

Molecular epidemiology is the application of biologically based hypotheses using molecular and epidemiologic methods and measures. New technologies continue to allow epidemiologic studies to improve the testing of biologically based hypotheses and to develop large datasets for hypothesis generation, most notably the application of various *–omics* technologies via next-generation sequencing (e.g., genomics, epigenomics, transcriptomics), proteomics, and metabolomics. The greatest challenge now is to develop methods that allow for analysis cutting across various technologies.[37–43] Recent advances now include the role of microRNA and long noncoding RNAs in tumor development and progression because of their impact on the regulation of gene expression.[44,45] Chemical effects on microRNAs and the resultant gene expression is currently being identified.[46] Using such technologies, emerging evidence is noting the importance of the microbiome and associated infections as a risk of human cancer.[47–50] The complexity of environmental exposure and how it interacts with humans to affect numerous biologic pathways has been characterized as the exposome, also expressed as a multidimensional complex dataset.[51] Therefore, the important goal remains: to characterize cancer risk based on gene–environment interactions. However, we remain challenged because cancer is a complex disease of diverse etiologies by multiple exposures causing damage in different genes; for example, gene^n–environment^n interactions, for which the variable n is not known.

Two fundamental principles underlie current studies of molecular epidemiology. First, carcinogenesis is a multistage process, and behind each stage are numerous genetic events that occur either due to an exogenous insult such as a chemical exposure or an endogenous insult, such as from free radicals generated via cellular processes or errors in DNA replication. Therefore, identifying a cancer risk factor can be challenging because of the multifactorial nature of carcinogenesis, given that any one risk factor occurs within a background of many risk factors. Second, wide interindividual variation in response to carcinogen exposure and other carcinogenic processes indicate that the human response is not homogeneous, so that experimental models and epidemiology (e.g., the use of a single cell clone to study a gene's effect experimentally or the assumption that the population responds similarly to the mean in epidemiology studies), might not be representative of susceptible and resistant groups within a population.

Genetic Susceptibility

In humans, the determination of genetic susceptibility can be assessed by phenotyping or genotyping methods. Phenotypes generally represent complex genotypes. Examples of phenotypes include the assessment of DNA repair capacity in cultured blood cells, mammographic breast density, or the quantitation of carcinogen-DNA adducts in a target organ. Phenotypes now also include profiles of methylation that affect gene expression, a so-called epigenetic effect, for example, identified though next-generation sequencing or other methods.[52] The contribution of genetics to cancer risk from chemical carcinogens can range from small to large, depending on its penetrance.[4] Highly penetrant cancer-susceptibility genes cause familial cancers, but account for less than 5% of all cancers. Low-penetrant genes cause common sporadic cancers, which have large public health consequences.

A genetic polymorphism (e.g., single nucleotide polymorphisms) is defined as a genetic variant present in at least 1% of the population. Because of the advent of improved genotyping methods that have reduced cost and increased high throughput, haplotyping and whole genomewide association studies are ongoing. Although haplotyping studies, facilitated through the International HapMap Project (www.hapmap.org), have not proven useful for predicting human cancers; high-density, whole genomewide, single nucleotide polymorphism association studies have shown remarkable consistency for many gene loci, although the risk estimates are only 1.0 to 1.4, which are not useful in the clinic for individual risk assessment.[6] For example, the contribution of genetic polymorphisms to cancer risk, at least for breast cancer, appears to improve risk modeling by only a few percent; known breast cancer risk factors account for about 58% of risk, and adding 10 genetic variants increases the risk prediction only to 62%.[53] Genes under study are from pathways that affect behavior, activate and detoxify carcinogens, affect DNA repair, govern cell-cycle control, trigger apoptosis, effect cell signaling, and so forth.

Biomarkers of Cancer Risk

The evaluation of dose and risk estimates in epidemiologic studies can include four components: namely, external exposure measurements, internal exposure measurements, biomarkers estimating the biologically effective dose, and biomarkers of effect or harm. The latter three measurements are biomarkers that improve on the first by quantifying exposure inside the individual and at the cellular level to characterize low-dose exposures in low-risk populations, providing a relative contribution of individual chemical

carcinogens from complex mixtures, and/or estimating total burden of a particular exposure where there are many sources.[54]

Chemicals cause genetic damage in different ways, namely in the formation of carcinogen-DNA adducts leading to base mutations or gross chromosomal changes. Adducts are formed when a mutagen, or part of it, irreversibly binds to DNA so that it can cause a base substitution, insertion, or deletion during DNA replication. Gross chromosomal mutations are chromosome breaks, gaps, or translocations. The level of DNA damage is the biologically effective dose in a target organ, and reflects the net result of carcinogen exposure, activation, lack of detoxification, lack of effective DNA repair, and lack of programmed cell death. A variety of assays have been used for determining carcinogen-macromolecular adducts in human tissues; for example, for assessing risk from tobacco smoking for lung cancer and aflatoxin and liver cancer.[55,56] Important considerations for the assessment of biomarkers include sensitivity, specificity, reproducibility, accessibility for human use, and whether it represents a risk measured in a target organ or surrogate tissue. No single biomarker has been considered to be sufficiently validated for use as a cancer risk marker in an individual as it relates to chemical carcinogenesis.[57] However, there is some evidence that DNA adducts are cancer risk factors in both cohort and case-control studies.[58]

People are commonly exposed to *N*-nitrosamine and other N-nitroso compounds from dietary and tobacco exposures, which are associated with DNA adduct formation and cancer. Exposure can occur through endogenous formation of *N*-nitrosamines from nitrates in food or directly from dietary sources, cosmetics, drugs, household commodities, and tobacco smoke. Endogenous formation occurs in the stomach from the reaction of nitrosatable amines and nitrate (used as a preservative), which is converted to nitrites by bacteria. The *N*-nitrosamines undergo metabolic activation by cytochrome P450s (CYP2E1, CYP2A6, and CYP2D6) and form DNA adducts. Biomarkers are available to assess *N*-nitrosamine exposure from tobacco smoke (e.g., urinary tobacco-specific nitrosamine levels) or DNA, including in target organs such as the lungs. Recent data indicate that increasing levels of tobacco-specific nitrosamine metabolites are associated with increased lung cancer risk.[55]

Heterocyclic amines are formed from the overheating of food with creatine, such as meat, chicken, and fish.[59] Heterocyclic amines, estimated based on consumption of well-done meat, have been associated with breast and colon cancer, presumably through metabolic activation mechanisms and DNA damage.[59] Aflatoxins, another food contaminant, are considered to be a major contributor to liver cancer in China and parts of Africa, especially interacting with hepatitis viruses, and urinary aflatoxin adduct levels are predictors of liver cancer risk.[56]

Aromatic amines are another class of human carcinogens. Aryl aromatic amines have been implicated in bladder carcinogenesis, especially in occupationally exposed cohorts (e.g., dye workers) and tobacco smokers.[60] These compounds are activated by cytochrome P4501A2 and excreted via the N-acetyltransferase 2 gene. They are genotoxic, and the quantitative assessment using biomarkers has been more difficult, but some persons have studied DNA adducts as well.[61]

Polycyclic aromatic hydrocarbons (PAH) are large, aromatic (three or more fused benzene rings) compounds that are a class of more than 200 chemicals. These compounds are ubiquitous in the environment and present in the ambient air. They are formed from overcooking foods, fireplaces, charcoal barbeques, burning of coal and crude oil, tobacco smoke, and can be found in various occupational settings. In order for PAHs to exert their toxic effect, they must undergo metabolic activation via cytochromes P4501A1 and P4503A4 to form DNA adducts, or are excreted via pathways involving the glutathione-S-transferase genes. PAHs are associated with an increased risk of lung and skin cancer in the occupational setting, although risk varies by type of industry and the individual being exposed.[62,63] Benzo(a)pyrene (BaP), the most frequently studied PAH, serves as a model for chemical carcinogens. The bay region diol epoxide binds to DNA, mostly as the N2-deoxyguanosine adduct. The evidence linking BaP-deoxyguanosine adducts with a carcinogenic effect in lung cancer is very strong, including site-specific hotspot mutations in the p53 tumor suppressor gene.[64–68] Various biomarkers of exposure have been developed for assessing PAH exposure. These include measuring DNA adducts, protein adducts, and urinary 1-hydroxypyrene; only the latter is a validated biomarker of exposure and no adducts have been validated as biomarkers of cancer risk. However, recent data indicate that PAH metabolites might be risk factors for lung cancer.[58]

Air pollution has been recently classified by the IARC as a known human lung carcinogen.[69] Studies that support the conclusion include cohort studies that use biomarkers of exposure.[70] Such markers include measurements of 1-hydroxypyrene, DNA adducts, chromosomal aberrations, micronuclei, oxidative damage to nucleobases, and methylation changes.[71]

Epidemiologic and experimental studies have linked benzene to hematologic toxicity, including aplastic anemia, myelodysplastic syndrome, and acute myeloid leukemia.[72–74] Benzene is metabolized by hepatic P4502E1 (CYP2E1), yielding benzene oxide and hydroquinone, among other reactive metabolites. Circulating hydroquinones may be further metabolized to reactive benzoquinones by myeloperoxidase in bone marrow white blood cell precursors and stroma. Benzene metabolites are reported to have a variety of biologic consequences on bone marrow cells, including covalent binding to DNA and protein, alterations in gene expression, cytokine and chemokine abnormalities, and chromosomal aberrations.[75] There are well-established biomarkers of exposure to benzene, but to date, biomarkers of toxicity have not been validated (except for high-level exposure workplaces and effects of peripheral blood counts).

ARISTOLOCHIC ACID AND UROTHELIAL CANCERS AS A MODEL FOR IDENTIFYING HUMAN CARCINOGENS

Aristolochic acids come from the Aristolochia genus of plants, which have been used for herbal remedies (e.g., birthwort, Dutchman's pipe). The case of the carcinogen aristolochic acid, which is identified as a Class 1 human carcinogen by the IARC (http://monographs.iarc.fr/ENG/Monographs/vol100A/mono100A-23.pdf), presents a powerful example of how the forces of epidemiology, classical chemical carcinogenesis, and genomics collaborate to unravel the pathogenesis and prevention of a specific human cancer.[76] In the 1990s, epidemiologists independently reported on three distinct unrelated population groups that developed nephrotoxicity (interstitial fibrosis) and an extraordinary high incidence of urothelial cancer of the upper urinary track after exposure for different reasons and in different parts of the world (Belgium, the Balkans, and China). In Belgian women ingesting an extract from plants of the Aristolochia species for weight reduction, which was provided to them in a weight loss clinic, nearly 50% developed this unusual syndrome. A similar clinical picture (so-called Balkan endemic nephropathy) was reported for residents farming around the Danube River and eating home-baked bread from wheat contaminated with seeds from Aristolochia weeds grown in the same fields. In China, the Aristolochia herbs have been used for centuries in Chinese medicine and are prominently prescribed in Taiwan, a nation with the highest incidence of urothelial cancer in the world, as remedies for ailments of the heart, liver, snake bites, arthritis, gout, childbirth, and others.

Common to all Aristolochia species are one of two major nitrophenanthrene carboxylic acid toxicants, namely, aristolochic acid I and II (http://monographs.iarc.fr/ENG/Monographs/vol100A/

mono100A-23.pdf).[77,78] The oral administration of aristolochic acid to rodents is highly carcinogenic, producing predominantly forestomach cancers and lymphomas, along with cancers of the lung, kidney, and urothelium (http://monographs.iarc.fr/ENG/Monographs/vol100A/mono100A-23.pdf). The major route of excretion of aristolochic acid is through the kidneys. These clinical and experimental observations inspired further analyses of the mechanism of action of these potent human carcinogens. Studies in intact mice and mice reconstituted with humanized P450 revealed that CYP1a and CYP2a were responsible for both the activation and the detoxification of aristolochic acid I and II, and that NAD(P)H:quinone oxidoreductase produced the ultimate reactive aristolactam I nitrenium species.[78] The molecular action of the ultimate carcinogen is remarkably specific, targeting purine nucleotides in DNA to form DNA adducts and binding at the exocyclic amino group of deoxyadenosine and deoxyguanosine with a far greater affinity for dA over dG (Fig. 7.1). DNA adducts from aristolochic acids have been found in both experimental animals and humans. Furthermore, unlike any other human carcinogen, the predominant mutagenic outcome is an A:T transversion with a marked preference for the nontranscribed strand of DNA, notably in the p53 gene.[77,79] The A:T to T:A transversions are extremely uncommon among the mutation spectrum in all eukaryotes. These unique properties of aristolochic acid DNA adducts appear to elude DNA repair mechanisms that commonly focus on transcribing DNA, resulting in persistent carcinogen-DNA adducts in human tissues and surgical tumor specimens, thus confirming the association of exposure with a biologic effect.[80] In experimental models in mice where human p53 is substituted for the mouse gene, multiple sites on p53 are mutated, almost all of which are those unusual A:T transversions.[81] Modern genomic techniques have unraveled other selective properties of this unusual but potent human chemical carcinogen. Whole genome and exome sequencing of multiple aristolochic-associated kidney cancers from patients confirmed the high frequency of the unusual A:T to T:A transversion mutations. Furthermore, an unusual pattern emerges where there is selectivity for mutations at splice sites with a preferable consensus sequence of T/CAG. Among the many mutations detected, certain targets stand out, particularly in p53, MLL2, and other genes the products of which function in regulating gene expression through higher chromosome order.[82,83] This cancer story covers the gamut of all elements of chemical carcinogenesis, and its illumination has opened a door for cancer prevention.

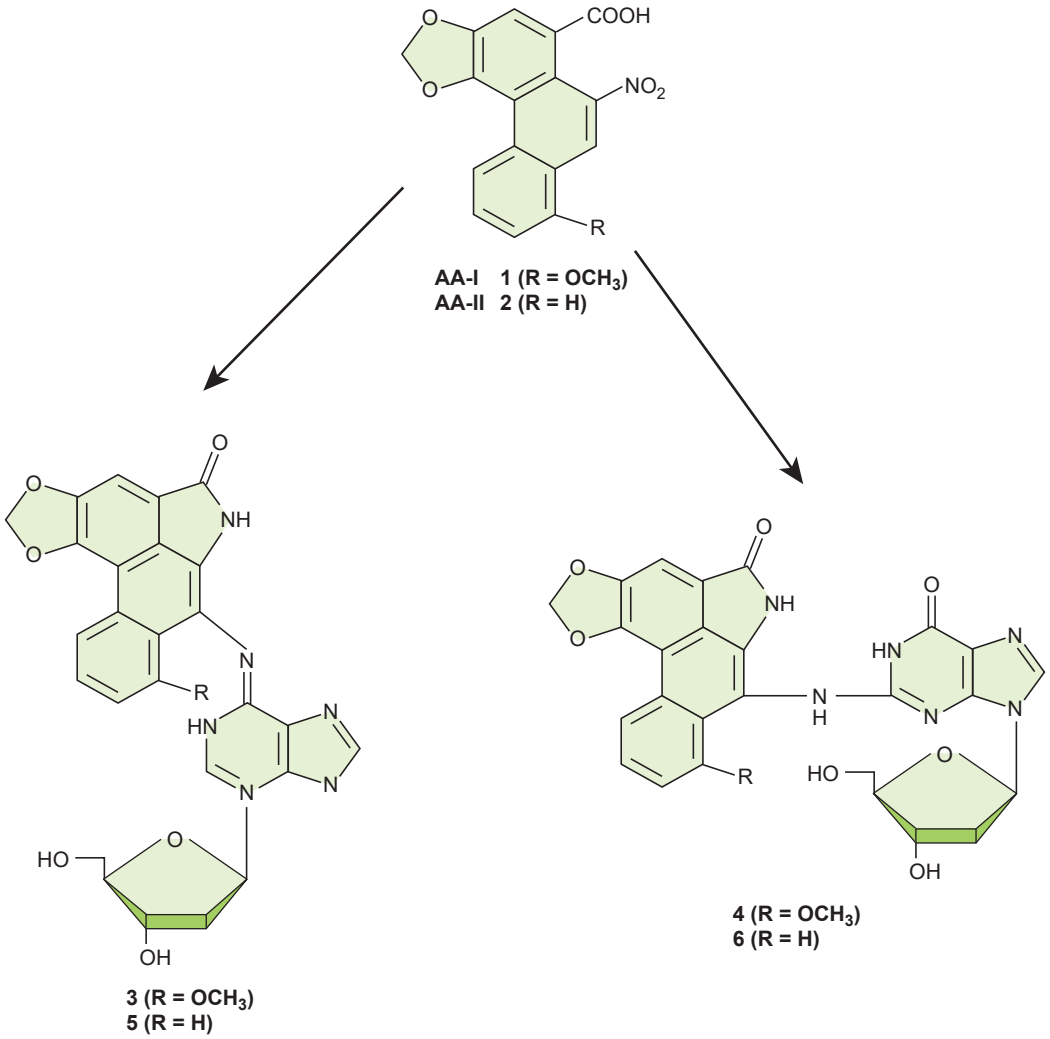


Figure 7.1 Aristolochic acid I and II form DNA adducts through the exocyclic amino group of deoxyadenosine and deoxyguanosine. The deoxyadenosine adduct is highly favored. For more detailed analysis of the complete metabolic profile, see Attaluri et al.[79]

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8 Physical Factors

Mats Ljungman

INTRODUCTION

Ionizing radiation (IR) and ultraviolet (UV) light have challenged the genetic integrity of all living organisms throughout time. By inducing DNA damage and subsequent mutations, these physical agents have promoted diversity through natural selection, and, as a result, organisms from all kingdoms of life carry genes that encode proteins that repair damaged DNA. In higher, multicellular organisms, many additional mechanisms of genome preservation have evolved, such as cell cycle checkpoints and apoptosis. Despite the many sophisticated mechanisms to safeguard the human genome from the mutagenic actions of DNA-damaging agents, not all exposed cells successfully restore the integrity of their DNA and some cells may subsequently progress into malignant cancer cells. Furthermore, through manmade activities, we are now exposed to many new physical agents, such as radiofrequency and microwave radiation, electromagnetic fields, asbestos, and nanoparticles, for which evolution has not yet had time to deliver genome-preserving response mechanisms. This chapter will highlight the molecular mechanisms by which these physical agents affect cells and how human exposure may lead to cancer.

IONIZING RADIATION

IR is defined as radiation that has sufficient energy to ionize molecules by displacing electrons from atoms. IR can be electromagnetic, such as x-rays and gamma rays, or can consist of particles, such as electrons, protons, neutrons, alpha particles, or carbon ions. Natural sources of IR make up about 80% of human exposure and medical sources make up about 20%.[1] The increased medical use of diagnostic x-rays and computed tomography (CT) scanning procedures likely translates into higher incidences of cancer. Of the natural sources, radon exposure is the most significant exposure risk to humans. Importantly, with better and more comprehensive screening techniques, the human exposure to radon could be dramatically lowered.

Mechanisms of Damage Induction

Linear Energy Transfer

The biologic effects of IR are unique in that the induced damage is clustered due to the local deposition of energy in radiation tracks. The distance between the depositions of energy is biologically very relevant and unique to the energy and the type of radiation. The term *linear energy transfer (LET)* denotes the energy transferred per unit length of a track of radiation. Electromagnetic radiation, such as x-rays or gamma rays, are sparsely ionizing and therefore classified as low LET radiation, whereas particulate radiation, such as neutrons, protons, and alpha particles, are examples of high LET radiation.[1]

Radiation Biochemistry

Radiation-induced damage to cellular target molecules, such as DNA, proteins, and lipids, can be either direct or indirect (Fig. 8.1). The *direct action* of radiation, which is the dominant mode of action of high LET radiation, is due to the deposition of energy directly to the target molecule, resulting in one or more ionization events. The *indirect action* of radiation is due to the radiolysis of water molecules, which, after initial absorption of radiation energy, become excited and generate different types of radiolysis products where the reactive hydroxyl radical (•OH), can damage both DNA and proteins. About two-thirds of the damage induced by low LET radiation is due to the indirect action of radiation. Since the hydroxyl radical is very reactive (half-life is 10^{-9} seconds), it does not diffuse more than a few nanometers after it is formed before it reacts with other molecules, and, thus, only radicals formed in close proximity to the target molecule will contribute to the damage of that target.[2] However, by chemical recombination of the primary radiolysis products, hydrogen peroxide (H_2O_2) is formed, which in turn can produce hydroxyl radicals at a later time through the Fenton reaction, involving free metals. Because H_2O_2 is not very reactive, it can diffuse long distances away from the initial site of energy deposition.

Radical scavengers normally present in cells, such as glutathione, can protect target molecules by reacting with the hydroxyl radical (see Fig. 8.1). Even after the target molecule has been hit and ionized, glutathione can contribute to cell protection by donating a hydrogen atom to the radical, allowing the unpaired electron present in the radical to pair up with the electron from the hydrogen atom. This is considered the simplest of all types of repair and is called *chemical repair*.[3] However, if oxygen molecules are present, they will compete with scavenger molecules for the ionized molecule, and if oxygen reacts with the ionized target molecule before the hydrogen donation occurs, the damage will be solidified as a peroxide, which is not amendable to chemical repair. Instead, this lesion will require enzymatic repair for the restoration of DNA. This augmenting biologic effect of oxygen is called the *oxygen effect* and is considered an important factor for the effectiveness of radiation therapy.[1]

Damage to DNA

The direct and indirect effects of radiation induce more or less identical types of lesions in DNA. However, the density of lesions induced in a stretch of DNA is higher for high LET radiation, and this increased complexity is thought to complicate the repair of these lesions. Radiation-induced lesions consist of more than 100 chemically distinct base lesions, such as the mutagenic lesions thymine glycol and 8-hydroxyguanine.[2,4,5] Furthermore, damage to the sugar moiety in the backbone of DNA and some types of base damage can result in single-strand breaks (SSB). Because the energy deposition of radiation is clustered even for low LET radiation, it is possible that two individual strand breaks are formed in close proximity on opposite strands, resulting in the formation of a double-strand break (DSB). It has been estimated that 1 Gy of ionizing radiation gives rise to about 40 DSBs, 1,000 SSBs, 1,000 base lesions, and 150 DNA-protein cross-links per cell.[2] For a similarly lethal dose of UV light, about 400,000 lesions are required, demonstrating that the lesions induced by IR are much more toxic

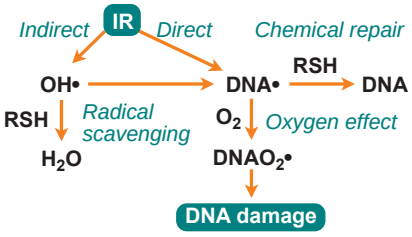

Figure 8.1 Factors affecting the induction of DNA damage by ionizing radiation (IR). Ionizing radiation can ionize DNA either by direct action or by indirect action, in which radiation energy is absorbed by neighboring molecules, such as water, leading to the generation of hydroxyl radicals that attack DNA. Sulfur-containing cellular molecules (RSH), such as glutathione, can scavenge hydroxyl radicals by hydrogen atom donations and thereby protect the DNA from the indirect action of radiation. Glutathione can also donate hydrogen atoms to ionized DNA, thereby restoring the integrity of DNA in a process termed *chemical repair*. Oxygen can compete with chemical repair in a process termed the *oxygen effect*, resulting in the enhancement of the biologic effect of ionizing radiation by the fixation of the initial DNA damage into DNA peroxides ($DNAO_2$•).

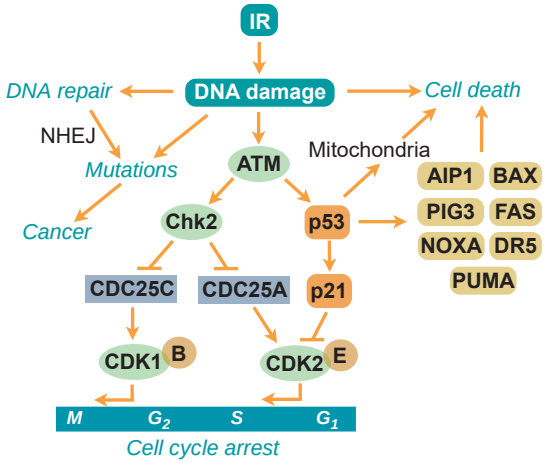

Figure 8.2 Cellular responses to ionizing radiation. Ionizing radiation induces predominantly base lesions and single- and double-strand breaks. Base lesions and single-strand breaks are repaired by base excision repair (BER), whereas double-strand breaks are repaired by nonhomologous end joining (NHEJ) and homologous recombination (HR). If DNA lesions are misrepaired by NHEJ or not repaired at all before cells enter S phase or mitosis, genomic instability is manifested as mutations or chromosome aberrations that promote carcinogenesis. In order for cells to assist DNA repair and safeguard against genetic instability and cancer, cells can induce cell cycle arrest or apoptosis. The ATM kinase is an early responder to DNA damage induced by ionizing radiation that activates the cell cycle checkpoint kinase Chk2 and the tumor suppressor p53. Chk2 inactivates the CDC25A and CDC25C phosphatases that are critical in promoting cell cycle progression by activating the cyclin-dependent kinases CDK2 or CDK1 and thereby arresting the cells at the G_1/S or G_2/M checkpoints. In addition, p53 can arrest cells at the G_1/S checkpoint by inducing the CDK inhibitor p21. p53 also plays a role in promoting apoptosis by inducing a number of proapoptotic proteins as well as translocating to mitochondria where it inhibits the actions of antiapoptotic factors. AIP1, actin interacting protein 1; BAX, bcl-2-like protein; PIG3, p53-inducible gene 3.

than lesions induced by UV light. It is believed that DSBs are the critical lesions that lead to cell lethality following exposure to ionizing radiation.[6]

Damage to Proteins

Although proteins and lipids are subject to damage following exposure to IR, the common belief is that DNA is the critical target for the biologic effects of radiation. Indeed, abrogation of DNA damage surveillance or repair processes in cells results in the enhanced induction of mutations and decreased cell survival following radiation.[5] However, studies of radiation-sensitive and radiation-resistant bacteria imply that mechanisms that suppress protein damage may also play important roles in radiation resistance.[7] *Deinococcus radiodurans* is a bacterium that can survive radiation exposures of up to 17,000 Gy, and its extreme radioresistance has been linked to high intracellular levels of manganese, which protect proteins from oxidation. The thought is that if a cell can limit protein oxidation, then its enzymes will remain active, and cellular functions such as DNA repair will be able to restore the integrity of DNA even after severe DNA damage.[8] It would be interesting to explore whether the concentration of manganese can be manipulated to sensitize tumor cells to radiation therapy. Furthermore, because protein damage due to reactive oxygen species (ROS) accumulate during the aging process, could supplements of manganese turn back the clock on aging?

Cellular Responses

DNA Repair

Ever since organisms started to utilize atmospheric oxygen for metabolic respiration many millions of years ago, they have been forced to deal with the cellular damage induced by ROS. Base excision repair (BER) evolved to remove many of the different types of oxidative base lesions and DNA SSBs induced by ROS. However, ROS seldom induce DSBs unless the generation of hydroxyl radicals is clustered near the DNA molecule. A more important source of intracellular generation of DSBs may instead be the process of DNA replication, and it is possible that homologous recombination (HR) repair primarily evolved to overcome DSBs sporadically induced during the replication process. The other major pathway of DSB repair is the nonhomologous end-joining (NHEJ) pathway, which is utilized by immune cells in the process of antibody generation. Although the HR pathway has high fidelity due to the utilization of homologous sister chromatids to ensure that correct DNA ends are joined, the NHEJ pathway lacks this control mechanism and therefore occasionally rejoins ends incorrectly. Thus, the NHEJ pathway may contribute to the generation of mutations following radiation (Fig. 8.2). However, NHEJ is the only mechanism available for DSB repair in postmitotic cells and cells in the G_1 phase of the cell cycle because no sister chromatids are available in these cells to support HR repair.

Ataxia-Telangiectasia Mutated and Cell Cycle Checkpoints

Due to the enormous task of replicating the whole genome during the S phase and segregating the chromosomes during mitosis, proliferating cells are generally much more vulnerable to radiation than stationary cells. To prevent cells with damaged DNA from entering into these critical stages of the cell cycle, cells can activate cell cycle checkpoints (see Fig. 8.2). The major sensor of radiation-induced damage in cells is the ataxia-telangiectasia mutated (ATM) kinase, which, following activation, can phosphorylate more than 700 proteins in cells.[9] Two ATM substrates, p53 and Chk2, are critical for the activation of cell cycle arrests at multiple sites in the cell cycle.[10,11] The kinase p53 regulates the gene expression of specific genes such as *p21*, which inhibits cyclin-dependent kinase (CDK)2- and CDK4-mediated phosphorylation of the retinoblastoma protein, resulting in a block in the progression from the G_1 phase to the S phase of the cell cycle.[12,13] The Chk2 kinase promotes checkpoint activation in G_1 by targeting the cell division cycle 25 homolog A (CDC25A) phosphatase[14] and, in G_2/M, by targeting the CDC25C phosphatase.[15] The activation of a cell cycle arrest following DNA damage provides the cell with additional time to repair the DNA before entering critical cell cycle stages,

which promotes genetic stability. Loss or defects in the *ATM* or *p53* genes result in abrogation of radiation-induced cell cycle checkpoints, which manifests itself as the highly cancer-prone human syndromes ataxia telangiectasia[16] or Li-Fraumeni,[17] respectively.

Radiation-Induced Cell Death

Terminally differentiated and stationary cells, such as kidney, lung, brain, muscle, and liver cells, are generally more resistant to radiation-induced killing than are cells with a high turnover rate, such as different epithelial cells, spermatogonia, and hair follicles. However, the spleen and thymus, which consist of mostly nondividing cells, are among the most radiosensitive tissues, implying that the rate of cell proliferation is not the sole determiner of the radiation sensitivity of a tissue. An important factor regulating the induction of programmed cell death (apoptosis) in tissues is the tumor suppressor p53.[18] The p53 protein is activated in cells following exposure to IR by the ATM kinase (see Fig. 8.2). When activated, it regulates the expression of multiple genes that have roles in DNA repair, cell cycle arrest, and apoptosis. p53 can also localize to mitochondria following irradiation, where it triggers apoptosis through the inactivation of antiapoptotic regulatory proteins.[19] Not all tissues induce the p53 response to the same degree after similar doses of IR, nor do they activate downstream pathways, such as DNA repair, cell cycle arrest, and apoptosis, in a similar way. For example, thymocytes have an intrinsic setting that favors apoptosis over cell cycle arrest following IR, whereas fibroblasts rarely induce apoptosis, but instead activate a strong and lasting cell cycle arrest.[18]

IR can induce cell death in tissues by many different mechanisms. Apoptosis can occur rapidly in a p53-dependent manner or later in a p53-independent manner. This later wave of radiation-induced apoptosis is often initiated by mitotic catastrophe, which occurs as a result of complications during chromosome segregation. Cell death induced by IR may in some cases be associated with autophagy, also called autophagocytosis, in which cells degrade cellular components via the lysosomal machinery. Whether autophagy is a programmed cell death or occurs in parallel with cell death is not clear. Interestingly, for some cell types, autophagy has been shown to actually protect the cells from radiation-induced death. Finally, tissue can undergo necrotic cell death following exposure to IR. Necrosis is a clinical problem following radiation therapy that can occur in normal tissues many months after treatment and can contribute to the inflammatory response.

Cancer Risks

It is clear from epidemiologic studies of radiation workers and atomic bomb and Chernobyl victims that IR can induce cancer.[20] Twenty years after the atomic bomb explosions in Japan during World War II, significant increases in the incidence of thyroid cancer and leukemia were observed. However, it took almost 50 years before solid tumors appeared in the population as a result of radiation exposure from the atomic bombs.[21] The incidences of solid tumors, such as breast, ovary, bladder, lung, and colon cancers, were estimated to have increased by a factor of 2 in the exposed group during this time period. The epidemiology studies following the nuclear power plant disaster in Chernobyl showed a clear increase in thyroid cancer as early as 4 years after the accident.[22] Young children were the most vulnerable to radiation exposure, with 1-year-old children being 237-fold more susceptible to thyroid cancer than the control group, while 10-year-old children were found to be sixfold more susceptible to thyroid cancer. Many of the thyroid cancers that developed following the Chernobyl disaster could have been prevented if the population had not consumed locally produced milk that was contaminated with radioactive iodine.

The molecular signatures of radiation-induced tumors are complex but involve point mutations that could lead to the activation of the *RAS* oncogene or inactivation of the tumor suppressor gene *p53*. Furthermore, IR induces DNA DSBs that may be unfaithfully repaired by the NHEJ pathway, leading to chromosome rearrangements. One such rearrangement found in 50% to 90% of the thyroid cancers examined following the Chernobyl accident involved the receptor tyrosine kinase c-RET, which promotes cell growth when activated.[22] Furthermore, a great majority of the thyroid cancers found in the exposed children harbored kinase fusion oncogenes affecting the mitogen-activated protein kinase (MAPK) signaling pathway.[23]

The correlation between high exposure to IR and cancer following the atomic bomb explosions and the Chernobyl accident is clear. What about the cancer risk following lower radiation exposures occurring in daily life? There are four theoretical risk models of radiation-induced cancer to consider. First, the *linear, no threshold* (LNT) *model* suggests that the induction of cancer is directly proportional to the dose of radiation, even at low doses of exposure. Second, the *sublinear* or *threshold model* suggests that below a certain threshold dose the risk of radiation-induced cancers is negligible. At these lower doses of radiation exposure, the DNA damage surveillance and repair mechanisms are thought to be fully capable of safeguarding the DNA to avoid the induction of mutations and cancer. Third, the *supralinear* or *stealth model* suggests that doses below a certain threshold or radiation with sufficiently low dose rates may not trigger the activation of DNA damage surveillance and repair mechanisms, resulting in suboptimal activation of cell cycle checkpoints and repair. This would be expected to lead to a higher rate of mutations and cancers than predicted by the LNT model, but may be balanced by a higher incident of cell death. Fourth, the *linear-quadratic model* suggest that radiation effects at low doses are due to a single track of radiation hitting multiple targets, resulting in a linear induction rate, whereas at higher doses, multiple radiation tracks hit multiple cellular targets, resulting in a quadratic induction rate.

The Biological Effects of Ionizing Radiation (BEIR) VII report, released by the Committee on Biological Effects of Ionizing Radiation of the National Academy of Sciences and commissioned by the US Environmental Protection Agency (EPA), is a review of published data regarding human health and cancer risks from exposure to low levels of IR. Although this topic is controversial and not fully settled, the BIER VII report favored the LNT model.[24] Thus, the "official" view is that no level of radiation is safe; therefore, a careful consideration of risks versus benefits is necessary to ensure that the general population only receives radiation doses as low as reasonably achievable. Furthermore, the BIER VII committee concluded that the heritable effects of radiation were not evident in the published data, indicating that an individual is not likely to develop cancer due to radiation exposure of his or her parents.

The largest source of radiation exposure to the population is radon, which is a natural radioactive gas formed as a decay product of radium in the decay chain of uranium. Radon gas can accumulate to high levels in poorly ventilated basements in houses built on rock containing uranium. The major risk with radon is that some of its radioactive decay products can attach to dust particles that accumulate in the lungs, leading to a continuous exposure of the lung tissues to high LET alpha particles. Due to this radiation exposure, the EPA claims that radon is the second leading cause of lung cancer in the United States. Another important source of human exposure to IR is medical x-ray devices, and there is a growing concern about the dramatically increased use of whole body CT scans for diagnostic purposes. For a typical CT scan, a patient will receive about 100-fold more radiation than from a typical mammogram.[24] It is recommended that the use of whole body CT scans for children be very restricted due to the elevated risk of developing radiation-induced cancer for this age group.

Cancer patients who receive radiation therapy are at risk of developing secondary tumors induced by the radiation therapy treatment.[1] This is particularly a concern for young patients since (1) children are more prone to radiation-induced cancer,

(2) children have a relatively good chance of surviving the primary cancer and would have long life expectancies so a secondary tumor would have plenty of time to develop, and (3) many childhood cancers are promoted by genetic defects in DNA damage response pathways, making these patients highly prone to the genotoxic effects of radiation and subsequent secondary cancers. The most sensitive tissues for the development of secondary cancer have been found to be bone marrow (leukemia), the thyroid, breast, and lung.[1]

ULTRAVIOLET LIGHT

Depending on the wavelength, UV light is categorized into UVA (320 to 400 nm), UVB (290 to 320 nm), and UVC (240 to 290 nm) radiation. Most of the UVC light emitted from the sun is absorbed by the ozone layer in the atmosphere, and, thus, living organisms are mostly exposed to UVA and UVB irradiation.

Mechanisms of Damage Induction

UVC light is more damaging to DNA than UVA and UVB because the absorption maximum of DNA is around 260 nm. UVB and UVC induce predominantly pyrimidine dimers and 6-4 photoproducts, which consist of covalent ring structures that link two adjacent pyrimidines on the same DNA strand.[5] The formation of these lesions results in the bending of the DNA helix, resulting in the interference with both DNA and RNA synthesis. UVA light does not induce pyrimidine dimers or 6-4 photoproducts but can induce ROS, which in turn can form SSBs and base lesions in DNA of exposed cells.

Cellular Responses

DNA Repair

The nucleotide excision repair (NER) pathway removes pyrimidine dimers and 6-4 photoproducts from cellular DNA.[5] This pathway involves proteins that recognize the DNA lesions, nucleases that excise the DNA strand that contains the lesion, a DNA polymerase that synthesizes new DNA to fill the gap, and a DNA ligase that joins the backbone in the newly synthesized strand. Genetic defects in the NER pathway result in the human syndrome xeroderma pigmentosum, with individuals more than 1,000-fold more prone to sun-induced skin cancer than normal individuals. In addition, human polymorphisms in certain NER genes are thought to predispose individuals to cancers such as lung cancer, nonmelanoma skin cancer, head and neck cancer, and bladder cancer, indicating that NER is responsible for safeguarding the genome against many types of DNA adducts in addition to UV-induced lesions.[5]

UV-induced lesions formed in the transcribed strand of active genes block the elongation of RNA polymerase II, and if a cell does not restore transcription within a certain time frame, it may undergo apoptosis (Fig. 8.3).[25,26] To rapidly restore RNA synthesis and avoid cell death, NER enzymes are recruited to the sites of blocked RNA polymerase II and the lesions are removed in a process called transcription-coupled repair (TCR).[27] Individuals with Cockayne syndrome (CS), trichothiodystrophy, or the UV-sensitive syndrome, are unable to utilize the TCR pathway following UV irradiation.[5] Cells from these individuals do not recover RNA synthesis following UV irradiation and are therefore very prone to UV-induced apoptosis. Interestingly, despite a clear DNA repair defect, these individuals are not predisposed to UV-induced skin cancer. It is thought that the inability of CS cells to remove the toxic lesions that block transcription following UV irradiation results in the suppression of tumorigenesis by the elimination of damaged cells by apoptosis. However, while protecting against tumorigenesis, the elevated level of apoptosis in these cells leads to increased cell loss, which in turn may lead to neurologic degeneration.[25,28] Persistent transcription-blocking lesions in the genome have also been linked to aging.[29–31]

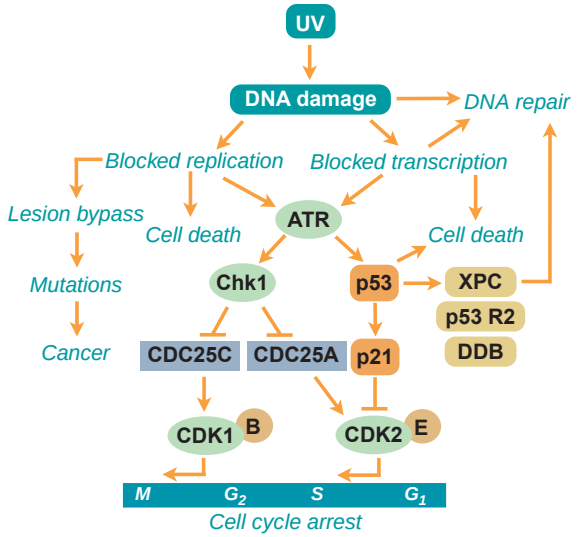

Figure 8.3 Cellular responses to ultraviolet (UV) light–induced DNA damage. UV light predominantly induces bulky DNA lesions that interfere with the processes of DNA replication and transcription. These lesions are removed from the global genome by global genomic nucleotide excision repair (GG-NER) and from transcribed DNA strands by transcription-coupled NER (TC-NER). Lesions blocking replication can be bypassed by exchanging processive DNA polymerases with less processive translesion DNA polymerases. While these polymerases allow cells to continue DNA synthesis and progress through the cell cycle, they have low fidelity, resulting in the potential induction of mutations promoting UV-induced carcinogenesis. To suppress mutations and support DNA repair efforts, the ataxia-telangiectasia and Rad3-related (ATR) kinase is activated in response to blocked replication or transcription. ATR activates the cell cycle checkpoint kinase Chk1, which, similar to Chk2, arrests cells in the G_1/S and G_2/M checkpoints by inhibiting CDC25A and CDC25C. ATR also activates p53, promoting G_1/S checkpoint activation via the induction of the Cdk-inhibitor p21. p53 also stimulates GG-NER by the transactivation of various NER genes and can promote apoptosis by the induction of proapoptotic factors and translocation to mitochondria. Finally, apoptosis is induced if cells do not recover transcription in a certain time frame, potentially due to the loss of survival factors or complications in the S phase when replication encounters stall the transcription complexes.

Translesion DNA Synthesis

Proliferating skin cells are very vulnerable to UV light because UV lesions block DNA replication (see Fig. 8.3). Cells that have entered the S phase and have initiated DNA synthesis have no choice but to finish replicating the whole genome or they will die. If DNA repair enzymes are not able to remove the blocking lesions from the template, the processive DNA polymerases may be exchanged for other, less processive DNA polymerases that can bypass the lesions. This is part of a "tolerance" mechanism, which allows cells to complete replication and eventually divide.[5] However, the translesion DNA polymerases do not have the same fidelity as the processive DNA polymerases; thus, mutations may occur. This is thought to be a major pathway by which UV light induces mutagenesis and, subsequently, cancer (see Fig. 8.3).

ATM and Rad3-Related Mediated Cell Cycle Checkpoints

In addition to utilizing the NER and BER pathways to repair UV-induced DNA damage, proliferating cells activate cell cycle checkpoints to allow more time for repair before entering critical

parts of the cell cycle, such as the S phase and mitosis. The ATM and Rad3-related (ATR) kinase is activated following UV irradiation by blocked replication or transcription (see Fig. 8.3).[32] ATR phosphorylates a large number of proteins, many of which are the same as those phosphorylated by ATM after exposure to ionizing radiation.[9] Two important substrates of ATR are p53 and Chk1, which are critical in promoting cell cycle arrest. When induced by ATR, p53 transactivates the gene that encodes the cell cycle inhibitor p21, leading to the arrest of cells in the G_1 phase of the cell cycle, while Chk1 phosphorylates the CDK-activating phosphatases CDC25A and CDC25C, which targets them for degradation, resulting in an S-phase or G_2-phase arrest (see Fig. 8.3).[33]

Activation of Cell Membrane Receptors

In addition to triggering cellular stress responses by inducing DNA damage, UV light can directly induce membrane receptor signaling by receptor phosphorylation. This is thought to be due to the direct UV-mediated inhibition of protein-tyrosine phosphatases that regulate the phosphorylation levels of various membrane receptors.[34] In addition, membrane receptors may physically aggregate following UV irradiation, leading to the activation of signal transduction pathways that regulate cell growth[35] or apoptosis.[36]

Cell Death

UV light effectively induces apoptosis in skin cells. The mechanism by which UV light induces cell death is not fully understood, but failure to adequately resume RNA synthesis following UV light exposure is strongly linked to apoptosis (see Fig. 8.3).[25] Many potential mechanisms of how blocked transcription results in apoptosis have been suggested, such as a physical clash during the S phase between elongating replication machineries and transcription complexes stalled at UV lesions. Another possible mechanism involves the preferential loss of survival factors coded by highly unstable mRNAs.[37] The induction of p53 may also contribute to UV-induced apoptosis,[38] although p53 appears to protect human fibroblasts[39] and keratinocytes[40] from UV-induced apoptosis. Although complications induced by DNA damage may be the predominant mechanism by which cells die following UV irradiation, UV light may induce apoptosis in certain cell types by directly promoting the physical aggregation of the death receptor Fas/APO1.[36]

Cancer Risks

The incidence of sun-induced skin cancer, especially melanoma, is on the increase due to higher rates of sun exposure in the general population. The link between UV light exposure and skin cancer is very strong, but the role of UV light in the etiology of nonmelanoma and melanoma skin cancer differs. Although the risk of nonmelanoma cancer relates to the cumulative lifetime exposure to UV light, the risk of contracting melanoma appears to be linked to high sunlight exposure during childhood.[41] What makes UV light such a potent carcinogen is that it can initiate carcinogenesis by inducing DNA lesions as well as suppressing the immune system, resulting in a greater probability that initiated cells will survive and grow into tumors.[42,43]

Nonmelanoma Skin Cancer

Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are the two most common skin cancer types. BCC and SCC occur predominantly in sun-exposed areas of the skin, but there are examples of these cancers forming in nonexposed areas as well (see Chapter 92). The tumor suppressor genes *p53* and *p16* are frequently inactivated in BCC and SCC, while the hedgehog-signaling pathway is activated primarily by mutations to the patched gene (percutaneous transhepatic cholangiography [*PTCH*]). This scenario promotes proliferation without the opposition of the cell cycle inhibitors p53 and p16.

Melanoma

Melanoma arises from mutations in epidermal melanocytes and is the most dangerous form of skin cancer because it has the highest propensity to metastasize (see Chapter 93). It is formed in both sun-exposed and shielded areas of the skin; therefore, the role of UV light as the major carcinogen in melanoma has been controversial.[41] Defects in the NER pathway do not seem to predispose the development of melanoma, suggesting that pyrimidine dimers or 6-4 photoproducts induced by UVB are not the initiators of melanoma carcinogenesis. Instead, ROS induced by UVA may be responsible for the development of melanoma.[41] However, a study using next-generation sequencing techniques to catalog all mutations in a melanoma cell line found a mutational spectrum of the over 33,000 mutations detected that strongly indicated that pyrimidine dimers and 6-4 photoproducts are the major mutagenic lesions in melanoma, whereas a subset of mutations may be induced by ROS.[44] The incidence of mutations in the *p16* and *ARF* genes is high, whereas *p53* and *RAS* mutations are fairly uncommon in melanoma (see Chapter 93).

Photoimmunosuppression

Studies of transplantation of mouse skin cancers into syngeneic mice revealed that prior UVB irradiation of recipient mice promoted tumor growth, whereas transplantation into naïve nonirradiated mice led to rejection.[43] These studies established that UV light has local immunosuppressing ability, and subsequent studies found that UV light preferentially depletes Langerhans cells from irradiated skin.[42] Langerhans cells play an important role in the immune response by presenting antigens to the immune cells, and, thus, depletion of these cells leads to local immunosuppression. In addition to local immunosuppression, UV light has been shown to promote systemic immunosuppression.[45] This response is complex, but it is known that UV-induced DNA lesions in skin cells contribute to the systemic immunosuppression response.[46] The secretion of the immunosuppressing cytokine interleukin (IL)-10 from irradiated keratinocytes as well as UV-induced structural alteration of the epidermal chromophore urocanic acid may mediate the long-range immunosuppressive effects of UV light.[42,45]

RADIOFREQUENCY AND MICROWAVE RADIATION

Radiofrequency radiation (RFR) is electromagnetic radiation in the frequency range 3 kHz to 300 MHz, whereas microwave radiation (MR) is in the frequency range between 300 MHz to 300 GHz. RFR and MR do not have sufficient energies to cause ionizations in target tissues. Rather, the radiation energy is converted into heat as the radiation energy is absorbed. Sources of radiofrequency and microwave radiation include mobile phones, radio transmitters of wireless communication, radars, medical devices, and kitchen appliances.

Mechanism of Damage Induction

Because human exposure to RFR has increased dramatically in recent years, it is important to know whether this type of radiation gives rise to genotoxic damage. Although there are many studies showing that RFR can induce ROS, leading to genetic damage in cell culture systems, other studies have generated conflicting results.[47] One confounding factor when assessing the genotoxic effect of RFR, and especially MR, is the heating effect that occurs

in the tissue when the radiation energy is absorbed. A recent study controlling for the potential heating effect of exposure found that RFR induces ROS and DNA damage in human spermatozoa in vitro, which is an alarming finding considering the potential hereditary implications.[48] It has been suggested that MR may affect the folding of proteins in cells that promote new protein synthesis.[49] Furthermore, exposure of cells to MR has been shown to lead to the phosphorylation of numerous cellular proteins largely through the activation of the p38/MAPK stress response pathway.[50] However, the biologic consequences of these cellular changes are not clear. Epidemiology studies that monitored the genetic effects in individuals exposed to high levels of RF have revealed evidence of increased induction of chromosome aberrations in lymphocytes.[51] However, there is a level of uncertainty in these studies about exposure levels, making it difficult to come to meaningful conclusions.

Cancer Risks

Because the population's exposure to RFR and MR has dramatically increased in recent years, it is of great importance to assess the potential cancer risks of these types of radiation so that appropriate exposure limits can be implemented. A number of studies have focused on the potential cancer risks from mobile phone usage, and some of these studies indicate that long-term mobile phone usage may be associated with increased risks of developing brain tumors (see the following). Other epidemiologic studies of cancer incidences in populations living near radio towers or mobile phone base stations are inconclusive. Some studies have shown a connection between proximity to mobile phone base stations and increased cancer incidence,[52] whereas another study found no association between exposure to RFR from mobile phone base stations and early childhood cancers.[53]

ELECTROMAGNETIC FIELDS

An electromagnetic field (EMF) is a physical field produced by electrically charged objects that can affect other charged objects in the field. Typical sources of EMFs are electric power lines, electrical devices, and magnetic resonance imaging (MRI) machines.

Mechanisms of Damage Induction

A low frequency EMF does not transmit energy high enough to break chemical bonds; therefore, it is not thought to directly damage DNA or proteins in cells. The data obtained from studies to assess the potential genotoxic effects of EMF do not provide a clear conclusion. Some of the results obtained in cell culture studies suggest a harmful effect of EMFs, but the concerns are that these effects may be related to heat production induced by EMFs rather than from the magnetic field itself. A recent in vitro study detected DNA strand breaks in cells exposed to EMFs, but this induction was thought to not be the result of ROS production, but rather due to indirect effects through interference with DNA replication and induction of apoptosis in a subset of cells.[54] A study using an MRI found no evidence of an induced formation of DNA DSBs in cell cultures.[55] EMFs have been shown to induce nongenotoxic effects in cells, such as interference with cellular signaling pathways,[56] which could contribute to neurodegeneration.[57]

Cancer Risks

Studies with rodents have largely failed to detect an association between exposure to EMFs and cancer. This is also true for numerous epidemiology studies, with the only exception being the association between EMF exposure and childhood leukemia where children exposed to doses of 0.4 mcT or above may have about a twofold increased risk of developing leukemia.[58,59] There is no strong link between EMF exposure and increased risks of contracting adult leukemia, brain tumors, or breast cancer.[60,61] Furthermore, a study investigating whether EMF exposure was associated with heritable effects found no correlation between parental exposure and childhood cancer.[62]

Potential Cancer Risks from Mobile Phone Usage

Mobile phones emit RFR and generate EMFs. The biggest health concern with mobile phone usage is its potential role in the development of brain tumors. During mobile phone use, the brain tissue is exposed to doses, giving peak specific absorption rates (SAR) of 4 to 8 W/kg. At these intensities, the induction of DNA damage has been detected in laboratory studies.[63] The current epidemiologic data are largely inconclusive on the association between mobile phone usage and brain tumor incidence. Meta-analysis studies of populations who had used mobile phones for more than 10 years concluded that mobile phone usage was associated with an elevated risk for brain tumors, such as acoustic neuroma and glioma cancer.[64–66] In contrast, other large prospective studies did not observe a correlation between mobile phone usage and incidences of glioma, meningioma, or non–central nervous system (CNS) cancers.[67,68] It is important to point out that, generally, it takes 30 to 40 years for brain tumors to develop, and because mobile phones have only been in general use for about 15 years, there has not been sufficient time to fully evaluate the brain cancer risks of mobile phone usage.

ASBESTOS

Asbestos is a class of naturally occurring silicate minerals that have been widely used in building materials for its heat, sound, and electrical insulating qualities. Asbestos becomes a serious health hazard if the fibers are inhaled over a long period of time, and these health effects are increased dramatically if the exposed individual is a smoker. It was first reported in 1935 that asbestos might be an occupational health hazard that could induce cancer.[69,70] However, it was not until 1986 that the International Labor Organization recommended banning asbestos.[71] The use of asbestos products peaked in the 1970s, yet remains a major health hazard in many places around the world today.

Mechanisms of Damage Induction

Asbestos fibers can enter cells and induce ROS, especially if they contain high levels of iron.[72] In addition, ROS can be generated by "frustrated" phagocytosis, and this in turn can lead to the release of proinflammatory cytokines with subsequent inflammation of the tissue. ROS have been implicated to originate from affected mitochondria leading to induction of SSBs and base damage, such as 8-hydroxyguanine in DNA.[73] Furthermore, if not successfully repaired, asbestos-induced DNA damage has been shown to result in chromosome aberrations, micronuclei formation, and increased rates of sister chromatid exchanges.[74]

Cellular and Tissue Responses

Asbestos-induced ROS cause base lesions and DNA strand breaks, which require base excision repair for the restoration of DNA and for minimizing mutagenesis. In addition to DNA repair, a number of cellular signaling pathways are activated by asbestos. These include the epidermal growth factor receptor (EGFR) and the MAPK pathway, leading to the activation of nuclear factor kappa B

(NF-κB) and transcription factor AP-1.[72,74] Activation of the NF-κB pathway leads to the induction of proinflammatory genes such as tumor necrosis factor (TNF), *IL-6*, *IL-8*, and proliferation-promoting genes such as *c-Myc*, leading to inflammation and increased cell proliferation. Asbestos exposure also stimulates the expression of the transforming growth factor beta (TGF-β), which, in turn, stimulates fibrogenesis in exposed tissues.[74]

Cancer Risks

Lung Cancer

Epidemiologic studies have found a strong link between asbestos exposure and lung cancer.[74] It has been estimated that about 5% to 7% of all lung cancers are attributable to asbestos exposure, and asbestos and tobacco smoking act in synergy to induce lung cancer. Mutational spectra due to 8-hydroxyguanine lesions formed by ROS can be linked to asbestos exposure, and point mutations in the tumor suppressor genes *p53* and *p16/INK4A* and in the *KRAS* oncogene have been found in tumors from asbestos-exposed individuals.

Mesothelioma

After being taken up by lung tissues, asbestos fibers can translocate into the pleura, the body cavity that surrounds the lungs. The pleura are covered with a protective lining, the mesothelium, which consists of squamouslike epithelial cells. Mesothelial cells can internalize asbestos fibers, resulting in the induction of ROS and inflammatory responses, subsequently leading to the initiation and progression of malignant mesothelioma.[75] Asbestos is considered one of the major causes of malignant mesothelioma, and frequent mutations are found in the *p16/INK4A* and *NF2* genes, whereas *p53* mutations are fairly rare.

NANOPARTICLES

Nanoparticles are defined as ultrafine particles of the size range 1 to 100 nm in diameter. Nanoparticle chemistry of a certain compound is different from bulk chemistry of that compound because of the high percentage of atoms at the surface of the particle. The production of nanoparticles has increased dramatically in recent years, and they are found in many industrial and consumer products such as paint, cosmetics, and sunscreens. They also have many potential medical applications, such as delivery vehicles for specific drugs to specific target tissues or tumors.

Mechanisms of DNA Damage Induction

Many of the cellular effects of nanoparticles are similar to the effects exerted by asbestos, such as the generation of ROS and inflammation.[72] Nanoparticles have been shown to induce oxidative DNA damage, such as DNA strand breaks and 8-hydroxyguanine lesions both in cell culture[76, 77] and in vivo.[78] Nanoparticle-induced DNA lesions are manifested as histone γ-H2AX nuclear foci, chromosome deletions, and micronuclei.

Cellular Responses

Nanoparticles induce ROS either directly or indirectly, resulting in DNA lesions, such as 8-hydroxyguanine–base damage and DNA strand breaks. These lesions are repaired by the base excision repair. The phosphorylation of histone H2AX has been shown to occur following exposure of cells to nanoparticles, suggesting that the DNA lesions trigger the activation of ATM or ATR stress kinases.[79] Nanoparticles have also been found to affect the immune system[80] and can induce the release of the proinflammatory cytokine TNF-α from cells.

Cancer Risks

Some nanoparticles, such as titanium dioxide, which is used as pigments in paint, have been classified by the International Agency for Research on Cancer (IARC) as a group 2B carcinogen, "possible carcinogenic to humans." However, rigorous epidemiologic data is lacking to fully evaluate the cancer-inducing potential of nanoparticles.[81]

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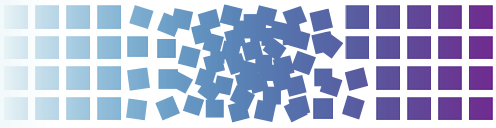
9 Dietary Factors



Karin B. Michels and Walter C. Willett

INTRODUCTION

Over two decades ago, Doll and Peto[1] speculated that 35% (range: 10% to 70%) of all cancer deaths in the United States may be preventable by alterations in diet. The magnitude of the estimate for dietary factors exceeded that for tobacco (30%) and infections (10%).

Studies of cancer incidence among populations migrating to countries with different lifestyle factors have indicated that most cancers have a large environmental etiology. Although the contribution of environmental influences differs by cancer type, the incidence of many cancers changes by as much as five- to tenfold among migrants over time, approaching that of the host country. The age at migration affects the degree of adaptation among first-generation migrants for some cancers, suggesting that the susceptibility to environmental carcinogenic influences varies with age by cancer type. Identifying the specific environmental and lifestyle factors most important to cancer etiology, however, has proven difficult.

Environmental factors such as diet may influence the incidence of cancer through many different mechanisms and at different stages in the cancer process. Simple mutagens in foods, such as those produced by the heating of proteins, can cause damage to DNA, but dietary factors can also influence this process by inducing enzymes that activate or inactivate these mutagens, or by blocking the action of the mutagen. Dietary factors can also affect every pathway hypothesized to mediate cancer risk–for example, the rate of cell cycling through hormonal or antihormonal effects, aiding or inhibiting DNA repair, promoting or inhibiting apoptosis, and DNA methylation. Because of the complexity of these mechanisms, knowledge of dietary influences on risk of cancer will require an empirical basis with human cancer as the outcome.

METHODOLOGIC CHALLENGES

Study Types and Biases

The association between diet and the risk of cancer has been the subject of a number of epidemiologic studies. The most prevalent designs are the case-control study, the cohort study, and the randomized clinical trial. When the results from epidemiologic studies are interpreted, the potential for confounding must be considered. Individuals who maintain a healthy diet are likely to exhibit other indicators of a healthy lifestyle, including regular physical activity, lower body weight, use of multivitamin supplements, lower smoking rates, and lower alcohol consumption. Even if the influence of these confounding variables is analytically controlled, residual confounding remains possible.

Ecologic Studies

In ecologic studies or international correlation studies, variation in food disappearance data and the prevalence of a certain disease are correlated, generally across different countries. A linear association may provide preliminary data to inform future research but, due to the high probability of confounding, cannot provide strong evidence for a causal link. Food disappearance data also may not provide a good estimate for human consumption. The gross national product is correlated with many dietary factors such as fat intake.[2] Many other differences besides dietary fat exist between the countries with low fat consumption (less affluent) and high fat consumption (more affluent); reproductive behaviors, physical activity level, and body fatness are particularly notable and are strongly associated with specific cancers.

Migrant Studies

Studies of populations migrating from areas with low incidence of disease to areas with high incidence of disease (or vice versa) can help sort out the role of environmental factors versus genetics in the etiology of a cancer, depending on whether the migrating group adopts the cancer rates of the new environment. Specific dietary components linked to disease are difficult to identify in a migrant study.

Case-Control Studies

Case-control studies of diet may be affected by recall bias, control selection bias, and confounding. In a case-control study, participants affected by the disease under study (cases) and healthy controls are asked to recall their past dietary habits. Cases may overestimate their consumption of foods that are commonly considered "unhealthy" and underestimate their consumption of foods considered "healthy." Giovannucci et al.[3] have documented differential reporting of fat intake before and after disease occurrence. Thus, the possibility of recall bias in a case-control study poses a real threat to the validity of the observed associations. Even more importantly, in contemporary case-control studies using a population sample of controls, the participation rate of controls is usually far from complete, often 50% to 70%. Unfortunately, health-conscious individuals may be more likely to participate as controls and will thus be less overweight, will consume fruits and vegetables more frequently, and will consume less fat and red meat, which can substantially distort associations observed.

Cohort Studies

Prospective cohort studies of the effects of diet are likely to have a much higher validity than retrospective case-control studies because diet is recorded by participants before disease occurrence. Cohort studies are still affected by measurement error because diet consists of a large number of foods eaten in complex combinations. Confounding by other unmeasured or imperfectly measured lifestyle factors can remain a problem in cohort studies.

Now that the results of a substantial number of cohort studies have become available, their findings can be compared with those of case-control studies that have examined the same relations. In

many cases, the findings of the case-control studies have not been confirmed; for example, the consistent finding of lower risk of many cancers with higher intake of fruits and vegetables in case-control studies has generally not been seen in cohort studies.[4] These findings suggest that the concerns about biases in case-control studies of diet, and probably many other lifestyle factors, are justified, and findings from such studies must be interpreted cautiously.

Randomized Clinical Trials

The gold standard in medical research is the randomized clinical trial (RCT). In an RCT on nutrition, participants are randomly assigned to one of two or more diets; hence, the association between diet and the cancer of interest should not be confounded by other factors. The problem with RCTs of diet is that maintaining the assigned diet strictly over many years, as would be necessary for diet to have an impact on cancer incidence, is difficult. For example, in the dietary fat reduction trial of the Women's Health Initiative (WHI), participants randomized to the intervention arm reduced their fat intake much less than planned.[5] The remaining limited contrast between the two groups left the lack of difference in disease outcomes difficult to interpret. Furthermore, the relevant time window for intervention and the necessary duration of intervention are unclear, especially with cancer outcomes. Hence, randomized trials are rarely used to examine the effect of diet on cancer but have better promise for the study of diet and outcomes that require a considerably shorter follow-up time (e.g., adenoma recurrence). Also, the randomized design may lend itself better to the study of the effects of dietary supplements such as multivitamin or fiber supplements, although the control group may adopt the intervention behavior because nutritional supplements are widely available. For example, in the WHI trial of calcium and vitamin D supplementation, two-thirds of the study population used vitamin D or calcium supplements that they obtained outside of the trial, again rendering the lack of effect in the trial uninterpretable.

Diet Assessment Instruments

Observational studies depend on a reasonably valid assessment of dietary intake. Although, for some nutrients, biochemical measurements can be used to assess intake, for most dietary constituents, a useful biochemical indicator does not exist. In population-based studies, diet is generally assessed with a self-administered instrument. Since 1980, considerable effort has been directed at the development of standardized questionnaires for measuring diet, and numerous studies have been conducted to assess the validity of these methods. The most widely used diet assessment instruments are the food frequency questionnaire, the 7-day diet record, and the 24-hour recall. Although the 7-day diet record may provide the most accurate documentation of intake during the week the participant keeps a diet diary, the burden of computerizing the information and extracting foods and nutrients has prohibited the use of the 7-day diet record in most large-scale studies. The 24-hour recall provides only a snapshot of diet on one day, which may or may not be representative of the participant's usual diet and is thus affected by both personal variation and seasonal variation. The food frequency questionnaire, the most widely used instrument in large population-based studies, asks participants to report their average intake of a large number of foods during the previous year. Participants tend to substantially overreport their fruit and vegetable consumption on the food frequency questionnaire.[6] This tendency may reflect social desirability bias, which leads to overreporting healthy foods and underreporting less healthy foods. Studies of validity using biomarkers or detailed measurements of diet as comparisons have suggested that carefully designed questionnaires can have sufficient validity to detect moderate to strong associations. Validity can be enhanced by using the average of repeated assessments over time.[7]

THE ROLE OF INDIVIDUAL FOOD AND NUTRIENTS IN CANCER ETIOLOGY

Energy

The most important impact of diet on the risk of cancer is mediated through body weight. Overweight, obesity, and inactivity are major contributors to cancer risk. (A more detailed discussion is provided in Chapter 10.) In the large American Cancer Society Cohort, obese individuals had substantially higher mortality from all cancers and, in particular, from colorectal cancer, postmenopausal breast cancer, uterine cancer, cervical cancer, pancreatic cancer, and gallbladder cancer than their normal-weight counterparts.[8] Adiposity and, in particular, waist circumference are predictors of colon cancer incidence among women and men.[9,10] A weight gain of 10 kg or more is associated with a significant increase in postmenopausal breast cancer incidence among women who never used hormone replacement therapy, whereas a weight loss of comparable magnitude after menopause substantially decreases breast cancer risk.[11] Regular physical activity contributes to a lower prevalence of being overweight and obesity and consequently reduces the burden of cancer through this pathway.

The mechanisms whereby adiposity increases the risk of various cancers are probably multiple. Being overweight is strongly associated with endogenous estrogen levels, which likely contribute to the excess risks of endometrial and postmenopausal breast cancers. The reasons for the association with other cancers are less clear, but excess body fat is also related to higher circulating levels of insulin, insulin-like growth factor (IGF)-1, and C-peptide (a marker of insulin secretion), lower levels of binding proteins for sex hormones and IGF-1, and higher levels of various inflammatory factors, all of which have been hypothesized to be related to risks of various cancers.

Energy restriction is one of the most effective measures to prevent cancer in the animal model. While energy restriction is more difficult to study in humans, voluntary starvation among anorectics and situations of food rationing during famines provide related models. Breast cancer rates were substantially reduced among women with a history of severe anorexia.[12] Although breast cancer incidence was higher among women exposed to the Dutch famine during childhood or adolescence, such short-term involuntary food rationing for 9 months or less was often followed by overnutrition.[13] A more prolonged deficit in food availability during World War II in Norway was associated with a reduction in adult risk of breast cancer if it occurred during early adolescence.[14]

Alcohol

Aside from body weight, alcohol consumption is the best established dietary risk factor for cancer. Alcohol is classified as a carcinogen by the International Agency for Research on Cancer. The consumption of alcohol increases the risk of numerous cancers, including those of the liver, esophagus, pharynx, oral cavity, larynx, breast, and colorectum in a dose-dependent fashion.[15] Evidence is convincing that excessive alcohol consumption increases the risk of primary liver cancer, probably through cirrhosis and alcoholic hepatitis. At least in the developed world, about 75% of cancers of the esophagus, pharynx, oral cavity, and larynx are attributable to alcohol and tobacco, with a marked increase in risk among drinkers who also smoke, suggesting a multiplicative effect. Mechanisms may include direct damage to the cells in the upper gastrointestinal tract; modulation of DNA methylation, which affects susceptibility to DNA mutations; and an increase in acetaldehyde, the main metabolite of alcohol, which enhances the proliferation of epithelial cells, forms DNA adducts, and is a recognized carcinogen. The association between alcohol consumption and breast cancer is notable because a small but significant risk has been found even

with one drink per day. Mechanisms may include an interaction with folate, an increase in endogenous estrogen levels, and an elevation of acetaldehyde. Some evidence suggests that the excess risk is mitigated by adequate folate intake possibly through an effect on DNA methylation.[16] Notably, for most cancer sites, no important difference in associations was found with the type of alcoholic beverage, suggesting a critical role of ethanol in carcinogenesis.

Dietary Fat

In recent years, reducing dietary fat has been at the center of cancer prevention efforts. In the landmark 1982 National Academy of Sciences review of diet, nutrition, and cancer, a reduction in fat intake to 30% of calories was the primary recommendation.

Interest in dietary fat as a cause of cancer began in the first half of the 20th century, when studies by Tannenbaum[17] indicated that diets high in fat could promote tumor growth in animal models. Dietary fat has a clear effect on tumor incidence in many models, although not in all; however, a central issue has been whether this is independent of the effect of energy intake. In the 1970s, the possible relation of dietary fat intake to cancer incidence gained greater attention as the large international differences in rates of many cancers were noted to be strongly correlated with apparent per capita fat consumption in ecologic studies.[2] Particularly strong associations were seen with cancers of the breast, colon, prostate, and endometrium, which include the most important cancers not due to smoking in affluent countries. These correlations were observed to be limited to animal, not vegetable, fat.

Dietary Fat and Breast Cancer

Breast cancer is the most common malignancy among women, and incidence has been increasing for decades, although a decline has been noted starting with the new millennium. Rates in most parts of Asia, South America, and Africa have been only approximately one-fifth that of the United States, but in almost all these areas rates of breast cancer are also increasing. Populations that migrate from low- to high-incidence countries develop breast cancer rates that approximate those of the new host country. However, rates do not approach those of the general US population until the second or third generation.[18] This slower rate of change for immigrants may indicate delayed acculturation; although because a similar delay in rate increase is not observed for colon cancer, it may suggest an origin of breast cancer earlier in the life course.

The results from 12 smaller case-control studies that included 4,312 cases and 5,978 controls have been summarized in a meta-analysis.[19] The pooled relative risk (RR) was 1.35 ($P < .0001$) for a 100-g increase in daily total fat intake, although the risk was somewhat stronger for postmenopausal women (RR, 1.48; $P < .001$). This magnitude of association, however, could be compatible with biases due to recall of diet or the selection of controls.

Because of the prospective design of cohort studies, most of the methodologic biases of case-control studies are avoided. In an analysis of the Nurses' Health Study that included 121,700 US female registered nurses, no association with total fat intake was observed, and there was no suggestion of any reduction in risk at intakes below 25% of energy.[20] Because repeated assessments of diet were obtained at 2- to 4-year intervals, this analysis provided a particularly detailed evaluation of fat intake over an extended period in relation to breast cancer risk. Similar observations were made in the National Institutes of Health (NIH)–American Association of Retired Persons (AARP) Diet and Health Study including 188,736 postmenopausal women[21] and in the European Prospective Investigation into Cancer and Nutrition (EPIC), which included 7,119 incident cases.[22] In a pooled analysis of seven prospective studies, which included 337,000 women who developed 4,980 incident cases of breast cancer, no overall association was seen for fat intake over the range of less than 20% to more than 45% energy (reflecting the current range observed internationally).[23] A similar lack of association was seen for specific types of fat. This lack of association with total fat intake was confirmed in a subsequent analysis of the pooled prospective studies of diet and breast cancer, which included over 7,000 cases.[24] Therefore, these cohort findings do not support the hypothesis that dietary fat is an important contributor to breast cancer incidence.

Endogenous estrogen levels have now been established as a risk factor for breast cancer. Thus, the effects of fat and other dietary factors on estrogen levels are of potential interest. Vegetarian women, who consume higher amounts of fiber and lower amounts of fat, have lower blood levels and reduced urinary excretion of estrogens, apparently due to increased fecal excretion. A meta-analysis has suggested that a reduction in dietary fat reduces plasma estrogen levels,[25] but the studies included were plagued by the lack of concurrent controls, the short duration, and the negative energy balance. In a large, randomized trial among postmenopausal women with a previous diagnosis of breast cancer, a reduction in dietary fat did not affect estradiol levels when the data were appropriately analyzed.[26]

The WHI Randomized Controlled Dietary Modification Trial similarly suggested no association between fat intake and breast cancer incidence,[5] but these results are difficult to interpret.[27] The data on biomarkers that reflect fat intake suggest little if any difference in fat intake between the intervention and control groups.[28] Even if dietary fat does truly have an effect on cancer incidence and other outcomes, this lack of adherence to the dietary intervention could explain the absence of an observed effect on total cancer incidence and total mortality. In another randomized trial in Canada that tested an intervention target of 15% of calories from fat, a small but significant difference in high-density lipoprotein (HDL) levels was observed after 8 to 9 years of follow-up suggesting a difference in fat intake in the two groups.[29] The incidence of breast cancer in the intervention and the control group did not differ significantly.

Some prospective cohort studies suggest an inverse association between monounsaturated fat and breast cancer. This is an intriguing observation because of the relatively low rates of breast cancer in southern European countries with high intakes of monounsaturated fats due to the use of olive oil as the primary fat. In case-control studies in Spain, Greece, and Italy, women who used more olive oil had reduced risks of breast cancer.

In a report of findings from the Nurses' Health Study II cohort of premenopausal women, a higher intake of animal fat was associated with an approximately 50% greater risk of breast cancer, but no association was seen with intake of vegetable fat.[30] This suggests that factors in foods containing animal fats, rather than fat per se, may account for the findings. In the same cohort, an intake of red meat and total fat during adolescence was also associated with the risk of premenopausal breast cancer.[31,32]

Dietary Fat and Colon Cancer

In comparisons among countries, rates of colon cancer are strongly correlated with a national per capita disappearance of animal fat and meat, with correlation coefficients ranging between 0.8 and 0.9.[2] Rates of colon cancer rose sharply in Japan after World War II, paralleling a 2.5-fold increase in fat intake. Based on these epidemiologic investigations and on animal studies, a hypothesis has developed that higher dietary fat increases the excretion of bile acids, which can be converted to carcinogens or act as promoters. However, evidence from many studies on obesity and low levels of physical activity increasing the risk of colon cancer suggests that at least part of the high rates in affluent countries previously attributed to fat intake is probably due to a sedentary lifestyle.

The Nurses' Health Study suggested an approximately twofold higher risk of colon cancer among women in the highest quintile of animal fat intake than in those in the lowest quintile.[33] In a multivariate analysis of these data, which included red meat intake and animal fat intake in the same model, red meat intake

remained significantly predictive of colon cancer risk, whereas the association with animal fat was eliminated. Other cohort studies have supported associations of colon cancer and the consumption of red meat and processed meats but not other sources of fat or total fat.[34–36] Similar associations were also observed for colorectal adenomas. In a meta-analysis of prospective studies, red meat consumption was associated with a risk of colon cancer (RR = 1.24; 95% confidence interval [CI], 1.09 to 1.41 for an increment of 120 g per day).[37] The association with the consumption of processed meats was particularly strong (RR = 1.36; 95% CI, 1.15 to 1.61 for an increment of 30 g per day).

The apparently stronger association with red meat consumption than with fat intake in most large cohort studies needs further confirmation, but such an association could result if the fatty acids or nonfat components of meat (e.g., the heme iron or carcinogens created by cooking) were the primary etiologic factors. This issue has major practical implications because current dietary recommendations support the daily consumption of red meat as long as it is lean.[38]

Dietary Fat and Prostate Cancer

Although further data are desirable, the evidence from international correlations, case-control[39] and cohort studies[40–44] provides some support for an association between the consumption of fat-containing animal products and prostate cancer incidence. This evidence does not generally support a relation with intake of vegetable fat, which suggests that either the type of fat or other components of animal products are responsible. Some evidence also indicates that animal fat consumption may be most strongly associated with the incidence of aggressive prostate cancer, which suggests an influence on the transition from the widespread indolent form to the more lethal form of this malignancy. Data are limited on the relation of fat intake to the probability of survival after the diagnosis of prostate cancer.

Dietary Fat and Other Cancers

Rates of other cancers that are common in affluent countries, including those of the endometrium and ovary, are also correlated with fat intake internationally. In prospective studies between Iowa and Canadian women, no evidence of a relation between fat intake and risk of endometrial cancer was found. Positive associations between dietary fat and lung cancer have been observed in many case-control studies. However, in a pooled analysis of large prospective studies that included over 3,000 incident cases, no association was observed.[45] These findings provide further evidence that the results of case-control studies of diet and cancer are likely to be misleading.

Summary

Largely on the basis of the results of animal studies, international correlations, and a few case-control studies, great enthusiasm developed in the 1980s that modest reductions in total fat intake would have a major impact on breast cancer incidence. As the findings from large prospective studies have become available, however, support for this relation has greatly weakened. Although evidence suggests that a high intake of animal fat early in adult life may increase the risk of premenopausal breast cancer, this is not likely to be due to fat per se because vegetable fat intake was not related to risk. For colon cancer, the associations seen with animal fat intake internationally have been supported in numerous case-control and cohort studies, but this also appears to be explained by factors in red meat other than simply its fat content. Further, the importance of physical activity and leanness as protective factors against colon cancer indicates that international correlations probably overstate the contribution of diet to differences in colon cancer incidence. At present, the available evidence most strongly suggests an association between animal fat consumption and risk of prostate cancer, particularly the aggressive form of this disease. As with colon cancer, the possibility remains that other factors in animal products contribute to risk.

Despite the large body of data on dietary fat and cancer that has accumulated since 1985, any conclusions should be regarded as tentative, because these are disease processes that are poorly understood and are likely to take many decades to develop. Because most of the reported literature from prospective studies is based on fewer than 20 years' follow-up, further evaluations of the effects of diet earlier in life and at longer intervals of observation are needed to fully understand these complex relations. Nevertheless, persons interested in reducing their risk of cancer could be advised, as a prudent measure, to minimize their intake of foods high in animal fat, particularly red meat. Such a dietary pattern is also likely to be beneficial for the risk of cardiovascular disease. On the other hand, unsaturated fats (with the exception of *transfatty* acids) reduce blood low-density lipoprotein cholesterol levels and the risk of cardiovascular disease, and little evidence suggests that they adversely affect cancer risk. Thus, efforts to reduce unsaturated fat intake are not warranted at this time and are likely to have adverse effects on cardiovascular disease risk. Because excess adiposity increases the risk of several cancers and cardiovascular disease, balancing calories from any source with adequate physical activity is extremely important.

Fruits and Vegetables

General Properties

Fruits and vegetables have been hypothesized to be major dietary contributors to cancer prevention because they are rich in potential anticarcinogenic substances. Fruits and vegetables contain antioxidants and minerals and are good sources of fiber, potassium, carotenoids, vitamin C, folate, and other vitamins. Although fruits and vegetables supply less than 5% of total energy intake in most countries worldwide on a population basis, the concentration of micronutrients in these foods is greater than in most others.

The comprehensive report of the World Cancer Research Fund and the American Institute for Cancer Research, published in 2007 and titled *Food, Nutrition, Physical Activity, and the Prevention of Cancer: A Global Perspective*, reached the consensus based on the available evidence: "findings from cohort studies conducted since the mid-1990s have made the overall evidence, that vegetables or fruits protect against cancers, somewhat less impressive. In no case now is the evidence of protection judged to be convincing."[15]

Fruit and Vegetable Consumption and Colorectal Cancer

The association between fruit and vegetable consumption and the incidence of colon or rectal cancer has been examined prospectively in at least six studies. In some of these prospective cohorts, inverse associations were observed for individual foods or particular subgroups of fruits or vegetables, but no consistent pattern emerged and many comparisons revealed no such links. The results from the largest studies, the Nurses' Health Study and the Health Professionals' Follow-Up Study, suggested no important association between the consumption of fruits and vegetables and the incidence of cancers of the colon or rectum during 1,743,645 person-years of follow-up.[46] In these two large cohorts, diet was assessed repeatedly during follow-up with a detailed food frequency questionnaire. Similarly, in the Pooling Project of Prospective Studies of Diet and Cancer, including 14 studies, 756,217 participants, and 5,838 cases of colon cancer, no association with overall colon cancer risk was found.[47]

Fruit and Vegetable Consumption and Stomach Cancer

At least 12 prospective cohort studies have examined the consumption of some fruits and vegetables and the incidence of stomach

cancer.[15] Seven of these studies considered total vegetable intake. Three found significant protection from stomach cancer, whereas three did not. All other comparisons were made for subgroups of vegetables and produced inconsistent results. Nine prospective cohort studies investigated the association between fruit consumption and stomach cancer risk. Four studies found an inverse association of borderline statistical significance.

Fruit and Vegetable Consumption and Breast Cancer

The most comprehensive evaluation of fruit and vegetable consumption and the incidence of breast cancer was provided by a pooled analysis of all cohort studies.[48] Data were pooled from eight prospective studies that included 351,825 women, 7,377 of whom developed incident invasive breast cancer during follow-up. The pooled relative risk adjusted for potential confounding variables was 0.93 (95% CI, 0.86 to 1.0; *P* for trend, .08) for the highest versus the lowest quartile of fruit consumption, 0.96 (95% CI, 0.89 to 1.04; *P* for trend, .54) for vegetable intake, and 0.93 (95% CI, 0.86 to 1.0; *P* for trend, .12) for total consumption of fruits and vegetables combined. The EPIC study confirmed this lack of association.[49] In a recent analysis within the Nurses' Health Study, an inverse association was seen between vegetable intake and the risk of estrogen receptor–negative breast cancer.[50] This observation was confirmed in the pooling project of prospective studies: The pooled relative risk for the highest vs. the lowest quintile of total vegetable consumption was 0.82 (95% CI 0.74 to 0.90) for estrogen-receptor negative breast cancer.[51]

Fruit and Vegetable Consumption and Lung Cancer

The relation between fruit and vegetable consumption and the incidence of lung cancer was examined in the pooled analysis of cohort studies.[52] Overall, no association was observed, although a modest increase in lung cancer incidence was evident among participants with the lowest fruit and vegetable consumption.

Fruit and Vegetable Consumption and Total Cancer

An analysis of the Nurses' Health Study and the Health Professionals' Follow-Up Study, including over 9,000 incident cases of cancer, did not reveal a benefit of fruit and vegetable consumption for total cancer incidence.[53] Observations from the EPIC cohort were essentially consistent with these findings.[54] Although there may be no or only a very weak protection conferred for cancer from consuming an abundance of fruits and vegetables, there is a substantial benefit for protection from cardiovascular disease.

Summary

The consumption of fruits and vegetables and some of their main micronutrients appear to be less important in cancer prevention than previously assumed. With an accumulation of data from prospective cohort studies and randomized trials, a lack of association of these foods and nutrients with cancer outcomes has become apparent. A modest association cannot be excluded because of an imperfect measurement of diet, and it remains possible that a high consumption of fruits and vegetables during childhood and adolescence is more effective at reducing cancer risk than consumption in adult life due to the long latency of cancer manifestation.

Conversely, it is possible that, with the fortification of breakfast cereal, flour, and other staple foods, the frequent consumption of fruits and vegetables has become less essential for cancer prevention. Nevertheless, an abundance of fruits and vegetables as part of a healthy diet is recommended, because evidence consistently suggests that it lowers the incidence of hypertension, heart disease, and stroke.

Fiber

General Properties

Dietary fiber was defined in 1976 as "all plant polysaccharides and lignin which are resistant to hydrolysis by the digestive enzymes of men."[55] Fiber, both soluble and insoluble, is fermented by the luminal bacteria of the colon. Among the properties of fiber that make it a candidate for cancer prevention are its "bulking" effect, which reduces colonic transit time, and the binding of potentially carcinogenic luminal chemicals. Fiber may also aid in producing short-chain fatty acids that may be directly anticarcinogenic. Fiber may also induce apoptosis.

Dietary Fiber and Colorectal Cancer

In 1969, Dennis Burkitt hypothesized that dietary fiber is involved in colon carcinogenesis.[56] While working as a physician in Africa, Burkitt noticed the low incidence of colon cancer among African populations whose diets were high in fiber. Burkitt concluded that a link might exist between the fiber-rich diet and the low incidence of colon cancer. Burkitt's observations were followed by numerous case-control studies that seemed to confirm his theories. A combined analysis of 13 case-control studies[57] as well as a meta-analysis of 16 case-control studies[58] suggested an inverse association between fiber intake and the risk of colorectal cancer. The inclusion of studies was selective, however, and effect estimates unadjusted for potential confounders were used for most studies. Moreover, recall bias is a severe threat to the validity of retrospective case-control studies of fiber intake and any disease outcome.

Data from prospective cohort studies have largely failed to support an inverse association between dietary fiber and colorectal cancer incidence. Initial analyses from the Nurses' Health Study and the Health Professionals' Follow-Up Study[36] found no important association between dietary fiber and colorectal cancer. A significant inverse association between fiber intake and incidence of colorectal cancer was reported from the EPIC study. The analysis presented on dietary fiber and colorectal cancer encompassed 434,209 women and men from eight European countries.[59] The analytic model used by the EPIC investigators included adjustments for age, height, weight, total caloric intake, sex, and center assessed at baseline and identified[60] a significant inverse association between fiber intake and colorectal cancer. Applying the same analytic model used in EPIC to data from the Nurses' Health Study and the Health Professionals' Follow-Up Study encompassing 1.8 million person-years of follow-up and 1,572 cases of colorectal cancer revealed associations similar to those found in the EPIC study.[61] After a more complete adjustment for confounding variables, however, the association vanished.[61] Results from the pooled analysis of 13 prospective cohort studies, including 8,081 colorectal cancer cases diagnosed during over 7 million person-years of follow-up, suggested an inverse relation between dietary fiber and colorectal cancer incidence in age-adjusted analyses, but this association disappeared after appropriate adjustment for confounding variables, particularly other dietary factors.[62] The NIH–AARP study, which included 2,974 cases of colorectal cancer, confirmed the lack of association between total dietary fiber and colorectal cancer risk.[63]

The association between dietary fiber and colorectal cancer appears to be confounded by a number of other dietary and nondietary factors. These methodologic considerations must be taken into account when interpreting the evidence. It is possible that other dietary factors such as folate intake are more important for colorectal cancer pathogenesis than dietary fiber.

Dietary Fiber and Colorectal Adenomas

In a few prospective cohort studies, the primary occurrence of colorectal polyps was investigated, but no consistent relation was found.

The study of fiber intake and colorectal adenoma recurrence lends itself to a randomized clinical trial design because of the relatively short follow-up necessary and because fiber can be provided as a supplement. A number of RCTs have explored the effect of fiber supplementation on colorectal adenoma recurrence. Evidence has fairly consistently indicated no effect of fiber intake.[64–68] In one RCT, an increase in adenoma recurrence was observed among participants randomly assigned to use a fiber supplement, which was stronger among those with high dietary calcium.[69]

Dietary Fiber and Breast Cancer

Investigators have speculated that dietary fiber may reduce the risk of breast cancer through a reduction in intestinal absorption of estrogens excreted via the biliary system.

Relatively few epidemiologic studies have examined the association between fiber intake and breast cancer. In a meta-analysis of 10 case-control studies, a significant inverse association was observed. However, these retrospective studies were likely affected by the aforementioned biases—selection and recall bias, in particular. Results from at least six prospective cohort studies consistently suggested no association between fiber intake and breast cancer incidence.[70–75]

Dietary Fiber and Stomach Cancer

The results from retrospective case-control studies of fiber intake and gastric cancer risk are inconsistent. In the Netherlands Cohort Study, dietary fiber was not associated with an incidence of gastric carcinoma.[76] Further investigations through prospective cohort studies must be completed before conclusions about the relation between fiber intake and stomach cancer incidence can be drawn.

Summary

The observational data presently available do not indicate an important role for dietary fiber in the prevention of cancer, although small effects cannot be excluded. The long-held perception that a high intake of fiber conveys protection originated largely from retrospectively conducted studies, which are affected by a number of biases, in particular, the potential for differential recall of diet, and from studies that were not well controlled for potential confounding variables.

OTHER FOODS AND NUTRIENTS

Red Meat

The regular consumption of red meat has been associated with an increased risk of colorectal cancer. In a recent meta-analysis, the increase in risk associated with an increase in intake of 120 g per day was 24% (95% CI, 9% to 41%).[37] The association was strongest for processed meat; the relative risk of colorectal cancer was 1.36 (95% CI, 1.15 to 1.61) for a consumption of 30 g per day.[37] No overall association has been observed between red meat consumption and breast cancer in a pooled analysis of prospective cohorts.[77] However, among premenopausal women in the Nurses' Health Study II, the risk for estrogen-receptor–positive and progesterone-receptor–positive breast cancer doubled with 1.5 servings of red meat per day compared to three or fewer servings per week.[78] No associations have been found in studies on poultry or fish.[15] Mechanisms through which red meat may increase cancer risk include anabolic hormones routinely used in meat production in the United States, heterocyclic amines, and polycyclic aromatic hydrocarbons formed during cooking at high temperatures, the high amounts of heme iron, and nitrates and related compounds in smoked, salted, and some processed meats that can convert to carcinogenic nitrosamines in the colon.

Milk, Dairy Products, and Calcium

Regular milk consumption has been associated with a modest reduction in colorectal cancer in both a pooling project[79] and a meta-analysis of cohort studies,[80] possibly due to its calcium content. In the pooling project of prospective studies of diet and cancer, a modest inverse association was also seen for calcium intake.[79] This finding is consistent with the results of a randomized trial in which calcium supplements reduced the risk of colorectal adenomas.[81] Associations with cheese and other dairy products have been less consistent.[79,80]

Conversely, in multiple studies, a high intake of calcium or dairy products has been associated with an increased risk of prostate cancer,[80,82–86] specifically fatal prostate cancer.[87,88] Similar observations were made in the NIH–AARP study, although the increase in risk there did not reach statistical significance.[89] While the Multiethnic Cohort[90] and the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial[91] did not find an important association between dairy consumption and prostate cancer, these cohort studies did not specifically include fatal prostate cancer cases. A meta-analysis of prospective studies generated an overall relative risk of advanced prostate cancer of 1.33 (95% CI, 1.00 to 1.78) for the highest versus the lowest intake categories of dairy products.[92] In another meta-analysis, no significant association was found for cohort studies on dairy or milk consumption, but relative risk estimates suggested a positive association.[93] Thus, although the findings are not entirely consistent and are complicated by the widespread use of prostate-specific antigen (PSA) screening in the United States, the global evidence suggests a positive association between the regular consumption of dairy products and the risk of fatal prostate cancer. Consuming three or more servings of dairy products per day has been associated with endometrial cancer among postmenopausal women not using hormonal therapy.[94] A high intake of lactose from dairy products has also been associated with a modestly higher risk of ovarian cancer.[95]

These observations are particularly important in the context of national dietary recommendations to drink three glasses of milk per day.[38] Possible mechanisms include an increase in endogenous IGF-1 levels[96] and steroid hormones contained in cows' milk.[97]

Vitamin D

In 1980, Garland and Garland[98] hypothesized that sunlight and vitamin D may reduce the risk of colon cancer. Since then, substantial research has been conducted in this area supporting an inverse association between circulating 25-hydroxyvitamin D (25[OH]D) levels and colorectal cancer risk.[99–103] A meta-analysis, including five nested case-control studies with prediagnostic serum, suggested a reduction of colorectal cancer risk by about half among individuals with serum 25(OH)D levels of more than 82 nmol/L compared to individuals with less than 30 nmol/L.[104] A subsequent meta-analysis including eight studies confirmed these associations.[105] These observations are supported by similar findings for colorectal adenomas.[106] Vitamin D levels may particularly affect colorectal cancer prognosis; colorectal cancer mortality was 72% lower among individuals with 25(OH)D concentrations of 80 nmol/L or higher.[107]

The evidence for other cancers has been less consistent. High plasma levels of vitamin D have been associated with a decreased risk of several other cancers, including cancer of the breast[108–111]; prostate, especially fatal prostate cancer[112]; and ovary.[113,114] Whether vitamin D plays a role in pancreatic cancerogenesis remains to be determined with one pooling project, suggesting a positive association,[115] whereas other prospective studies[116] and a pooling project of cohort studies found inverse associations.[117]

The activation of vitamin D receptors by $1,25(OH)_2D$ induces cell differentiation and inhibits proliferation and angiogenesis.[118] Solar ultraviolet B radiation is the major source of plasma

vitamin D, and dietary vitamin D without supplementation has a minor effect on plasma vitamin D. To achieve sufficient plasma levels through sun exposure, at least 15 minutes of full-body exposure to bright sunlight is necessary. Physical activity has to be considered as possible confounder of studies on plasma levels of vitamin D and cancer. Sunscreen effectively blocks vitamin D production. Populations who live in geographic areas with limited or seasonal sun exposure may benefit from a vitamin D supplementation of 1,000 IU per day.

Folate

Folate is a micronutrient commonly found in fruits and vegetables, particularly oranges, orange juice, asparagus, beets, and peas. Folate may affect carcinogenesis through various mechanisms: DNA methylation, DNA synthesis, and DNA repair. In the animal model, folate deficiency enhances intestinal carcinogenesis.[119] Folate deficiency is related to the incorporation of uracil into human DNA and to an increased frequency of chromosomal breaks. A number of epidemiologic studies suggest that a diet rich in folate lowers the risk of colorectal adenomas and colorectal cancer.[15] Because the folate content in foods is generally relatively low, is susceptible to oxidative destruction by cooking and food processing, and is not well absorbed, folic acid from supplements and fortification plays an important role. Pooled results from 13 prospective studies suggests that intake of 400 to 500 μg per day is required to minimize risk.[120]

Potential interactions among alcohol consumption, folic acid intake, and methionine intake have been described. Although alcohol consumption has been fairly consistently related to an increase in breast cancer incidence, the potential detrimental effect of alcohol seems to be eliminated in women with high folic acid intake.[16] A similar folic acid or methionine–alcohol interaction has been observed for colorectal cancer risk.[119]

Genetic susceptibility may also modify the relation between folate intake and cancer risk. A polymorphism of the *methylenetetrahydrofolate reductase* (*MTHFR*) gene (cytosine to thymine transition at position 677) may result in a relative deficiency of methionine. Individuals with the common C677T mutation appear to experience the greatest protection from high folic acid or methionine intake and low alcohol consumption.[121] Although the interaction between this polymorphism and dietary factors needs to be investigated further, the consistently observed association between this polymorphism and the risk of colorectal cancer supports a role of folate in the etiology of colorectal cancer.

Folate levels also affect the availability of methyl groups via S-adenosylmethionine in the one-carbon metabolism.[122] Low red blood cell folate levels are associated with low DNA methylation status among homozygous *MTHFR* 677T/T mutation carriers, whereas at high red blood cell folate levels, the amount of methylated cytosine in DNA is similar to that of the heterozygote *MTHFR* C677T genotype.[123]

Conversely, evidence from animal and human studies suggests that a high folate status may promote the progression of existing neoplasias.[122,124,125] The randomization of folic acid supplements among individuals with a history of colorectal adenoma resulted in either no effect on recurrent adenoma recurrence[126] or an increase in recurrence with over 6 to 8 years of follow-up.[127] The high proliferation rate of neoplastic cells requiring increased DNA synthesis is likely supported by folate, which is necessary for thymidine synthesis.[122,125] The effects of folate on de novo methylation and subsequent gene silencing have been insufficiently studied. An increase in colorectal cancer rates has been observed in the United States and Canada concurrent with the introduction of the folic acid fortification program, but this could be an artifact due to increased use of colonoscopies.[128] The lack of increase in mortality, but an acceleration in a long-term downward trend suggests the latter explanation (http://progressreport.cancer.gov/).

Carotenoids

Carotenoids, antioxidants prevalent in fruits and vegetables, enhance cell-to-cell communication, promote cell differentiation, and modulate immune response. In 1981, Doll and Peto[1] speculated that beta-carotene may be a major player in cancer prevention and encouraged testing its anticarcinogenic properties. Indeed, subsequent observational studies, mostly case-control investigations, suggested a reduced cancer risk—especially of lung cancer—with a high intake of carotenoids. In contrast, clinical trials randomizing the intake of beta-carotene supplements have not revealed the evidence of a protective effect of beta-carotene. In fact, beta-carotene was found to increase the risk of lung cancer and total mortality among smokers in the Finnish Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study.[129] However, these adverse affects disappeared during longer periods of follow-up.[130] In a detailed analysis of prospective studies, no association was seen between the intake of beta-carotene and the risk of lung cancer.[131]

The pooled analysis of 18 cohort studies including more than 33,000 breast cancer cases suggested inverse associations between the intake of several carotenoids (beta-carotene, alpha-carotene, luteine/zeaxanthin) and estrogen-receptor–negative breast cancer incidence, whereas no association was found for estrogen-receptor–positive tumors.[132] Similarly, in a pooled analysis of data from eight prospective studies including about 3,055 breast cancer cases, blood levels of carotenoids were inversely related to estrogen-receptor–negative mammary tumor incidence.[133] Women in the highest quintile of beta-carotene levels had about half the risk of developing estrogen-receptor–negative breast cancer than women in the lowest quintile (hazard ratio [HR] = 0.52; 95% CI, 0.36 to 0.77).

The particularly pronounced antioxidant properties of lycopene, a carotenoid mainly found in tomatoes, may explain the inverse associations with some cancers. The frequent consumption of tomato-based products has been associated with a decreased risk of prostate, lung, and stomach cancers.[134] The bioavailability of lycopene from cooked tomatoes is higher than from fresh tomatoes, making tomato soup and sauce excellent sources of the carotenoid.

Selenium

Selenium has long been of interest in cancer prevention due to its antioxidative properties. Its intake is difficult to estimate because food content depends on the selenium content of the soil it is grown in. Selenium enriches in toenails, which provide an integrative measure of intake during the previous year and therefore are popular biomarkers in epidemiologic studies. Inverse associations with toenail selenium levels have been found in several prospective studies, especially for fatal protate cancer.[135–137] In a recent meta-analysis, plasma/serum selenium was also inversely correlated with prostate cancer.[138] In the Selenium and Vitamin E Cancer Prevention Trial (SELECT), no protective effect of selenium was found for prostate cancer. However, the trial was terminated prematurely after 4 years, which is a short period in which to expect a reduction in cancer.[139]

Soy Products

The role of soy products has been considered for breast carcinogenesis. In Asian countries, which traditionally have a high consumption of soy foods, breast cancer rates have been low until recently. In Western countries, soy consumption is generally low, and between-person variation may be insufficient to allow meaningful comparisons. Soybeans contain isoflavones, which are phytoestrogens that compete with estrogen for the estrogen receptor. Hence, soy consumption may affect estrogen concentrations differently depending on the endogenous baseline level. This mechanism may also contribute to

the equivocal results of studies on soy foods and breast cancer risk. In a recent meta-analysis of 18 epidemiologic studies, including over 9,000 breast cancer cases, frequent soy intake was associated with a modest decrease in risk (odds ratio = 0.86; 95% CI, 0.75 to 0.99).[140] Wu et al. [141] observed that childhood intake of soy was more relevant to breast cancer prevention than adult consumption.

Carbohydrates

The Warburg hypothesis postulated in 1924 that tumor cells mainly generate energy by the nonoxidative breakdown of glucose (glucolysis) instead of pyrovate.[142] Carbohydrates with a high glycemic load increase blood glucose levels after consumption, which results in insulin spikes increasing the risk for type 2 diabetes. Several cancers, including colorectal cancer[143] and breast cancer,[144] have been associated with type 2 diabetes. The evidence on the consumption of sucrose and refined, processed flour and cancer incidence is heterogeneous.[145] Whereas in some prospective cohort studies an increase in colon cancer incidence was observed,[146] this was not found in other studies.[147] In large cohort studies, associations have been observed for pancreatic[148] and endometrial[145] cancer risk, but not for postmenopausal breast cancer.[149] Especially in obese, sedentary individuals, abnormal glucose and insulin metabolism may contribute to tumorigenesis.

DIETARY PATTERNS

Foods and nutrients are not consumed in isolation, and, when evaluating the role of diet in disease prevention and causation, it is sensible to consider the entire dietary pattern of individuals. Public health messages may be better framed in the context of a global diet than individual constituents.

The role of vegetarian diets for cancer incidence has been examined in a few studies. In the Adventist Health Study-2, vegetarians had an 8% lower incidence of cancer than nonvegetarians (95% CI, 1 to 15%).[150] The protective association was strongest for cancers of the gastrointestinal tract with 24% (95% CI, 10 to 37%). Vegans had a 16% (95%, 1 to 28%) lower incidence of cancer, with a particular protection conferred to female cancers of 34% (95% CI, 8 to 53%). A combined analysis of data from the Oxford Vegetarian Study and EPIC similarly suggest a 12% (95% CI, 4 to 19%) reduction in cancer incidence among vegetarians compared to meat eaters.[151]

During the past decade, dietary pattern analyses have gained popularity in observational studies. The most commonly employed methods are factor analyses and cluster analyses, which are largely data-driven methods, and investigator-determined methods such as dietary indices and scores. The search for associations between distinct patterns such as the "Western pattern," which is characterized by a high consumption of red and processed meats; high fat dairy products, including butter and eggs; and refined carbohydrates, such as sweets, desserts, and refined grains, and the "prudent pattern," which is defined by the frequent consumption of a variety of fruits and vegetables, whole grains, legumes, fish, and poultry, and the risk of cancer has been largely disappointing. Notable exceptions were the link between a Western dietary pattern and colon cancer incidence and an inverse relation between a prudent diet[152] and estrogen-receptor–negative breast cancer.[153] These findings were subsequently in the California Teachers Study.[154] The general lack of association between global dietary patterns and cancer supports a more modest role of nutrition during adult life in carcinogenesis than previously assumed.

DIET DURING THE EARLY PHASES OF LIFE

Some cancers may originate early in the course of life. A high birth weight is associated with an increase in the risk of childhood leukemia,[155] premenopausal breast cancer,[156] and testicular cancer.[157] Tall height is an indicator of the risk of many cancers and is in part determined by nutrition during childhood.[15] Until recently, most studies focused on the role of diet during adult life. However, the critical exposure period for nutrition to affect cancer risk may be earlier, and because the latent period for cancer may span several decades, diet during childhood and adolescence may be important. However, relating dietary information during early life and cancer outcomes prospectively is difficult because nutrition records from the remote past are not available. Studies in which recalled diet during youth is used have to be interpreted cautiously due to misclassification, although recall has been found reasonably reproducible and consistent with recalls provided by participants' mothers.[158,159] The role of early life diet has been explored in only a few studies in relation to breast cancer risk. In a study nested in the Nurses' Health Study cohorts that used data recalled by mothers, frequent consumption of french fries was associated with an increased risk of breast cancer, whereas whole milk consumption was inversely related to risk.[160] Similarly, an inverse association with milk consumption during childhood was found among younger women (30 to 39 years), but not among older premenopausal women (40 to 49 years) in a Norwegian cohort.[161] Dietary habits during high school recalled by adult participants of the Nurses' Health Study II (but before the diagnosis of breast cancer) suggested a positive association of total fat and red meat consumption.[31,32] More data are needed in this promising area of research.[162]

DIET AFTER A DIAGNOSIS OF CANCER

The role of diet in the secondary prevention of cancer recurrence and survival is generally of great interest to cancer patients because they are highly motivated to make lifestyle changes to optimize their prognosis. The compliance of cancer patients makes the RCTs a more feasible design to evaluate the role of diet than among healthy individuals. However, concurrent cancer treatments may make any effect of diet more difficult to isolate.

Most evidence is available for breast cancer, colorectal, and prostate cancer. Observational data suggest a limited role of diet in the prevention of breast cancer recurrence and survival. The Life After Cancer Epidemiology (LACE) Cohort supported a beneficial role for vitamin C and E supplement use but the effect of other health-seeking behaviors is difficult to exclude.[163] In a pooled analysis, alcohol consumption after a diagnosis did not affect survival.[164] Several randomized trials have addressed the role of diet in breast cancer prognosis. In the Women's Intervention Nutrition Study (WINS), 2,437 women with early stage breast cancer were randomized to a dietary goal of 15% of calories from fat or maintenance of their usual dietary habits.[165] The intervention group received dietary counseling by registered dieticians and, according to self-reports, a difference of 19 g in daily fat intake was maintained between the intervention and the control group after 60 months of follow-up. However, at that time, women in the intervention group were also 6 pounds lighter, making it difficult to separate an effect of dietary fat from a nonspecific effect of intensive dietary intervention, which quite consistently produces weight loss. Breast cancer recurrence was 29% lower in the intervention group (95% CI, 6% to 47%), whereas overall survival was not affected. In the Women's Healthy Eating and Living (WHEL) RCT, 3,088 early stage breast cancer patients were randomly assigned to a target of five vegetable servings, three fruit servings, 30 g fiber per day, and 15% to 20% of calories from fat.[166] After 72 months, the intervention versus control group reports were 5.8 versus 3.6 servings of vegetables, 3.4 versus 2.6 servings of fruit, 24.2 versus 18.9 g fiber per day, and 28.9% versus 32.4% of calories from fat. The total plasma carotenoid concentration, a biomarker of vegetable and fruit intake, was 43% higher in the intervention group than the comparison group after 4 years ($p<0.001$). Neither recurrence

rates nor mortality were affected by the intervention after the 7.3-year follow-up. Overall, diet is unlikely a major factor influencing breast cancer prognosis. However, because the prognosis for breast cancer is relatively good, women diagnosed with breast cancer remain at risk for cardiovascular disease and other causes of death that affect those without breast cancer. Thus, among women in the Nurses' Health Study diagnosed with breast cancer, a higher diet quality, which was assessed by the Alternative Healthy Eating Index, was not associated with mortality due to breast cancer but was associated with substantially lower mortality due to other causes.[167] Similarly, among over 4,000 women with breast cancer, intakes of saturated and trans fat, but not of total fat, were associated with significantly greater total mortality but not specifically breast cancer mortality. Thus, there is good reason for women with breast cancer to adopt a healthy diet even if it does not affect the prognosis of breast cancer.

In a systematic review, no consistent association between individual dietary components and colorectal cancer prognosis outcome was found.[168] However, in an observational study including 1,009 patients with stage III colon cancer, a Western dietary pattern was associated with lower rates of disease-free survival, recurrence-free survival, and overall survivals.[169] In the same patient population, higher dietary glycemic load and total carbohydrate intake were significantly associated with an increased risk of recurrence and mortality.[170] These findings support a possible role of glycemic load in colon cancer progression.

In the Physician's Health Study, whole milk consumption among men with incident prostate cancer was associated with double the risk of progression to fatal disease.[171] Among men with nonmetastatic prostate cancer in the Health Professionals' Follow-up Study, replacing 10% of energy intake from carbohydrates with vegetable fat was associated with a lower risk of lethal prostate cancer.[172] A marginally increased risk of progression of localized to lethal prostate cancer among these men was also associated with postdiagnostic poultry and processed red meat consumption,[173] whereas postdiagnostic consumption of fish and tomato sauce were inversely related with a risk of progression.[174] In an intervention study, 93 patients with early stage prostate cancer (PSA = 4 to 10 ng per mililiter and Gleason score <7) were randomized to comprehensive lifestyle changes, including a vegan diet based on 10% of calories from fat and consisting predominantly of vegetables, fruit, whole grains, legumes, and soy protein.[175] Other interventions included moderate exercise, stress management, and relaxation. After 1 year, PSA values decreased 4% in the intervention group, but increased 6% in the control group. Six patients in the control group, but none in the experimental group, underwent conventional prostate cancer treatment. Although the impact of the different intervention components are difficult to separate in this study, further data on diet and the prognosis for patients with localized prostate cancer are needed.

SUMMARY

A considerable proportion of cancers are potentially preventable through lifestyle changes. Besides a curtailment of smoking, the most important strategies are maintaining a healthy body weight and regular physical activity, which contribute to a lower prevalence of being overweight and obesity. The avoidance of a positive energy balance and becoming overweight are the most important nutritional factors in cancer prevention.

Although dietary patterns, including frequent fruit and vegetable consumption, appear to play a modest role in cancer prevention, knowledge gained about some specific foods and nutrients might inform a targeted approach. Vitamin D is a strong candidate to counter carcinogenesis, thus supplementation could be a feasible and safe route to avoid several types of cancer. Although the data on vitamin D and cancer incidence are not conclusive, the prevention of bone fractures is a sufficient reason to maintain good vitamin D status.

Limiting or avoiding red meat, processed meat, and alcohol reduces the risk of breast, colorectal, stomach, esophageal, and other cancers. Although the role of dairy products and milk remains to be more fully elucidated, current evidence suggests a probable increase in the risk of prostate cancer with frequent milk consumption, and possibly endometrial cancer, which raises concern regarding current dietary recommendations of three glasses of milk per day. The relation of calcium and dairy intake to cancer is complex, as the evidence for a reduction in the risk of colorectal cancer is strong, but high intakes appear likely to increase the risk of fatal prostate cancer. The consumption of tomato-based products may contribute to the prevention of prostate cancer. Finally, diet may influence the prognosis of colorectal and prostate cancer, but more data are needed in this area. Because most people with cancer remain at risk of cardiovascular disease and other common conditions related to unhealthy diets, an overall healthy diet can be recommended while further research on diet and cancer survival is ongoing.

LIMITATIONS

Studying the role of diet in health and disease requires overcoming a number of hurdles. Because biomarkers reflecting nutrient intake with sufficient accuracy are largely lacking, assessing nutrition in a population-based study has to rely on self-reports by individuals, which inevitably leads to imprecision or error in the diet assessment. Such misclassification may produce spurious associations in case-control studies or may lead to an underestimation of true associations in prospective cohort studies. Ideally, hypotheses relating dietary factors to cancer risks would be tested in large randomized trials. Besides being extremely expensive, maintaining adherence to assigned diets has been challenging; for example, in the WHI trial that focused on dietary fat reduction, there were no differences between intervention and control groups in blood lipid fractions that are known to change with a reduction in fat intake, indicating a failure to test the hypothesis.[28]

Most observational studies are conducted within populations or countries. Although reasonable variations in nutritional habits exist within populations, allowing for the detection of substantial dietary risk factors for cardiovascular disease and diabetes, these contrasts may be too limited to detect small relative risks as they may exist for cancer. The pooled analysis of large prospective cohort studies across countries and continents attempts to overcome this limitation. Studies taking advantage of the large between-population variation in diets across developed and developing countries would appear to be advantageous, but would be plagued by confounding by other differences in lifestyle factors that might be difficult to assess and control adequately.

Few epidemiologic studies repeatedly capture dietary habits over time and thus account for potential changes in diet over time. Furthermore, the length of follow-up in prospective studies may not be sufficient to capture the impact of diets assessed at baseline. In case-control studies, a recall of dietary habits prior to the disease onset may be influenced by current disease status; moreover, the relevant time for nutrition to act may be decades earlier, which is more difficult to remember.

Most epidemiologic studies of diet and cancer have assessed intake among adults. Due to greater susceptibility to genotoxic influences earlier in life, it is possible that data on diet during childhood or early adolescence are more relevant for carcinogenesis and cancer prevention. Studies that have collected dietary data during childhood and followed the subjects for cancer incidence would be most informative but are virtually nonexistent and will be challenging to conduct.

Finally, data on special diets including organic foods, whole foods, raw foods, and a vegan diet are limited.

FUTURE DIRECTIONS

Some of the most promising research at present is in the areas of vitamin D, milk consumption, and the effect of diet early in life on cancer incidence. Recent nutrition changes in countries previously maintaining a more traditional diet such as Japan and some developing countries have already been followed by increased rates of some cancers (but declines in stomach cancer), providing a setting to study the effect of change over time. Additional insight may come from studies on gene–nutrient interaction and epigenetic changes induced by the diet. To improve observational research methods, refined dietary assessment methods, including the identification of new biomarkers, will be advantageous.

RECOMMENDATIONS

A wealth of data are available from observational studies on diet and cancer, and the current evidence supports suggestions made by Doll and Peto[1] that approximately 30% to 40% of cancers may be avoidable with changes in nutrition; however, much of this risk of cancer is related to being overweight and to inactivity. Excessive energy intake and lack of physical activity, marked by rapid growth in childhood and being overweight, have become growing threats to population health and are important contributors to risks of many cancers. Nevertheless, the cumulative incidence for many cancers has decreased over the past decade, in part due to the decreasing prevalence of smoking and use of hormone therapy.

Dietary recommendations must integrate the goal of overall avoidance of disease and maintenance of health and, thus, should not focus singularly on cancer prevention. The strength of the evidence and magnitude of the expected benefit should also be considered in recommendations. With these considerations in mind, the following recommendations are outlined, which are largely in agreement with the guidelines put forth by the American Cancer Society in 2012:[176]

1. *Engage in regular physical activity.* Physical activity is a primary method of weight control and it also reduces risk of several cancers, especially colon cancer, through independent mechanisms. Moderate to vigorous exercise for at least 30 minutes on most days is a minimum and more will provide additional benefits.
2. *Avoid being overweight and weight gain in adulthood.* A positive energy balance that results in excess body fat is one of the most important contributors to cancer risk. Staying within 10 pounds of body weight at age 20 may be a simple guide, assuming no adolescent obesity.
3. *Limit alcohol consumption.* Alcohol consumption contributes to the risk of many cancers and increases the risk of accidents and addiction, but low to moderate consumption has benefits for coronary heart disease risk. The individual family history of disease as well as personal preferences should be considered.
4. *Consume lots of fruits and vegetables.* Frequent consumption of fruits and vegetables during adult life is not likely to have a major effect on cancer incidence, but will reduce the risk of cardiovascular disease.
5. *Consume whole grains and avoid refined carbohydrates and sugars.* A regular consumption of whole grain products instead of refined flour and a low consumption of refined sugars lower the risk of cardiovascular disease and diabetes. The effect on cancer risk is less clear.
6. *Replace red meat and dairy products with fish, nuts, and legumes.* Red meat consumption increases the risk of colorectal cancer, diabetes, and coronary heart disease and should be largely avoided. Frequent dairy consumption may increase the risk of prostate cancer. Fish, nuts, and legumes are excellent sources of valuable mono- and polyunsaturated fats and vegetable proteins and may contribute to lower rates of cardiovascular disease and diabetes.
7. *Consider taking a vitamin D supplement.* A substantial proportion of the population, especially those living at higher latitudes, are vitamin D deficient. Most adults may benefit from taking 1,000 IU of vitamin D_3 per day during months of low sunlight intensity. Vitamin D supplementation will, at a minimum, reduce bone fracture rates, probably colorectal cancer incidence, and possibly other cancers.

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10 Obesity and Physical Activity

Yani Lu, Jessica Clague, and Leslie Bernstein

INTRODUCTION

Evidence showing that physical activity is associated with decreased cancer risk and that obesity is associated with increased cancer risk at certain sites is rapidly accumulating. It is not yet known whether these two factors are interrelated or independent. Physical activity may act to decrease cancer risk primarily by preventing weight gain and obesity. However, physical activity may also have independent effects on cancer risk. In this chapter, we present a summary of the current epidemiologic literature on the possible associations between physical activity and obesity and risk of cancer at several organ sites.

Physical activity is defined as any movement of the body that results in energy expenditure. In this chapter, we focus on recreational physical activity, also called leisure-time physical activity or exercise, and occupational physical activity, including household activity.[1] Occupational physical activity typically occurs over a longer period of time and generally requires less energy expenditure per hour than bouts of strenuous or moderate recreational physical activity. The distinction between recreational and occupational activity is important because increasing mechanization and technologic advances have led to decreased occupational physical activity in developed areas of the world, perhaps contributing to a decrease in overall physical activity.

Obesity is defined as the condition of being extremely overweight. In epidemiologic studies, the usual, but not necessarily the best, measure of body mass in adults is Quetelet's Index, or body mass index (BMI), which is measured as weight in kilograms (kg) divided by the square of height in meters (m^2). In the year spanning 2009 to 2010, the prevalence of obesity, defined by having a BMI of 30 kg/m^2 or greater, in the US population was 35.5% for adult men and 35.8% for adult women.[2] Physical inactivity has likely contributed to the high prevalence of obesity in the United States; data from the 2003 to 2004 National Health and Nutritional Examination Survey, a cross-sectional study of a sample of the civilian, noninstitutionalized population of the United States, has indicated that less than 5% of US adults achieve 30 minutes per day of physical activity, and that men are more physically active than women.[3]

Epidemiologic evidence on the associations of physical activity and obesity with cancer come from observational studies, including cohort studies, which follow populations forward in time after collecting exposure information, and case-control studies, which optimally identify a population-based series of newly diagnosed cases and healthy control subjects, collecting information retrospectively on exposures. In both study designs, physical activity information is usually self-reported and measures vary substantially with respect to timing and level of detail. Studies have measured lifetime or long-term physical activity, activity at defined ages or time points in life, and/or current or recent activity. Ideally, a study would capture activity by type (recreational, occupational, or other, such as an activity related to transportation), duration (minutes per session), frequency (sessions per day), and intensity (low, moderate, or strenuous as defined by examples of activity types) across the lifetime. These studies have often measured height and weight by self-report at one time point, such as at the time of study entry. Some studies have collected other or more detailed anthropometric information, such as waist circumference, hip circumference, or weight at an additional time point like at age 18. Anthropometrics are directly measured by trained study personnel in only a few studies.

Epidemiologic evidence for a role of physical activity or obesity in relation to cancer risk exists for cancers of the breast, colon, endometrium, esophagus, kidney, and pancreatic cancer. Evidence is accumulating to link at least one of these "exposures" to the incidence of gallbladder cancer, non-Hodgkin lymphoma (NHL), and advanced prostate cancer. The evidence for an association between either physical activity or obesity and lung and ovarian cancer is inconclusive.

In addition to specific biologic mechanisms pertinent to physical activity or to obesity at each specific organ site, several global mechanisms have been implicated in both relationships across a number of these organ sites. The steroid hormone and insulin/insulinlike growth factor (IGF) pathways are two such global mechanisms hypothesized to be involved in the links between physical activity or obesity and cancer.[4] The role of steroid hormones as a mediator in these relationships is perhaps best understood in the context of breast cancer and endometrial cancer, and will be discussed in those sections. The roles of the insulin and IGF pathways have been discussed in depth with respect to colon cancer and, thus, will be presented in that context. Other global mechanisms have been proposed that have more generalized anticancer impacts and may explain associations between physical activity and several cancer sites; these include heightening immune surveillance, reducing inflammation, increasing insulin sensitivity, controlling growth factor production and activation, decreasing obesity and central adiposity, optimizing DNA repair capacity, and reducing oxidative stress.[5,6] Further, obesity has been shown to produce a proinflammatory state and, thus, inflammation may mediate the relationship between obesity and cancer risk.[7] It is highly plausible that several of these mechanisms act simultaneously and that they interact synergistically to mediate the associations between physical activity, obesity, and cancer.

BREAST CANCER

Low level of physical activity is an established breast cancer risk factor among postmenopausal women and, to a lesser extent, premenopausal women.[4,8,9] The evidence for an association between physical activity and breast cancer has been classified as convincing, with a 20% to 40% reduced risk among physically active women.[10] Obesity appears to have a paradoxical relationship with breast cancer risk in that it is an established breast cancer risk factor among postmenopausal women, but may offer some protection for breast cancer among premenopausal women.[4]

The epidemiologic literature has shown with relative consistency that breast cancer risk is reduced by increasing one's amount of physical activity.[4,8,9,11–13] One of the earliest studies, a case-control study of women age 40 years or younger, showed a dramatic reduction in risk of approximately 50% among women who averaged about 4 hours of activity per week during their

reproductive years.[14] Similarly, among postmenopausal women, those with higher levels of recreational physical activity during their lifetimes have been shown to have lower breast cancer risk.[15] A meta-analysis of 29 case-control studies and 19 cohort studies published between 1994 and 2006 provided strong evidence for an inverse association between physical activity and risk of breast cancer, citing that the evidence for an association between physical activity and premenopausal breast cancer was not as strong as that for postmenopausal breast cancer.[8] The conclusion of the meta-analysis was that each additional hour of physical activity per week decreases breast cancer by approximately 6%.

Epidemiologists require that a risk factor demonstrate consistency across populations before considering it as accepted. Recently, studies have been published on the association between physical activity and breast cancer risk among Japanese,[16] Chinese,[17] Mexican,[18] Tunisian,[19] and African American women.[20] All studies showed a decreased risk of breast cancer with increasing physical activity. Interestingly, both Suzuki et al.[17] and Pronk et al.[21] observed the strongest associations among "heavier" women (BMI $\geq$25 kg/m^2 and 23.73 kg/m^2, respectively). In the California Teachers Study (CTS), a prospective cohort study of over 133,000 female public school professionals, a variable combining strenuous and moderate long-term recreational physical activity was associated with a reduced risk of estrogen receptor (ER)-negative but not ER-positive invasive breast cancer.[11] On the contrary, the Women's Health Initiative (WHI) observed decreases in breast cancer risk associated with recreational physical activity among postmenopausal women with ER-positive breast cancer and triple negative breast cancer, with only results for ER-positive breast cancer demonstrating a 15% statistically significant reduced risk (when comparing the highest versus lowest tertile of moderate-intensity physical activity).[22] Similar but not statistically significant results were observed for strenuous recreational physical activity.[22] A major limitation to this and previous studies stratifying by hormone receptor status is the inability to comprehensively classify triple negative breast cancer due to missing HER2 status (unknown in 40% of cases in the WHI study). The use of hormone therapy did not alter the inverse association between recreational physical activity and invasive breast cancer in the Women's Contraceptive and Reproductive Experiences (CARE) Study.[23] Most recently, in the American Cancer Society Cancer Prevention Study II Nutrition Cohort, it was observed that postmenopausal women who engage in at least 7 hours of walking over the course of a week had a modest decreased risk of breast cancer, even in the absence of more vigorous exercise.[24] Further, this association did not differ by ER status, BMI, adult weight gain, postmenopausal hormone therapy use, or time spent sitting.[24]

Lastly, whether physical activity reduces breast cancer risk by impacting preinvasive disease has been studied by assessing the associations with in situ breast cancer and benign breast disease. In the CTS cohort, increasing levels of long-term strenuous recreational physical activity were associated with a decreasing risk of in situ breast cancer.[11] Furthermore, a report from the Nurses' Health Study II cohort showed that lifetime recreational physical activity was associated with a decreased risk of benign breast disease and columnar cell lesions, which may be precursors to breast cancer.[25]

In summary, epidemiologic studies investigating the association between physical activity and breast cancer risk have produced relatively consistent results showing a reduction in breast cancer risk with increasing level of physical activity. Results to date suggest that moderate-to-strenuous activity may be required for the effect between physical activity and breast cancer risk to be clear; however, clarification of other key details, such as the importance of timing and intensity of activity or variation in effects by tumor characteristics, is pending.

Adult obesity and adult weight gain have both been associated with increased breast cancer risk among postmenopausal women, especially among women who were not current users of menopausal hormone therapy.[4,26,27] Most studies among postmenopausal women show a 1.5- to 2-fold increase in risk of invasive breast cancer when comparing the most obese women or those with the largest weight gain to normal-weight women (BMI: 18.5 to 24.9 kg/m^2) or those with the least weight gain.[4] Paradoxically, overweight or obese premenopausal women have a slightly decreased risk of breast cancer compared with normal-weight or thinner women. Whether larger waist circumference is more important than BMI has been studied in order to separate overall weight gain from abdominal obesity (i.e., visceral fat, which is one element of metabolic syndrome); however, most studies have reported a null association between waist circumference, used as a surrogate for visceral fat, and risk of postmenopausal breast cancer after adjustment for BMI.[26] In contrast to the results for postmenopausal women, waist circumference and a positive association with premenopausal breast cancer was found after adjustment for BMI.[26] A recent analysis of the Nurses' Health Study suggests that self-rated body fatness during youth and BMI at age 18 years are both inversely associated with breast cancer risk, with similar results for premenopausal and postmenopausal breast cancer.[28]

Hormones are central to the discussion of biologic mechanisms linking both physical activity and obesity with breast cancer risk. Physical activity can alter menstrual cycle patterns in premenopausal women, and hormone profiles in both premenopausal and postmenopausal women. Physical activity may lower body fat among children,[29] which in turn may delay age at menarche.[30] Later age at menarche has been associated with reduced breast cancer risk.[31] Physical activity may reduce the frequency of ovulatory cycles.[32] Having less frequent and therefore fewer cumulative ovulatory cycles is likely to reduce the lifetime exposure of the breast to endogenous ovarian hormones,[31] which are proven proliferative agents.[33] Physical activity also can have a direct impact on circulating estrogen levels among postmenopausal women.[34]

In the postmenopausal period, adipose tissue is the primary source of endogenous hormones via aromatization of androstenedione to estrone.[35] Thus, heavier postmenopausal women have higher levels of circulating estrogen than women with less adipose tissue. The involvement of estrogen in the relationship between obesity and breast cancer risk is supported by the observation that obesity does not independently increase breast cancer risk among menopausal hormone therapy users[27]; the obesity-related increase in estrogen over that provided by exogenous estrogens is negligible. The breast tissue of overweight or obese perimenopausal and postmenopausal women with relatively high risk of breast cancer has been shown to have cytologic abnormalities and higher epithelial cell counts than that of normal-weight women.[36] In contrast, obese premenopausal women experience menstrual cycle disturbances, including anovulatory cycles and secondary amenorrhea, thereby lowering their cumulative exposure to estradiol and progesterone.[31] A possible explanation for the inverse association between youth body fatness and breast cancer risk is that youth body size is inversely associated with adult IGF-1 levels.[28]

Other likely mechanisms that may link physical activity[37,38] and obesity[39,40] with breast cancer risk include aspects of immune function, inflammatory mechanisms, oxidative stress and DNA repair capability, metabolic hormones, and growth factors.

COLON AND RECTAL CANCER

An inverse association between physical activity and colon cancer risk has been consistently observed among epidemiologic studies; however, the evidence for rectal cancer remains inconclusive. Historically, comprehensive reviews have estimated that physical activity may reduce colon cancer risk by 20% to 25% when comparing individuals with the highest levels to those with the lowest levels of activity.[41] Risk reductions are greater for case-control studies (24%) than for cohort studies (17%), and risk reductions for occupational activity (22%) and recreational activity (23%) are similar.[41] In cohort studies, colon cancer risk reduction associated with physical activity is greater for men than for women, which

may be due to the influence of hormone therapy on colon cancer risk,[42] although case-control studies suggest similar benefits for men and women.[43]

Whether physical activity preferentially protects against proximal or distal colon cancer is of interest. A meta-analysis including 21 cohort and case-control studies that examined associations between physical activity and the risks of proximal colon and distal colon cancers produced results suggesting that physical activity is associated with a reduced risk of both proximal colon and distal colon cancers, and that the magnitude of the association does not differ by subsite.[44]

Although the majority of previous studies have not found an association between physical activity and rectal cancer,[41] the National Institutes of Health (NIH)–AARP Diet and Health Study observed a modest reduction in rectal cancer risk for men but not for women after 6.9 years of follow-up.[45] Further, in a case-control study conducted in Australia, rectal cancer risk was reduced among men but not among women who participated in vigorous recreational physical activity averaging at least 6 metabolic equivalent task (MET)-hours per week during their adult years.[46]

An emphasis has been made on trying to identify risk factors for colon adenomas, which are considered precursor lesions for colon cancer; these are detected and removed during colonoscopy or sigmoidoscopy. Wolin et al. conducted a meta-analysis of 20 studies published through April 2010 that investigated the association between recreational physical activity and colon adenomas.[47] Adenoma risk was reduced by 19% among men and by 13% among women and, when combining men and women, the inverse association with physical activity was strongest for large/advanced polyps.

Obesity is an established risk factor for colon cancer in both men and women, although the relative risks for men have been higher than those for women.[4,26] The adverse impact of being overweight or obese on colon cancer risk is stronger for distal than for proximal colon cancers. In addition, visceral adiposity appears to confer greater risk than general adiposity.[26] In the European Prospective Investigation into Cancer and Nutrition (EPIC) study, abdominal obesity as well as adult weight gain were strongly associated with colon cancer risk in both men and women.[48,49] No association between these adiposity measures and colon cancer risk was evident among postmenopausal women who had used menopausal hormone therapy, and no association was observed between any measure of adiposity and rectal cancer risk.[48] The positive association between obesity and risk of colon cancer was further supported by the findings that both general obesity and abdominal obesity increase the risk of colon adenomas[47] with one study of women indicating that the distal colon is the main target site.[50]

Given that a higher BMI and lack of physical activity are both risk factors for colon cancer, several statistical approaches have been employed to tease apart their joint and independent effects on colon cancer risk. In the Netherlands Cohort Study,[51] colorectal cancer risk was increased at each subsite among larger women in the lowest recreational activity category (<30 minutes per day) than in smaller women in the highest recreational activity category (>90 minutes per day); however, the interaction between physical activity and body size was statistically significant only for proximal tumors. Using different fatness measures for men, the only similar finding was that men with low levels of physical activity whose trouser size was below the median of that for the cohort had an increased risk of distal colon cancer; no differences in risk were noted for other subsites or for men with larger trouser sizes.[51]

The mechanisms explaining the relationship between physical activity and colon cancer are not clearly established, but include the impact on insulin sensitivity and IGF profiles, and inflammation, as well as some colon-specific mechanisms. Physical activity may stimulate stool transit in the colon, thereby decreasing the exposure of colonic mucosa to carcinogens in the stool.[6] Alternatively, physical activity–induced decreases in prostaglandin E_2 may decrease colonic cell proliferation rates and increase colonic motility.[6] In addition to steroid hormones, which have been clearly implicated as biologic modifiers of the effect of physical activity and obesity on colon cancer risk, the insulin and IGF pathways may mediate the associations between these exposures and colon cancer risk. For obesity in particular, the link can be inferred because obesity can lead to insulin resistance,[52] a syndrome characterized by high circulating insulin levels. High insulin levels appear to promote cell proliferation and tumor growth in the colon[7] and may also suppress the expression of IGF-binding proteins 1 and 2, leading to increased bioavailable IGF-1 levels.[53] Another possible mechanism is obesity-enhanced inflammation in which increases in adipose tissue macrophages lead to the secretion of inflammatory cytokines associated with colon cancer risk (e.g., tumor necrosis factor [TNF]-α, monocyte chemoattractant protein [MCP]-1, and interleukin [IL]-6).

ENDOMETRIAL CANCER

The evidence for an association between physical activity and endometrial cancer risk is accumulating[4,54–58] but is not definitive. A meta-analysis of prospective cohort studies results published through 2009 indicates that recreational physical activity lowers endometrial cancer risk by 27%, and occupational activity lowers risk by 21%.[59] Adjustments for BMI minimally change relative risk estimates, suggesting that physical activity is independently associated with endometrial cancer. Although physical activity is associated with a decreased risk of endometrial cancer in both normal-weight and obese women, two recent studies have suggested that this association is more pronounced for obese women.[54,58]

Two meta-analyses of the association between physical activity and endometrial cancer have identified some inconsistencies in dose-response relationships, indicating the importance of differences in activity type and intensity.[55,56] Little evidence exists on how long-term or lifetime physical activity and activity patterns during different life periods might influence endometrial cancer risk; it has been suggested that recent or long-term activity might be more important than activity at early ages.[56] In the CTS, higher levels of recent (at cohort formation) strenuous recreational physical activity was associated with lower levels of endometrial cancer risk; among women exercising >3 hours per week per year, risk was approximately 25% lower than that of women exercising <0.5 hour per week per year.[60] This inverse association was limited to overweight and obese women (BMI ≥25 kg/m^2). Finally, sitting time has been independently associated with increased endometrial cancer risk.[59]

Epidemiologic studies have established a strong association between obesity and endometrial cancer risk.[26] Recent studies have suggested a linear trend between increasing body weight or BMI and increasing endometrial cancer risk among postmenopausal women, whereas among premenopausal women, no trend is observed, but rather, only obese women have an increased risk.[26] Furthermore, the strong association among postmenopausal women is only observed among those who are not using hormone therapy.[26] Finally, BMI appears to exert an effect on the risk of endometrial cancer that is independent of physical activity.[55]

Physical activity and obesity are likely to influence endometrial cancer risk by altering endogenous hormone profiles.[31,53] Heavier postmenopausal women have higher circulating levels of estrogen than do lighter postmenopausal women because of the aromatization of androstenedione to estrone in adipose tissue. This is pertinent to endometrial cancer risk because this aromatization occurs in the absence of progesterone, which opposes the proliferative effects of estrogen on endometrial tissue. Physical activity may counter the proliferative effects of estrogen either directly or by restricting weight gain. Some evidence also links elevated insulin levels and diabetes to endometrial cancer risk.[61] Physical inactivity and obesity play a role in the development of insulin insensitivity and diabetes, providing another mechanism by which they may influence endometrial cancer risk.

ADENOCARCINOMA OF THE ESOPHAGUS

Several case-control studies[62–64] and one cohort study[65] have examined the association between physical activity and risk of adenocarcinoma of the esophagus. Zhang et al.[62] reported a modest association between participation in recreational physical activity more than once per week and a decreased risk of all esophageal cancer (adenocarcinomas and squamous cell tumors), although the result was not statistically significant. Lagergren et al.[63] reported no association between total, usual recreational and occupational physical activity and esophageal adenocarcinoma. Vigen et al.[64] showed that lifetime occupational physical activity was modestly associated with a lower risk of adenocarcinoma of the esophagus: the average annual level of occupational physical activity before age 65 years was associated with an approximately 40% reduction in risk of esophageal adenocarcinoma when the highest was compared with the lowest occupational physical activity category. Results from the NIH–AARP Diet and Health Study also support the hypothesis that physical activity lowers the risk of esophageal adenocarcinoma, but no association between physical activity and the risk of squamous cell esophageal cancer was found.[65]

Obesity is strongly associated with an increased risk of esophageal adenocarcinoma.[66,67] A pooled analysis of existing data showed that individuals with severe obesity (BMI ≥40 kg/m^2) had a 4.8-fold greater risk than individuals who were not overweight (BMI <25 kg/m^2), with similar risk estimates for men and women.[68] Several studies have examined the effect of abdominal adiposity, which have suggested that the risk associated with obesity is driven primarily by abdominal fatness.[26]

It is likely that obesity impacts esophageal adenocarcinoma risk because it is associated with the risk of gastroesophageal reflux disease (GERD). GERD may cause changes in the esophageal epithelium, leading to Barrett esophagus, a well-established precancerous condition for esophageal adenocarcinoma. On the other hand, obesity is associated with a systemic inflammatory state, which includes the exposure to adipocytokines and procoagulant factors released by adipocytes in central fat, which may also contribute to the development of esophageal adenocarcinoma.[67] Physical activity may influence the risk of esophageal adenocarcinoma by increasing digestive track transit time, thus reducing exposure of the esophagus to putative cancer-causing agents.

KIDNEY/RENAL CELL CANCER

Physical activity has been studied in relation to renal cell carcinoma in part because of the known deleterious effects of high BMI and hypertension on the risk of renal cell cancer; however, no association has been firmly established. A review of physical activity and risk of genitourinary cancers noted significant protective effects in 8 of 15 studies of physical activity in relation to renal cell carcinoma, with an average 8% reduction in risk when comparing individuals with the highest level of physical activity to those with the lowest level of activity.[69] Reductions in risk were greater for recreational than for other forms of activity and for activity performed later in life.

Obesity, in addition to high blood pressure and diabetes, is an established risk factor for kidney cancer.[26] It is still uncertain whether a gender difference exists, however. A meta-analysis has suggested a similar impact of BMI on kidney cancer risk among women and men, with an approximate 7% increase in risk per unit increase in BMI.[26] The effect of obesity may differ by histology; a recent study reported an increased risk observed for clear cell and chromophobe cancers, but not papillary renal cell cancer.[70]

PANCREATIC CANCER

Pancreatic cancer is generally diagnosed at an advanced stage and is associated with high mortality rates. A meta-analysis of 28 studies of pancreatic cancer showed that higher total lifetime physical activity and occupational activity were associated with a lower risk.[71] Nonsignificant reductions in risk were observed for recreational physical activity and transportation (walking and cycling as a form of commuting). Significant heterogeneity was present across the studies, making it difficult to find a definitive answer.

Evidence indicating that obesity is a risk factor for pancreatic cancer is convincing. Three large pooled analyses and three of four meta-analyses that encompass a range of well-designed, independent observational epidemiologic studies have demonstrated a positive association between obesity and pancreatic cancer risk.[72,73] Effects were relatively consistent across studies, with an approximate 10% or greater increase in risk for every 5 kg/m^2 increase in BMI. Two of the pooled analyses and one of the meta-analyses assessed measures of adiposity such as waist circumference or waist-to-hip ratio (WHR); each of the results suggested positive associations with pancreatic cancer risk.[72,74,75] The pooled analyses reported at least a 35% greater risk when the fourth quartile of WHR was compared to the first quartile. The meta-analysis study reported an 11% increase in risk associated with each 10-cm increase in waist circumference and a 19% increase in risk for each 0.1-unit increment in WHR.

GALLBLADDER CANCER

Gallbladder cancer occurs more frequently in women than in men, and the major risk factor is a history of gallstones,[10] which has been associated with the use of exogenous estrogens.[76] To date, we have found no epidemiologic literature investigating the possible association of physical activity and gallbladder cancer, although several studies have suggested a positive association between obesity and gallbladder cancer. In a meta-analysis comprised of 3,288 cases derived from eight cohort studies and three case-control studies, obesity was associated with a 66% increased risk of gallbladder cancer, and the increase in risk was larger for women than for men.[77] Further, two studies found that WHR was positively associated with gallbladder cancer risk among men and women with and without a history of gallstones, suggesting that abdominal obesity may be important in the etiology of this disease.[78,79]

NON-HODGKIN LYMPHOMA

Studies addressing physical inactivity and obesity as potential risk factors for NHL have been mixed, in part because they have not had a sufficient number of cases to assess risk by NHL subtype. Generally, studies have shown no overall association between physical activity and NHL risk.[4] The results of four cohort studies, the CTS,[80] WHI,[81] EPIC,[82] and the American Cancer Society Prevention Study-II[83] have been unconvincing, with WHI showing a nonstatistically significant positive association, whereas the other studies showed no association.

In 2008, the International Lymphoma Epidemiology Consortium (InterLymph) published a pooled analysis of 18 case-control studies with more than 10,000 cases reporting no association between BMI around the time of diagnosis and NHL risk overall, but an increased risk of diffuse NHL for severe obesity (BMI ≥40 kg/m^2).[84] The results from meta-analyses of cohort studies suggested a weak positive association overall and for diffuse NHL.[85,86] An analysis of two cohort studies has suggested that body size in early adulthood may be more predictive of NHL risk than that later in life for all NHL and for the diffuse and follicular subtypes.[87]

PROSTATE CANCER

More than 20 studies have assessed the potential association between physical activity and prostate cancer.[4,88,89] Regardless of the

different approaches used, the populations studied, or the sample sizes of the studies, the majority of studies have suggested a modest reduction in risk with an increased level of physical activity.[4] In a review of the literature, Friedenreich and Orenstein[88] concluded that prostate cancer risk is reduced 10% to 30% when comparing the most active with the least active men and suggested that it may be high levels of physical activity earlier in life that are most relevant to this disease. An update to this review, based on 22 additional studies, indicates that the majority of recent research studies observed protective effects.[90] Leitzmann and Rohrmann[91] added that the associations with reduced risk may be most apparent for fatal prostate cancer. A current systematic review and meta-analysis, including 19 cohort and 24 case-control studies, agrees.[92] A pooled 19% reduction in risk was observed for occupational physical activity, and a 5% reduction was observed for recreational physical activity comparing the most physically active men to the least active.[92] An issue that somewhat reduces our confidence in these estimates is that considerable heterogeneity between studies was observed. Further, it is not yet clear whether these results reflect a true causal association or whether they are due to confounding by prostate-specific antigen testing, which may be more common among physically active men.

The early epidemiologic literature on the potential association between obesity and prostate cancer provided no consistent evidence of any relationship.[4] Recent studies have suggested that obesity may have a dual effect on prostate cancer risk. One meta-analysis reported that the risk of early-stage prostate cancer decreased by 6%, whereas the risk of advanced prostate cancer increased 9% per 5-kg/m^2 increase in BMI.[93] Another possibility is that obesity may decrease the likelihood of diagnosis of less aggressive prostate cancer. Proposed mechanisms include the paradoxical effects of testosterone on low-grade versus more advanced prostate cancer and alterations in insulin and circulating IGF-1.[94]

LUNG CANCER

Physical activity may reduce lung cancer risk by 30% to 40%,[88] but no definitive conclusion can be drawn because one cannot ignore potential residual confounding or effect modification due to smoking as an explanation for any observed association. Recent studies have attempted to address this issue by estimating risk within subgroups defined by smoking status. A recent review suggests an inverse relationship between heavy lifetime physical activity and lung cancer in former and current smokers that is consistent across all histologies, but is not observed among never smokers.[5] A small case-control study of current and former smokers enrolled in the Cologne Smoking Study came to a similar conclusion, observing a lower risk of lung cancer among participants who were physically active compared to those who were not.[95] In the large NIH–AARP Diet and Health Study, no associations were observed between occupational or recreation physical activity and lung cancer risk among those who never smoked.[96]

Due to sex differences in lung cancer pathology, risk factors, and prognosis, current research has also begun to investigate the association for men and women separately.[97] The recent literature consists of small case-control studies,[98] which lack statistical power to examine risks in subgroups defined by histology, smoking status, or sex, and which may be affected by survival bias in that rapidly fatal cases or those who are too ill to be interviewed are excluded from the study population.

Several studies have suggested the existence of an inverse association between increasing BMI and lung cancer risk.[99–102] Nevertheless, this inverse effect may have been due to residual confounding by smoking because the inverse association was restricted to ever smokers. One meta-analysis showed an inverse association between BMI and lung cancer in nonsmokers[103]; however, caution should be exercised when interpreting the results due to concerns about heterogeneity of risk estimates across studies, the quality of the original studies, and confounding by smoking.[104]

OVARIAN CANCER

The literature on ovarian cancer risk in relation to physical activity and obesity has been inconclusive. More than 18 studies have assessed the impact of physical activity on ovarian cancer risk. A meta-analysis of 12 studies found an approximate 20% decrease in ovarian cancer risk associated with physical activity when the highest category of exercise was compared to the lowest.[105] Four[106–109] of five[110] additional studies found no association; the fifth study found a nonsignificant 10% to 20% reduction in ovarian cancer risk for women who participated in at least 1 hour per week of recreational aerobic activity.

The evidence for an association between obesity and increased ovarian cancer risk is weak, with few studies showing a statistically significant result.[4,111] A meta-analysis of 16 studies indicated that adult obesity increases the risk for ovarian cancer; the overall pooled effect estimate was a 30% increase in ovarian cancer risk associated with adult obesity with a possible dose-response effect, but no variation in risk estimates across histologic subtypes.[111] In contrast, the results from the Ovarian Cancer Association Consortium, based on original data from 15 case-control studies, suggest that obesity only increases the risk of the less common histologic subtypes of ovarian cancer; obesity does not increase risk of high-grade invasive serous cancers, the most common subtype.[112] A pooled analysis of 12 cohort studies reported that BMI was not associated with ovarian cancer risk in postmenopausal women, but was positively associated with risk in premenopausal women.[113] Another meta-analysis, using 47 studies, showed that the positive association between BMI and ovarian cancer was restricted to women who had never used hormone therapy; among these women, risk increased by 10% with every 5 kg/m^2 increase in BMI.[114]

CONCLUSIONS

Table 10.1 illustrates the strength of evidence regarding increased physical activity as a protective factor and obesity as a risk factor

TABLE 10.1

Summary of the Strength of the Observational Epidemiologic Evidence for Physical Activity as a Protective Factor and Obesity as a Risk Factor for Cancer, By Type of Cancer

	Physical Activity	Overweight/ Obesity
Breast, postmenopausal	+++	+++
Breast, premenopausal	++	++ (protection)
Colon	+++	+++
Endometrium	+	+++
Esophagus, adenocarcinoma	?	+++
Kidney/renal cell	?	+++
Gallbladder	?	++
Pancreas	?	+++
Non-Hodgkin lymphoma	?	+
Prostate, aggressive	+	+
Lung	+	?
Ovary	?	?

+++, evidence is convincing; ++, evidence is probable; +, evidence is possible; ?, evidence remains insufficient/inconclusive.

for cancer. The strength of evidence for each exposure is classified as convincing (+++), probable (++), possible (+), or insufficient and inconclusive (?). Overall, for physical activity, convincing evidence exists for an association with postmenopausal breast cancer and colon cancer; for obesity, the evidence is convincing for breast, colon, endometrial, esophageal, and kidney/renal cell cancer. Evidence for associations between these exposures and several other cancer sites is accumulating. Despite some convincing evidence of the effects of physical activity and obesity on the risk of certain cancers, it is difficult to make recommendations as to appropriate changes in lifestyle that will reduce a person's chances of developing cancer. We have no physical activity prescriptions to give at this time. Many questions remain to be answered: What are the ages at which physical activity will provide the most benefit? What types of activity should one do and at what intensity, frequency (times per week), and duration (hours per week)? Similarly, for BMI, is there some threshold below which the individual will not have excess cancer risk? Does purposeful weight loss during the adult years lower the risk associated with being overweight or obese? Finally, necessary research is ongoing to identify the biologic mechanisms that account for these effects and to determine whether all persons are affected equally. For instance, it is possible that genetically defined subgroups of the population respond to physical activity or obesity differently. Understanding mechanisms and population variation in these effects will illuminate appropriate prescriptions for lifestyle change.

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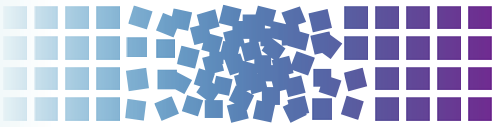
11 Epidemiologic Methods



Xiaomei Ma and Herbert Yu

INTRODUCTION

Epidemiology is the study of the distribution and determinants of health-related states or events in specified populations and the application of this study to control health problems.[1] Epidemiologic principles and methods have long been applied to cancer research, with the assumptions that cancer does not occur at random and the nonrandomness of carcinogenesis can be elucidated through systematic research. An example of such applications is the lung cancer study conducted by Doll and Hill in the early 1950s, which linked tobacco smoking to an increased mortality of lung cancer in over 40,000 medical professionals in the United Kingdom.[2] The observation from this study and many other studies, in conjunction with laboratory findings regarding the underlying biologic mechanisms for the effect of tobacco smoking, helped establish the role of tobacco smoking in the etiology of lung cancer. Epidemiologic methods are also used in clinical settings, where trials are conducted to evaluate the efficacy of new treatment protocols or preventive measures and where observational studies of prognostic factors are done.

Epidemiologic studies can take different forms, but generally they can be classified into two broad categories, observational studies and experimental studies (Fig. 11.1). In experimental studies, an investigator allocates different study regimens to the subjects, usually with randomization (experimental studies without randomization are sometimes referred to as "quasi-experiments").[3] Experimental studies can be individual based or community based. An experimental study most closely resembles laboratory experiments in that the investigator has control over the study condition. Experimental studies can be used to evaluate the efficacy of a treatment protocol (e.g., low-dose compared with standard-dose chemotherapy for non-Hodgkin's lymphoma)[4] or preventive measures (e.g., tamoxifen for women at an increased risk of breast cancer).[5] Although experimental studies are often considered the "gold standard" because of well-controlled study situations, they are only suitable for the evaluation of effects that are beneficial or at least not harmful due to ethical concerns. Experimental studies are discussed in detail in other chapters of this book. This section will focus on observational studies.

Observational studies do not involve the artificial manipulation of study regimens. In an observational study, an investigator stands by to observe what happens or happened to the subjects, in terms of exposure and outcome. Observational studies can be further divided into descriptive and analytical studies (see Fig. 11.1). Descriptive studies focus on the *distribution* of diseases with respect to person, place, and time (i.e., who, where, and when), whereas analytical studies focus on the *determinants* of diseases. Descriptive studies are often used to *generate* hypotheses, whereas analytical studies are often used to *test* hypotheses. However, the two types of studies should not be considered mutually exclusive entities; rather, they are the opposite ends of a continuum. Descriptive studies are discussed in detail in other chapters of this book.

ANALYTICAL STUDIES

Ecologic Studies

As in experimental studies, the unit of analysis can be individuals or groups of people in observational studies. Studies that use groups of people as the unit of analysis are called ecologic studies, which are relatively easy to carry out when group level measures are available. However, a relationship observed between variables on a group level does not necessarily reflect the relationship that exists at an individual level. For example, the fraction of energy supply from animal products was found to be positively correlated with breast cancer mortality in a recent ecologic study, which used preexisting data on both dietary supply and breast cancer mortality rates from 35 countries.[6] Because the data were country based, no reliable inference can be made at an individual level. Within each country, it could be that the people who had a low fraction of energy supply from animal products were actually dying from breast cancer. Results from ecologic studies are useful for inference at an individual level only when the within-group variability of the exposure is low so that a group-level measure can reasonably reflect exposure at an individual level. Alternatively, if the implications for prevention or intervention are at a group level (e.g., taxation of cigarettes to reduce smoking), results from ecologic studies are very useful.

Cross-Sectional Studies

There are three main types of analytical studies in which the unit of analysis is individuals: cross-sectional, cohort, and case-control studies. In a cross-sectional study, the information on various factors is collected from the study population at a given point in time. From a public health perspective, data collected in cross-sectional studies can be of great value in assessing the general health status of a population and allocating resources. For example, the National Health and Nutrition Examination Survey has provided valuable national estimates of health and nutritional status of the US civilian, noninstitutionalized population.[7] Findings from cross-sectional studies can also help generate hypotheses that may be tested later in other types of studies. However, it should be noted that cross-sectional studies have serious methodologic limitations if the research purpose is etiologic inference. Because exposures and disease status are evaluated simultaneously, it is usually not possible to know the temporality of events unless the exposure cannot change over time (e.g., blood type, skin color, race, country of birth). If one observes that more brain cancer patients are depressed than people without brain cancer in a cross-sectional study, the correlation does not necessarily mean that depression causes brain cancer. Depression may simply have resulted from the pathogenesis and diagnosis of brain cancer, or depression may

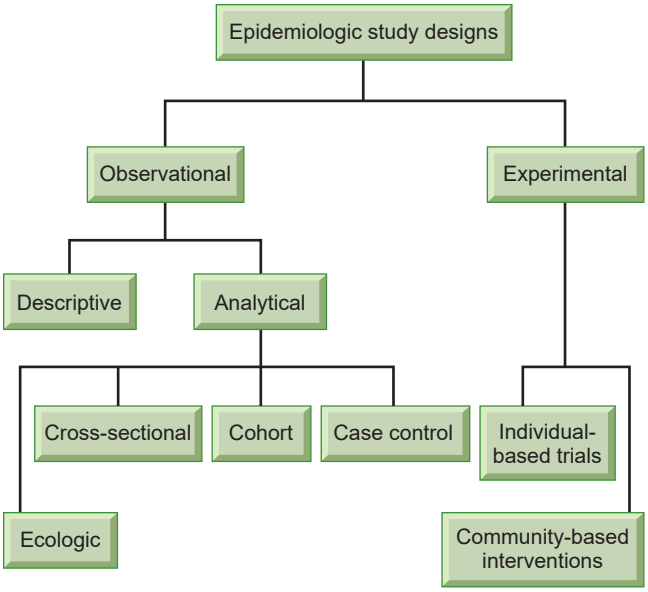


Figure 11.1 Classification of epidemiologic study designs.

have caused brain cancer in some patients and resulted from brain cancer in other patients. Without additional information on the timing of events, no conclusions can be made. Another concern in cross-sectional studies is the enrollment of prevalent cases, who survived different lengths of time after the incidence of disease. Factors that affect survival may also influence incidence. Prevalent cases may not be representative of incident cases, which makes etiologic inferences based on cross-sectional studies suspect at best.

Cohort Studies

In a cohort study, a study population free of a specific disease (or any other health-related condition) is grouped based on their exposure status and followed up for a certain period of time. Then the exposed and unexposed subjects are compared with respect to disease status at the end of the follow-up. The objective of a cohort study is usually to evaluate whether the incidence of a disease is associated with an exposure. The cohort design is fundamental in observational epidemiology and is considered "ideal" in that, if unbiased, cohort data reflect the real-life cause/effect sequence of disease.[8] Subjects in cohort studies may be a sample of the general population in a geographic area, a group of workers who are exposed to certain occupational hazards in a specific industry, or people who are considered at a high risk for a specific disease. A cohort study is considered prospective or concurrent if the investigator starts following up the cohort from the present time into the future, and retrospective or historical if the cohort is established in the past based on existing records (e.g., an occupational cohort based on employment records) and the follow-up ends before or at the time of the study. Alternatively, a cohort study can be ambidirectional in that data collection goes both directions.[9] Whether a cohort study is prospective, retrospective, or ambidirectional, the key feature is that all the subjects were free of the disease at the beginning of the follow-up and the study tracks the subjects from exposure to disease. Follow-up time, ranging from days to decades, is an essential element in cohort studies.

In a cohort study, the incidence of disease in the exposed group and the unexposed group is compared. The incidence measure can be cumulative incidence or incidence density, depending on the availability of data. When comparing the incidence in the two groups, both relative differences and absolute differences can be assessed. In cohort studies, the relative risk of developing the disease is expressed as the ratio of the cumulative incidence in the exposed group to that in the unexposed group, which is also called cumulative incidence ratio or risk ratio. If we have data on the exact person-time of follow-up for every subject, we can also calculate an incidence density ratio (also called rate ratio) in a similar way. The numeric value of the risk or rate ratio reflects the magnitude of the association between an exposure and a disease. For example, a risk ratio of 2 would be interpreted as exposed individuals have a doubled risk of developing a disease than unexposed individuals, whereas a risk ratio of 5 indicates that exposed individuals have 5 times the risk of developing a disease compared with unexposed individuals. To put in another way, a factor with a risk ratio of 5 has a stronger effect than another factor with a risk ratio of 2. In addition to risk ratio and rate ratio, another relative measure called probability odds ratio can be calculated in cohort studies. The probability odds of disease is the number of subjects who developed a disease divided by the number of subjects who did not develop the disease, and the probability odds ratio is the probability odds in the exposed group divided by the probability odds in the unexposed group. Many investigators prefer risk ratio or rate ratio to probability odds ratio in cohort studies, because the ability to directly measure the risk of developing a disease is one of the most significant advantages in cohort studies. In practice, however, a probability odds ratio is often used as an approximation for risk or rate ratio, especially when multivariate logistic regression models are employed to adjust for the effect of other factors that may influence the relationship between an exposure and a disease.

As for absolute differences, a commonly used measure is called attributable risk in the exposed, which is the incidence in the exposed group minus the incidence in the unexposed group. Attributable risk reflects the disease incidence that could be attributed to the exposure in exposed individuals and the reduction in incidence that we would expect if the exposure can be removed from the exposed individuals, provided that there is a causal relationship between the exposure and the disease. Another absolute measure called population attributable risk extends this concept to the general population; it estimates the disease incidence that could be attributed to an exposure in the general population. Because both relative and absolute differences can be assessed in cohort studies, a natural question to ask is what measures to choose. In general, the relative differences are used more often if the main research objective is etiologic inference, and they can be used for the judgment of causality. Once causality is established, or at least assumed, measures of absolute differences are more important from a public health perspective. This point can be illustrated using the following hypothetical example. Assume the following: toxin X in the environment triples the risk of bladder cancer and toxin Y doubles the risk of bladder cancer, the effects of X and Y are entirely independent of each other, the prevalence of exposure to toxin Y in the general population is 20 times higher than the prevalence of exposure to toxin X, and there are only resources available to reduce the exposure to one toxin. It would be more effective to use the resources to reduce the exposure to toxin Y instead of toxin X. This is because the population attributable risk due to Y is higher than that due to X, although the risk ratio associated with toxin Y is smaller than that associated with toxin X.

Cohort studies have many advantages. A cohort design is the best way to study the natural history of a disease.[9] There is usually a clear temporal relationship between an exposure and a disease because all the subjects are free of the disease at the beginning of the follow-up (it can be a problem if a subject has a subclinical disease such as undetected prostate cancer). Furthermore, multiple diseases can be studied with respect to the same exposure. On the other hand, cohort studies, especially prospective cohort studies, are costly in terms of both time and money. A cohort design requires the follow-up of a large number of study participants over a sometimes extremely lengthy period of time and usually extensive data collection through questionnaires, physical measurements, and/or biologic specimens at regular intervals. Participants may be

"lost" during the follow-up because they became tired of the study, moved away from the study area, or died from some causes other than the disease under study. If the subjects who were lost during the follow-up are different from those who remained under observation with respect to exposure, disease, or other factors that may influence the relationship between the exposure and the disease, results from the study may be biased. To date, cohort studies have been used to study the etiology of a wide spectrum of diseases, including different types of cancer. If a cohort study is conducted to evaluate the etiology of cancer, usually the study sample size would need to be very large (such as the National Institutes of Health-AARP Diet and Health Study, which included more than half million subjects[10]) and the follow-up time would need to be long, unless the cohort selected is a high-risk population.

For simplicity, we have discussed cohort studies in which the outcome of interest is the incidence of a specific disease and there are only two exposure groups. In practice, any health-related event can be the outcome of interest, and multiple exposure groups can be compared.

Case-Control Studies

Case-control design is an alternative to cohort design for the evaluation of the relationship between an exposure and a disease (or any other health condition). A case-control approach compares the odds of past exposure between cases and noncases (controls) and uses the exposure odds ratio as an estimate for relative risk. A primary goal in a case-control study is to reach the same conclusions as what would have been obtained from a cohort study, if one had been done.[11] If appropriately designed and conducted, a case-control study can optimize speed and efficiency as the need for follow-up is avoided.[8] The starting point of a case-control study is a source population from which the cases arise. Instead of obtaining the denominators for the calculation of risks or rates in a cohort study, a control group is sampled from the entire source population. After selecting control subjects, who ideally would have become cases had they developed the disease, an investigator collects data on past exposures from both the cases and the controls and then calculates an odds ratio, which is the odds of exposure in the cases divided by the odds of exposure in the controls.

There are two main types of case-control studies: case-based case-control studies and case-control studies within defined cohorts.[8] Some variations of the case-control design also exist. For instance, if the effect of an exposure is transient, sometimes a case can be used as his/her own control (case cross-over design). In case-based case-control studies, cases and controls are selected at a given point in time from a hypothetical cohort (e.g., at the end of follow-up). A cross-sectional ascertainment of cases will result in a case group that mostly contains prevalent cases who may have survived for different lengths of time after disease incidence. Cases who died before an investigator began subject ascertainment would not be eligible to be included in the study. As a result, the cases finally included in the study may not be representative of all the cases from the entire hypothetical cohort. Another disadvantage of enrolling prevalent cases is that cases that were diagnosed a long time ago will likely have difficulties recalling exposures that occurred before the disease incidence. In case-control studies, it is preferable to ascertain incident cases as soon as they are diagnosed and to select controls as soon as cases are identified. Case-control studies that enroll only incident cases are sometimes called *prospective* case-control studies because the investigators need to wait for the incident cases to develop and get diagnosed. For cancer studies, the cases can be ascertained from population-based cancer registries or hospitals. A major advantage of using a cancer registry is the completeness of case ascertainment; however, the reporting of cancer cases to registries is usually not instantaneous. There could be a lag time of several months or even over a year, and some cases could have died during the lag time. If the cancer under study has a poor survival rate and/or clinical specimens need to be obtained in a timely manner, it may be preferable to identify cases directly from hospitals using a rapid ascertainment protocol. As for the selection of controls, the key issue is that controls should be representative of the source population from which the cases arise and, theoretically, the controls would have been ascertained as cases had they developed the disease. The most common types of controls include population-based controls (often selected through random digit dialing in case-control studies of cancer etiology), hospital controls, and friend controls. The advantages and disadvantages of different types of controls have been nicely summarized by Wacholder et al.[12] Because no follow-up is involved in case-based case-control studies, the incidence risk or rate cannot be calculated directly for case and control groups. The odds ratio will be a good estimate of relative risk if the disease is uncommon.

In addition to case-based case-control studies, there are also case-control studies within defined cohorts (also known as hybrid or ambidirectional designs), including case-cohort studies and nested case-control studies. In case-cohort studies, cases are identified from a well-defined cohort after some follow-up time, and controls are selected from the baseline cohort. In nested case-control studies, cases are also identified from a cohort, but controls are selected from the individuals at risk at the time each case occurs (i.e., incidence density sampling).[8] In these types of designs, controls are a sample of the cohort and the controls selected can theoretically become cases at some point. The possibility of selection bias in case-control studies within defined cohorts is lower than that in case-based case-control studies because the cases and the controls are selected from the same source population. Because of an increased awareness of the methodological issues inherent in the design of case-based case-control studies and the availability of a growing number of large cohorts, case-control studies within defined cohorts have become more common in recent years. The advantage of case-control studies within cohorts over traditional cohort studies is mainly the efficiency in additional data collection. For instance, a recent nested case-control study evaluated the relationship between endogenous sex hormones and prostate cancer risk.[13] Instead of measuring the serum hormones levels of the entire cohort (over 12,000 subjects), investigators chose to measure 300 cases and 300 controls selected from the cohort. Doing so not only significantly reduced the cost of measurements and the time it took to address the research question, but also helped preserve valuable serum samples for possible analyses in the future. In a case-cohort design, an odds ratio estimates risk ratio; in a nested case-control design, an odds ratio estimates rate ratio. In both designs, the disease under study does not have to be rare for the odds ratio to be a good estimate of the risk ratio or rate ratio.[8,14]

The biggest advantage of a case-control design is the speed and efficiency of obtaining data. It is claimed that investigators implement case-control studies more frequently than any other analytical epidemiologic study.[15] Because most types of cancer are uncommon and take a long time to develop, to date, most epidemiologic studies of cancer have been case-control instead of cohort in design. A case-control study can be conducted to evaluate the relationship between many different exposures and a specific disease, but the study will have limited statistical power if the exposure is rare. In general, a case-control design tends to be more susceptible to biases than a cohort design. Such biases include, but are not limited to, selection bias when choosing and enrolling subjects (especially controls) and recall bias when obtaining data from the subjects. The status of the subjects—that is, case or control—may affect how they recall and report previous exposures, some of which occurred years or even decades ago. It is important for investigators to explicitly define the diagnostic and eligibility criteria for cases, to select controls from the same population as the cases independent of the exposures of interest, to blind data collection staff to the case or control status of subjects and/or the main hypotheses of the study, to ascertain exposure in a similar manner from cases and controls, and to take into account other

factors that may influence the relationship between an exposure and a disease.[15]

INTERPRETATION OF EPIDEMIOLOGIC FINDINGS

We have discussed measures of effects in various study designs. However, a risk ratio of 3 from a cohort study or an odds ratio of 2.5 from a case-control study does not necessarily mean that there is an association between an exposure and a disease. Several alternative explanations need to be assessed, including chance (random error), bias (systematic error), and confounding. Potential interaction also needs be evaluated.

Statistical methods are required to evaluate the role of chance. A usual way is to calculate the upper and lower limits of a 95% confidence interval around a point estimate for relative risk (risk ratio, rate ratio, or odds ratio). If the confidence interval does not include one, one would say that the observed association is statistically significant; if the confidence interval includes one, one would say that the observed relationship is not statistically significant. The width of a confidence interval is directly related to the number of participants in a study, which is called sample size. A larger sample size leads to less variability in the data, a tighter confidence interval, and a higher possibility in finding a statistically significant association if one truly exists. A 95% confidence interval means that if the data collection and analysis could be replicated many times, the confidence interval should include the correct value of the measure 95% of the time.[16] It is better to consider a confidence interval to be a general guide to the amount of random error in the data but not necessarily a literal measure of statistical variability.[16]

Bias can be defined as any systematic error in an epidemiologic study that results in an incorrect estimate of the association between exposure and disease, and it can occur in every type of epidemiologic study design. There are two main types of bias: selection bias and information bias. Selection bias is present when individuals included in a study are systematically different from the target population. For example, a selection bias would occur if a study aimed to generate a sample representing all women in the United States, but of the women contacted, more with a family history of breast cancer agreed to participate. This sample would be at a higher risk for breast cancer than the target population. Refusal to participate poses a constant challenge in epidemiologic studies. As individuals have become more concerned about privacy issues and as studies have become more demanding of time, biologic specimens, and other impositions, participation rates have dropped substantially in recent years. If nonparticipants are different from the participants with respect to study-related characteristics, the validity of the study is threatened. Information bias occurs when the data collected from the study subjects are erroneous. Information bias is also known as misclassification if the variable is measured on a categorical scale and the error causes a subject to be placed in a wrong category. Misclassification can happen to both exposure and disease. For example, in a case-control study of previous reproductive history and ovarian cancer, a woman who had an extremely early pregnancy loss might not even realize that she was ever pregnant and would mistakenly report no pregnancy, and another woman who has only subclinical presentations of ovarian cancer might be mistakenly selected as a control. Misclassification can be differential or nondifferential. An exposure misclassification is considered differential if it is related to disease status and nondifferential if not related to disease status. Similarly, a disease misclassification is considered differential if it is related to exposure status and nondifferential if not related to exposure status. If a binary exposure variable and a binary disease variable are analyzed, a nondifferential misclassification will result in an underestimate of the true association. Differential misclassification can either exaggerate or underestimate a true effect. Usually not much can be done to control or correct bias at the data analysis stage; therefore, it is important to establish research protocols that are not prone to bias. The evaluation of potential bias is critical to the interpretation of study results. An invalid estimate is worse than no estimate.

Confounding refers to a situation in which the association between an exposure and a disease (or any health-related condition) is influenced by a third variable. This third variable is considered a confounding variable or confounder. A confounder must fulfill three criteria: (1) be associated with the exposure, (2) be associated with the disease independent of the exposure, and (3) not be an intermediate step between the exposure and the disease (i.e., not on the causal pathway). Unlike bias, which is primarily introduced by the investigator or study participants, confounding is a function of the complex interrelationship between various exposures and disease.[17] In a hypothetical case-control study of the effect of alcohol drinking on lung cancer, we may observe an odds ratio of 2.5 (usually called a "crude" odds ratio in the sense that no other variables were taken into account), which indicates that alcohol drinking increases the risk of lung cancer by 1.5-fold. However, if we classify all study subjects into two strata based on a history of cigarette smoking and then calculate the odds ratio in the two strata (smokers and nonsmokers) separately, we may have two stratum-specific odds ratios both equal to one, indicating that alcohol drinking is not associated with lung cancer risk. In this example, the crude odds ratio calculated to estimate the association between alcohol drinking and lung cancer without considering smoking is simply misleading. Being associated with both the exposure (i.e., alcohol drinking) and the disease (i.e., lung cancer), smoking acted as a confounder in this example. A stratified analysis is needed to evaluate the potential confounding effect of a third variable, whether it is done with pencil and paper or statistical modeling. Usually data are stratified based on the level of a third variable. If the stratum-specific effect measures are similar to each other but different from the crude effect measure, confounding is said to be present. In this section, we have illustrated basic epidemiologic principles using an overly simplified scenario and only considered a single exposure. In practice, most if not all diseases, cancer included, have a multifactorial etiology. Consequently, it is usually necessary to assess the potential confounding effect of a group of variables simultaneously using multivariate statistical models. The effect measure derived from a multivariate model will then be called an "adjusted" one in the sense that the effect of other factors was also adjusted for. Without controlling for the potential effect of other variables, an investigator cannot really judge whether an observed association between a given exposure and a specific disease is spurious.

If the effect of an exposure on the risk of a disease is not homogeneous in strata formed by a third variable, the third variable is considered an effect modifier, and the situation is called interaction or effect modification. Put in other words, interaction exists when the stratum-specific effect measures are different from each other. In the lung cancer example given previously, if the odds ratio for alcohol drinking is 1 in smokers but 3 in nonsmokers, then there is interaction and smoking is an effect modifier. The evaluation of interaction is essentially a stratified analysis, which is similar to the evaluation of confounding. Confounding and interaction can be both present in a given study. However, when interaction occurs, the stratum-specific effect measures should be reported. It is no longer appropriate to report a summary measure in the presence of interaction. Unlike confounding, which is a nuisance that an investigator hopes to remove, interaction is a more detailed description of the true relationship between an exposure and a disease.

CANCER OUTCOMES RESEARCH

The discussion of epidemiologic methods in this section focuses primarily on etiological research, which aims at identifying the risk factors of cancer. However, similar principles and methods are applicable to cancer outcomes research, which aims at studying

a variety of factors related to the early identification, treatment, prognosis, health related quality of life, and cost of care. Cancer outcomes research can be experimental or observational in nature. For example, randomized clinical trials have been conducted to assess the impact of screening on prostate cancer mortality[18] and to compare the effect of radical prostatectomy versus observation in patients with localized prostate cancer.[19] Observational studies of cancer outcomes, especially those that build upon preexisting resources,[20,21] can be carried out in a large group of patients with relatively little cost to capture the patterns and cost of care and to address many other research questions that have important clinical implications. Although the findings of such observation studies are subject to bias and confounding inherent in an observational design, these studies are complementary to experimental studies and have their unique value. Given an increasing interest in improving the effectiveness and value of cancer care, more cancer outcomes research is to be expected in the future.

MOLECULAR EPIDEMIOLOGY

Molecular epidemiology involves multidisciplinary and transdisciplinary research that entails not only traditional epidemiology and biostatistics, but also genetics, molecular biology, biochemistry, cellular biology, analytical chemistry, toxicology, pharmacology, and laboratory medicine. Unlike traditional epidemiology research of cancer, which focuses on exposures or risk factors ascertained through questionnaire-based interviews or surveys, molecular epidemiology studies expand the assessment of exposure to a much broader scope that includes an analysis of biomarkers underlying internal exposure of exogenous and endogenous carcinogenic agents or risk factors, molecular alterations in response to exposure, and genetic susceptibility to cancer. The biomarkers often measured in molecular epidemiology research include DNA, RNA, proteins, chromosomes, compound molecules (e.g., DNA and protein adducts), and various metabolites as well as other endogenous and exogenous substances (e.g., steroids, nutrients, chemical or biologic toxins, and phytochemicals). Molecular markers can reflect different aspects of the tumorigenic process, which include biomarkers of internal exposure, biomarkers of molecular or cellular changes in response to exposure, and biomarkers of precursor lesions or early diseases.[22,23] Depending on the source of molecules and location of diseases, surrogates are often used in epidemiologic studies. When using a surrogate marker or tissue, the relevance of a proxy to its underlying target needs to be established or justified.[23] This justification is especially important when conducting population-based epidemiologic studies that focus on organ-specific cancers, because assessing biomarkers in target tissue is difficult for controls; molecular markers from blood samples are often used as substitutes. If a biomarker in the blood does not travel to or act on the tissue or organ of interest, an association between the circulating marker and the cancer may not be relevant. Thus, establishing a close link between a surrogate and its target is crucial in molecular epidemiology research.

Gene-environment interaction plays an essential role in cancer development.[24] Common genetic variations are considered an important determinant of host susceptibility and are a major focus of molecular epidemiology research. Depending on the biologic mechanism involved, genetic variations can influence every aspect of the carcinogenic process, ranging from external and internal exposure to carcinogens or risk factors to molecular and cellular damage, alteration, and response.[22,23] Currently, single nucleotide polymorphisms (SNPs) are the most studied genetic variations. It is believed that even if SNPs confer a small risk, they may still be important at the population level because these variations are common in the general population. It is also important that the impacts of SNPs on cancer are considered under the context of gene–gene and gene–environment interactions. As genotyping technology has advanced substantially with respect to its analytic quality, capacity, and cost, research of genetic polymorphisms has evolved rapidly from investigations of a single SNP to studies of haplotypes and tag SNPs, and from a pathway-based candidate gene approach to genome-wide association studies (GWAS).[25] A GWAS analyzes hundreds of thousands of SNPs simultaneously for hundreds or even thousands of study subjects. When these data are further combined with questionnaire information such as environmental exposures, lifestyle factors, dietary habits, and medical history, enormous information is generated, which requires a huge sample size to allow for a reliable and complete assessment of these variables individually and jointly. A single epidemiologic study can no longer provide sufficient power for this type of investigation. Multicenter investigations or study consortia that pool study information and specimens together are developed to address the sample size issue.[26] False-positive findings resulting from multiple comparisons constitute a major challenge in epidemiologic studies of genetic associations with cancer.[27] A meta-analysis or pooled analysis can be used to address this problem if sufficient studies are already published and available for evaluation. To address this issue at the time of study design, one may adopt a two- or multiphase study design in which study subjects are divided into two or multiple groups for genotyping and data analysis. Selected or genomewide SNPs are first screened in one group of the study subjects (discovery phase), and then the significant findings determined by stringent statistical criteria (usually p values less than 1×10^{-5} or 1×10^{-7}) are reanalyzed in one or several other groups of subjects for verification (validation phase). This study design also lowers the cost of genotyping. False-positive findings can also be addressed with various statistical methods, such as bootstrap, permutation test, estimate of false positive report probability, prediction of false discovery rate, and the use of a much more stringent p value to accommodate multiple comparisons. For epidemiologic studies that are not population based or not conducted strictly following epidemiology principles, population stratification is a potential source of bias that may distort genetic associations.[28]

A large number of GWAS have been completed in search for SNPs that influence host susceptibility to cancer. Considering that more than 5 million SNPs are present in the human genome, the numbers of SNPs that are found to be associated with cancer risk after rigorous validation are much fewer than what one would have anticipated. In addition, the risk associations detected are quite weak, with most of the odds ratios ranging from 1.1 to 1.5, and the functional relevance or biologic implications are unclear for most of the SNPs. Furthermore, not many SNPs associated with cancer risk are located in protein-coding regions, and even fewer are in the loci of candidate genes suspected to be involved in tumorigenesis, such as oncogenes, tumor suppressor genes, DNA repair genes, and xenobiotic metabolizing or detoxification genes. Genes where SNPs are found to be linked to cancer by GWAS include *FGFR2*, *MAP3K1*, *MRPS30*, *LSP1*, *TNRC9*, *TOX3*, *STXBP1*, and *RAD51L1* for breast cancer[29–31]; *JAZF1*, *HNF1B*, *MSMB*, *CTBP2*, and *KLK2/KLK3* for prostate cancer[29,32]; *SMAD7*, *CRAC1*, *EIF3H*, *BMP4*, *CDH1*, and *RHPN2* for colorectal cancer[29,33]; *CHRNA3* and *CHRNA5* for lung cancer[34,35]; *ABO* for pancreatic cancer[36]; *TACC3* and *PSCA* for bladder cancer[37,38]; and *KRT5* for basal cell carcinoma.[39] Among these genes identified by GWAS, two findings are considered especially interesting. One is the association of lung cancer with *CHRNA3* and *CHRNA5*, which encode neuronal nicotinic acetylcholine receptor subunits. Different genotypes of these receptor subunits appear to influence individual's addiction to tobacco, which further leads to different smoking exposure and lung cancer risk.[40,41] Another is the link of the *ABO* gene to pancreatic cancer. The association between pancreatic cancer risk and ABO blood type was observed 50 years ago. The GWAS finding not only confirms the relationship, but also provides new clues for understanding the underlying biologic mechanism.

Besides intragenic SNPs, GWAS also found many intergenic SNPs in association to cancer risk, which include those in the regions of 8q24, 5p15, 1p11, 1p36, 1q42, 2p15, 2q35, 3p12, 3p24,

3q28, 6p21, 6q25, 7q21, 7q32, 9p21, 9p22, 9p24, 9q22, 10p14, 11q13, 11q23, 14q13, 18q23, and 20p12.[29–31,33,39,42–48] Of these loci, SNPs in 8q24 are associated with several cancer sites, including prostate, breast, colon, and bladder.[29,31–33,47–49] Further analysis of 8q24 indicates that there are nine SNPs in five regions and each region is independently related to different types of cancer, with SNPs in regions 1, 4, and 5 associated exclusively with prostate cancer, a SNP in region 2 related to breast cancer, and SNPs in region 3 linked to prostate, colon, and ovarian cancers.[50] No known genes are located within the region of 8q24, but an oncogene *c-MYC* resides about 330 kb downstream of the region.[51] An initial investigation found no evidence of the SNPs' influence on *c-MYC* expression,[47] but a later study suggests that the SNPs in 8q24 may be distal enhancers of *c-MYC*, interacting with its promoter through a chromatin loop.[52] Another genomic region that is associated with the risk of multiple cancer sites is 5p15, a region involving telomerase reverse transcriptase (TERT) cleft lip and palate transmembrane protein 1–like protein (CLPTM1L). Five types of cancer are found to be linked to this region, including basal cell carcinoma, lung, bladder, prostate, and cervical cancers.[53] TERT extends the length of telomere and is associated with cell proliferation and abnormal telomere maintenance.[54] The risk alleles of TERT are associated with shorter telomere length among the elderly and with higher DNA adduct in the lungs.[53,55]

GWAS has demonstrated its value in identifying disease-related SNPs in unknown regions of the genome, which provides new clues for investigators to interrogate and understand different regions of the human genome, especially in the gene-desert areas. Despite the strength, the low yield of significant findings from the GWAS has raised concerns in several areas, including the SNP coverage in the genome (rare SNPs and SNP representativeness in unknown regions), associations with low statistical significance (p value between 0.01 and 1×10^{-5}, the GWAS cutoff), other forms of genetic variations (copy number variation and other structural variations), cancer subtypes, and genetic interplay with environmental factors (gene–environment interaction).[56,57] To address these issues, investigators propose to perform fine-mapping and resequencing to examine genetic regions more specifically and meticulously. Epidemiologists suggest that detailed environmental exposure and lifestyle factors should be included in the next wave of GWAS. Furthermore, to make the study more reliable and compelling, DNA specimens, instead of convenient samples, should come from well-designed and well-executed epidemiologic studies that pay close attention to the selection of study subjects and the measurement of environmental and lifestyle factors to eliminate or minimize selection bias and measurement errors.

As described earlier, analytical epidemiology has two major study designs: the case-control study and the cohort study. It is important that investigators choose an appropriate study design to investigate molecular markers in epidemiologic studies. Two types of molecular markers, genotypic and phenotypic markers, can be considered. Genotypic markers refer to nucleotide sequences of genomic DNA, and all other molecules are considered phenotypic markers, including most of the chemical modifications on DNA, such as cytosine methylation. The distinction between the two is a marker's status in relation to an outcome variable, usually a disease. Genotypic markers generally do not change over time and are not affected by the development of a disease, whereas phenotypic markers are likely to change over time or be influenced by the presence of a disease, either itself or the treatment associated with it. If measurements of a phenotypic marker are made from the specimens that are collected after or at the time of cancer diagnosis, investigators will have difficulties determining the status of the phenotypic marker before the cancer was diagnosed. A disease condition, however, does not affect genotypic markers such as SNPs; therefore, a temporal relationship can be easily established even if the samples are collected after the disease is diagnosed. Based on this distinction, one can evaluate genotypic markers either in case-control or cohort studies, but a case-control study would be the design of choice because of efficiency and cost-effectiveness. A prospective cohort study design is ideal for phenotypic markers. Investigators, however, may use other study designs if they can demonstrate that the disease status does not influence the phenotypic markers of interest. To reduce study cost, investigators usually use nested case-control or case-cohort designs to avoid analyzing specimens from the entire cohort. The main purpose in choosing a cohort study design for a molecular epidemiology investigation is to ensure that biospecimens are collected before the development of a disease so that a temporal relationship between a marker and disease development can be established.

The differences between molecular epidemiology and genetic epidemiology are the scope of the molecular analysis and the emphasis on heredity. Sometimes molecular and genetic epidemiology both investigate genetic factors in association with cancer risk, but each has its own emphasis. The former assesses genetic involvement, but not necessarily inheritance, whereas the latter focuses mainly on heredity. Because of the difference in focus, study populations are different between the two types of investigation. Molecular epidemiology studies unrelated individuals, whereas genetic epidemiology investigates family members in the format of pedigrees, parent–child trios, or sibling pairs. Given the different research focus between genetic and molecular epidemiology, these investigations evaluate different genetic markers. Genetic epidemiology research is designed to identify genetic markers with high penetrance (strong association with an underlying disease) but low prevalence in the general populations, whereas a molecular epidemiology investigation targets low penetrance markers that are commonly present in the general population. Given the difference in study design, the analysis of genetic marker's link to cancer is also different between the studies. Relative risks or odds ratios are calculated in molecular epidemiology studies because study participants are unrelated individuals, whereas linkage analysis is used in genetic epidemiology because individuals in the study are genetically related family members. Recently, both genetic and molecular epidemiology study designs have been considered in GWAS to improve study validity and to minimize false positive findings. Another difference between genetic and molecular epidemiology research is that molecular epidemiology also studies nongenetic molecules. Thus, the scope of molecular analysis is much broader in molecular epidemiology research than in genetic epidemiology studies.

A laboratory analysis of molecular markers is another integral part of molecular epidemiology research, which has unique features that are different from basic science research. Collecting biologic specimens is difficult and expensive in population-based epidemiologic studies. It not only increases the study cost, but also imposes constraints to multiple areas of epidemiology research. Specimen collection may adversely influence the response rate of study participants, potentially compromising study validity. For organ-specific cancer research, investigating molecular markers in target tissue is difficult. Blood is the most common and versatile specimen used in molecular epidemiology research; other specimens used include urine, stool, nail, hair, sputum, buccal cells, and saliva. Tissue samples, either fresh frozen or chemically fixed, are also used, but the availability of these samples is highly limited to patients or selected subgroups of a general study population. Comparability and generalizability are always problems in epidemiologic studies involving tissue specimens, except for those investigations that focus on cancer prognosis or treatment in which only cancer patients are involved. Attempts have been made to use special body fluids for epidemiologic research, such as nipple aspirate and breast or pulmonary lavage, but the difficulty in specimen collection and preparation makes these samples impractical in large population-based studies.

Given the research value of biologic specimens and the difficulty in collecting them for population-based studies, technical issues related to specimen collection, processing, and storage become especially important in molecular epidemiology research.

These include time and conditions for specimen transportation and processing, a sample aliquot and labeling system, a sample special treatment for storage and analysis, a sample storage and tracking system, as well as backup plans and equipment for unexpected adverse events during long-term storage (e.g., power failure, earthquake, flooding). Laboratory methods used to analyze biomarkers are also important in molecular epidemiology. Because large numbers of specimens are involved, laboratory methods are required to be robust, reproducible, high throughput, low cost, and easy to use. These requirements are often met in the analysis of nucleotide sequences that serve as genotypic markers. However, for phenotypic markers, many methods do not readily meet these requirements. Moreover, many phenotypic markers, such as proteins, require both qualitative and quantitative assessments. An ideal laboratory method should be quantitative (able to measure a wide range of values), sensitive (able to detect a small amount of analyte), specific (able to detect only the molecule of interest, no other molecules), reproducible (high precision and low variation), and versatile (easy to use). In addition, investigators need to implement appropriate quality assurance procedures during sample processing and testing as well as include appropriate quality control samples in specimen analysis.

Host–environment interaction is believed to play a key role in the etiology of most types of cancer. Genetic factors, including mutations and polymorphisms, are initially considered important host factors, but recent developments in cancer research has indicated that epigenetic factors may also play a critical role in cancer as a host factor involved in host–environment interaction. Epigenetic factors, which regulate the function of human genome without altering the physical sequences of nucleotides, include pretranscription regulation through nucleotide modification (e.g., cytosine methylation at CpG sites), chromosome modification (e.g., histone acetylation), and posttranscription regulation by noncoding small RNA (e.g., microRNAs). These epigenetic factors have two unique features that have captured the attention of cancer researchers, especially cancer epidemiologists who are interested in the gene–environment interaction. It is known that epigenetic factors are heritable, but these inherited features are readily modifiable by environmental and lifestyle factors. Monozygotic twins have an identical genome as well as epigenome at birth, but the latter undergoes substantial changes over time, resulting in distinct epigenetic profiles that depend heavily on their environmental exposures.[58] Animal studies also indicated that the maternal intake of dietary nutrients involving one-carbon metabolism could influence offsprings' growth phenotypes, which are regulated by DNA methylation.[59] As evidence mounts on epigenetic involvement in cancer, molecular epidemiologists will start to look for clues in human populations that can link epigenetic factors to both lifestyle factors and cancer risk. Given that epigenetic regulation is tissue specific and time dependent, investigators face challenges in accurately assessing these phenotypic markers in etiologic studies. However, progress in the analysis of circulating methylation markers and microRNAs may provide an alternative to study epigenetic regulation in human cancer. Furthermore, methods for a genome-wide analysis of DNA methylation have been developed and applied in epidemiologic studies, which can substantially accelerate the search for cancer-related DNA methylation. Together with the high-throughput, high-dimensional analysis of DNA methylation, two other evolving fields that will have significant impacts on molecular epidemiology of cancer research are metagenomics and metabolomics. The former focuses on environmental genomics of the microbiome that resides in our body and influences one's biologic functions and health status. The latter refers to the analysis of hundreds or thousands of metabolites in a biologic specimen, including tissue, blood, urine, body fluids, and fecal samples. These new analyses will add tremendous value to epidemiologic studies.

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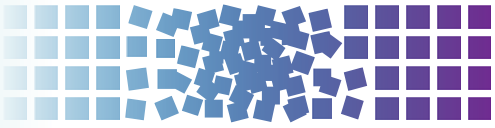
12 Trends in United States Cancer Mortality



Tim E. Byers

INTRODUCTION

Cancer incidence registries now cover nearly all of the US population. State-based vital records systems and aggregate national systems regularly report trends in both cancer incidence and mortality, and national surveys routinely monitor cancer-related risk factors in the population. These surveillance systems have documented substantial changes in both risk factors for cancer and in cancer incidence and mortality rates in the United States over the past 3 decades. In 1996, the American Cancer Society (ACS) set an ambitious challenge for the United States: to reduce cancer mortality rates from their apparent peak in 1990 by 50% in the 25-year period ending in 2015.[1] In 1998, the ACS then challenged the United States to also reduce cancer incidence rates from their peak in 1992 by 25% by the year 2015.[2] In this chapter, we will examine trends in cancer risk factors as well as trends in cancer incidence and mortality rates in the United States over the 25-year period between 1990 and 2015.

CANCER SURVEILLANCE SYSTEMS

Collecting cancer incidence rates is largely a state-based activity in the United States, because cancer is a reportable disease in all states. The Centers for Disease Control and Prevention (CDC) organizes all state-based cancer registries within the National Program of Cancer Registries, which now reports collective data on cancer incidence from over 40 different state-based registries, providing data that meets strict quality standards.[3] The National Cancer Institute has supported high-quality cancer incidence and outcomes registration in selected states and cities since 1973 within the Surveillance, Epidemiology, and End Results (SEER) Program.[4] The most precise measures of long-term trends in cancer incidence come from SEER-9, a set of nine SEER registries that together include about 10% of the US population. The populations included in the SEER-9 registries document the most detailed history of cancer trends beginning in the 1970s based on highly standardized cancer case ascertainment, staging, treatment, and outcomes. Deaths from cancer are well ascertained in all states via state-based vital records, which are aggregated into annual national mortality reports by the CDC's National Center for Health Statistics. [5] Each year, the ACS, the National Cancer Institute, and the CDC publish a *Report to the Nation* on trends in cancer incidence and mortality in the United States.[6] Trends in the prevalence of behavioral factors that affect cancer risk are tracked by the Health Interview Survey, an ongoing, in-person interview of a nationally representative sample of adults, and in annual reports by the Behavioral Risk Factor Surveillance System, a continuously operating telephone-based survey operated by state departments of health and organized by the CDC.[7]

MAKING SENSE OF CANCER TRENDS

Understanding the reasons for cancer trends requires understanding trends in cancer-related risk factors. For factors like tobacco, relating trends in exposure to trends in rates is easy, because those effects are large and single. However, for many other cancer risk factors, because effects are much smaller and multifactorial, simple correlations over time are less apparent. In most situations, all that maybe possible are crude qualitative relationships between temporal trends in cancer risk factors and subsequent trends in cancer rates. Statistical methods such as linear regression joinpoint analysis can tell us when inflections in cancer trends occur, but accounting for the precise reasons for changing rates is often impaired by our incomplete knowledge about the interacting impacts of variations in cancer screening, diagnosis, and treatment, and by uncertainties about latencies between interventions and outcomes.[8]

TRENDS IN CANCER RISK FACTORS AND SCREENING

Trends in major cancer risk factors have been mixed (Table 12.1). Although the downward trends in tobacco smoking among adults that began in the 1960s slowed after 1990, there has been a continuing downward trend in the number of cigarettes smoked per day by continuing smokers.[9] Obesity trends have been adverse among both men and women since the 1970s, with more than a doubling of the prevalence of obesity between 1990 and 2010. Long-term trends in the use of hormone replacement therapy (HRT) are not routinely monitored in the Behavioral Risk Factor Surveillance System (BRFSS), but HRT use increased substantially in the last 2 decades of the 20th century. Then, following the 2002 publication of the Women's Health Initiative trial, which showed clear adverse effects of HRT, there was a rapid and substantial drop in HRT use.[10,11] The use of endoscopic screening for colorectal cancer (sigmoidoscopy or colonoscopy) has increased substantially in recent years, approximately doubling since the mid 1990s, so that, as of 2010, about two-thirds of Americans age 50 and older reported ever having had an endoscopic examination. Mammography use increased progressively through the 1990s, but mammogram rates then leveled off after 2000.[12] Widespread prostate-specific antigen (PSA) testing began in the mid to late 1980s, then increased substantially during the 1990s. By 2002, a majority of US men age 50 and older reported having been tested.

CANCER INCIDENCE AND MORTALITY

In this chapter, we describe and discuss cancer trends for the time period 1990 through 2010 using cancer incidence data from the SEER-9 registry (Table 12.2 and Fig. 12.1) and US cancer mortality data from the National Center for Health Statistics (Table 12.3).[4,5] All rates were age-adjusted to the US 2000 standard population by the direct method, using 10-year age intervals.

Lung Cancer

The lung is the second leading site for cancer incidence and the leading site for cancer death among both men and women in the

TABLE 12.1

Trends in Risk Factors and Cancer Screening Practices in the United States, 1990–2010[a]

	Men		Women		Both Genders	
	Smoking	PSA Screening	Smoking	Mammography	Obesity	CRC Screening
1990	24.9	—	21.3	58.3	11.6	—
1991	25.1	—	21.3	62.2	12.6	—
1992	24.2	—	21.0	63.1	12.6	—
1993	24.0	—	21.1	66.5	13.7	—
1994	23.9	—	21.6	66.6	14.4	—
1995	24.8	—	20.9	68.6	15.8	29.4
1996	25.5	—	21.9	69.2	16.8	—
1997	25.4	—	21.1	70.3	16.6	32.4
1998	25.3	—	20.9	72.3	18.3	—
1999	24.2	—	20.8	72.8	19.7	43.7
2000	24.4	—	21.2	76.1	20.1	—
2001	25.4	—	21.2	—	21.0	—
2002	25.7	53.9	20.8	75.9	22.1	48.1
2003	24.8	—	20.2	—	—	—
2004	23.0	52.1	19.0	74.7	23.2	53.0
2005	22.1	—	19.2	—	24.4	—
2006	22.2	53.8	18.4	76.5	25.1	57.1
2007	21.2	—	18.4	—	26.3	—
2008	20.3	54.8	16.7	76.0	26.6	61.8
2009	19.5	—	16.7	—	27.1	—
2010	18.5	53.2	15.8	75.2	27.5	65.2

CRC, colorectal cancer; PSA, prostate-specific antigen.

[a] Median percent of the population across all states in the Behavioral Risk Factor Surveillance System. The survey covered such areas as body mass index and was based on self-reported height and weight. Questions included: Are you a regular cigarette smoker? Have you ever had a sigmoidoscopy or proctoscopic examination? For women age 40 and older, the following question was included: Have you had a mammogram in the past 2 years? For men aged 50 and older, the following question was included: Have you had a PSA test in the last 2 years? (From Centers for Disease Control and Prevention. Behavioral Risk Factor Surveillance System Web site. http://cdc.gov/brfss.)

United States.[6] There are now more deaths from lung cancer in the United States than from the sum of colorectal, breast, and prostate cancers. Trends in lung cancer incidence and mortality have been nearly identical because there are few effective treatments for lung cancer, and survival time remains short. Lung cancer trends follow historic declines in tobacco use, lagged by about 20 years.[13] Between 1965 and 1985, tobacco use among US adults dropped substantially, and more in men than in women. Lung cancer mortality rates began to decline among men in 1990, but rates increased among women throughout the 1990s. The stabilization of lung cancer incidence trends among women from 2000 to 2005 and the beginning of a decline in the period 2005 to 2010 foretells a coming persistent decline in lung cancer mortality among women in the United States.

The effectiveness of annual examinations by use of chest radiographs in reducing lung cancer mortality was studied as part of the Prostate, Lung, Colorectal, Ovary (PLCO) trial, and the effectiveness of annual screening by low-dose computed tomography (LDCT) of the lung fields was studied in the National Lung Screening Trial (NLST).[14,15] In brief, screening with standard chest radiography finds more cancers earlier but does not affect mortality, whereas screening with LDCT reduces the risk of death from lung cancer by at least 20%.[14,15] Therefore, both the ACS and the US Preventive Services Task Force have issued recommendations that favor informed decision making for lung cancer screening using LDCT.[16,17]

The major factor that will determine lung cancer incidence in the coming decade is the past history of tobacco use, but future screening will also reduce future mortality rates. Considering all factors, it is likely that over the coming decade the downward trends in mortality from lung cancer will continue at about the same rate among men, and soon will become more apparent among women.

Colorectal Cancer

The colorectum is the third leading site for cancer incidence and the second leading site for cancer death in the United States.[6] Colorectal cancer incidence rates increased until 1985, when they began to decline. The reasons for this decline are not clear, but could be related to downward trends in cigarette smoking and the increasing use of both nonsteroidal anti-inflammatory drugs (NSAIDs) and HRT.[18] The rapid decline in HRT use following the publication of the Women's Health Initiative trial results in 2002 may adversely affect colorectal trends among women in the coming years, because HRT reduces the risk for colorectal cancer among women.[11] Recent trials have demonstrated the potential for NSAIDs to reduce colorectal neoplasia, but adverse effects from these agents will limit their widespread use for that explicit purpose. Nonetheless, even the common sporadic use of NSAIDs for other indications will contribute to continuing declines in colorectal cancer incidence in the coming years.

Screening with either sigmoidoscopy or colonoscopy leads to the identification and removal of adenomas, thus preventing the development of colorectal cancer.[19,20] Medicare included

TABLE 12.2

Trends in Age-Adjusted Cancer Incidence Rates in the United States by Cancer Site, 1990–2010[a]

	Men		Women		Both Genders	
	Lung	Prostate	Lung	Breast	Colorectal	All Sites
1990	96.9	171.0	47.8	131.8	60.7	482.0
1991	97.2	214.8	49.6	133.9	59.5	503.0
1992	97.2	237.4	49.9	132.1	58.0	510.6
1993	94.0	209.5	49.2	129.2	56.8	493.4
1994	90.9	180.3	50.5	131.0	55.6	483.5
1995	89.8	169.3	50.4	132.6	54.0	476.9
1996	88.0	169.5	50.2	133.7	54.8	479.1
1997	86.3	173.5	52.6	138.0	56.4	486.4
1998	88.0	171.0	53.0	141.4	56.8	488.2
1999	84.6	183.4	52.4	141.5	55.5	490.4
2000	82.1	183.0	51.2	136.4	54.1	486.0
2001	81.4	184.8	51.7	138.7	53.6	489.7
2002	80.4	182.2	52.5	135.6	53.1	487.5
2003	81.0	169.6	53.0	126.8	50.8	475.2
2004	76.2	165.7	52.0	128.0	50.0	476.1
2005	75.8	156.5	53.7	126.4	47.8	471.9
2006	74.2	171.5	53.4	126.0	46.8	475.0
2007	73.5	174.3	53.4	127.9	46.3	480.5
2008	72.0	157.0	51.6	128.0	45.2	473.4
2009	70.2	153.7	51.8	130.3	43.0	470.5
2010	66.8	145.1	49.2	126.0	40.6	457.5
Average annual % change 1990–2010	−1.8	−0.5	+0.2	−0.2	−2.0	−0.2

[a] Data source is the Surveillance, Epidemiology, and End Results-9 populations for cancer incidence. Rates are age-adjusted to the year 2000 population standard. The annual percent change is the mean percent change per year across the 20-year period, 1990 to 2010. (From National Cancer Institute. Surveillance, Epidemiology, and End Results Program Web site. http://seer.cancer.gov.)

coverage for all recommended colorectal screening methods in 2001, and national publicity has substantially increased public interest in screening.[21] Colorectal screening rates have increased over time, now with about two-thirds of adults over age 50 reporting having ever been screened by lower gastrointestinal endoscopy (see Table 12.1).

Decreasing rates of colorectal cancer incidence are occurring in spite of the obesity epidemic, which is an adverse force on colorectal cancer risk, because obesity may account for as much as 20% of colorectal cancer in the United States.[22] Recently, however, obesity trends have stabilized in the United States. [23] As a result of the increased use of lower gastrointestinal endoscopy for colorectal screening and this stabilization of obesity trends, the incidence of colorectal cancer may exceed the ACS goal for 2015 of a 25% reduction, and there is a high likelihood that the rate of decline in deaths from colorectal cancer will be steep enough to reach the 2015 ACS mortality reduction goal of 50%.

Breast Cancer

The breast is the leading site of cancer incidence and the second leading site for cancer death among women in the United States.[6] Over the period 1990 to 2001, no substantial changes in incidence rates were observed, but after 2000, breast cancer incidence began to decline. The decline in breast cancer incidence observed after 2002 seems to have been the result of the sudden decline in the use of HRT following the 2002 publication of the Women's Health Initiative results.[10,11] It is likely that persisting lower rates of HRT use will cause a continued decline in breast cancer incidence in the coming years. Countering this favorable trend, however, are the adverse effects of the obesity epidemic. Obesity, a major risk factor for postmenopausal breast cancer, increased substantially between 1990 and 2005, now with over 25% of US women being obese. However, the slowing of the obesity epidemic since 2005 may have substantial beneficial effects on the future trends in breast cancer incidence.

After persistent increases in the use of mammography over a 20-year period, mammography rates declined modestly between 2000 and 2004, and then leveled off. The downgrading of the evidence recommendations by the US Preventive Services Task Force for mammography for women age 40 to 49 and recommendations for every other year mammographies for women age 50 and older have resulted in lower mammogram utilization, which is likely to continue into the coming decade.[17] This trend will have an adverse effect on breast cancer mortality, but will tend to reduce breast cancer incidence somewhat because of a lack of detection of very early stage cancers.

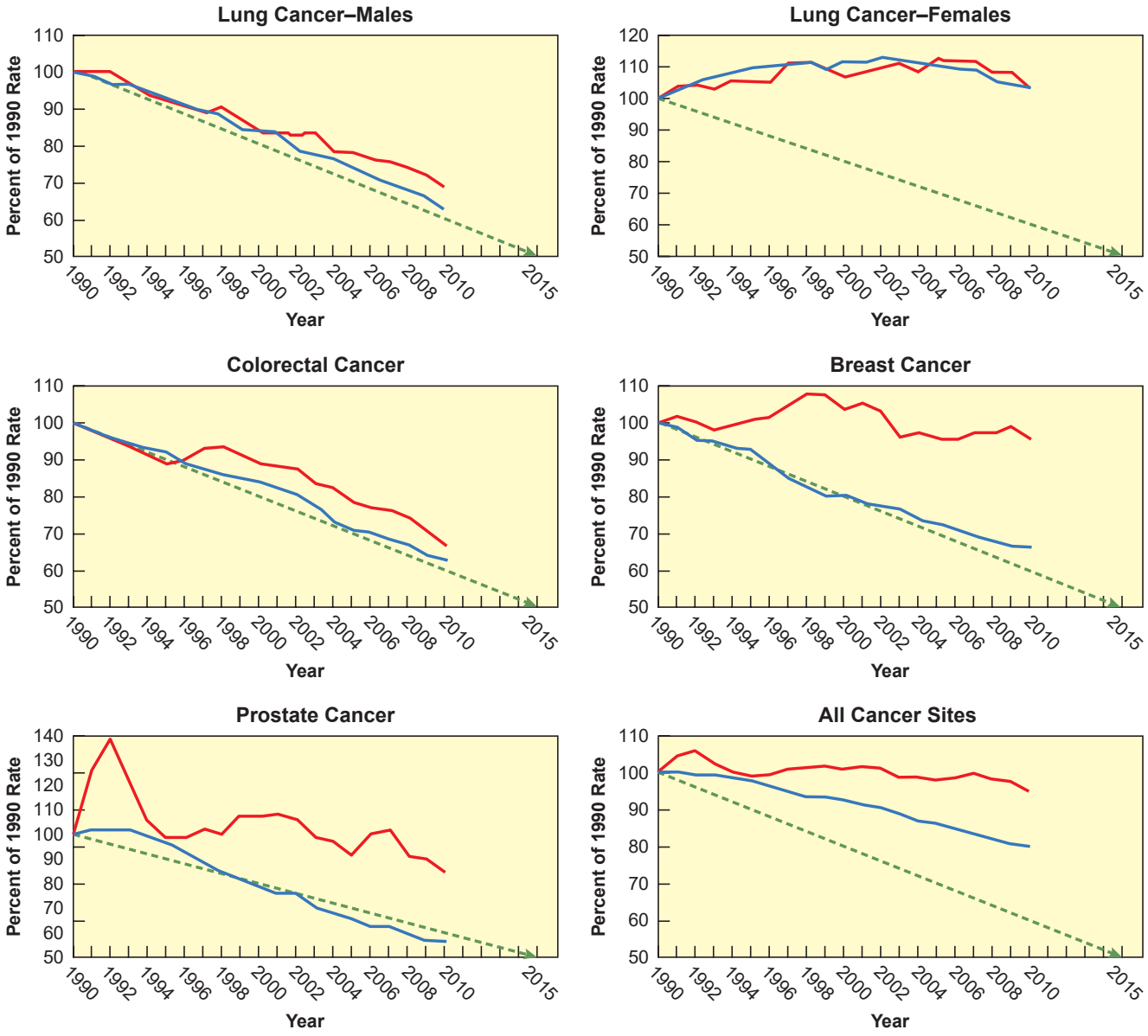


Figure 12.1 **(A–F)** Trends in cancer incidence and mortality between 1990 and 2010. Incidence rates are for the populations in the Surveillance, Epidemiology, and End Results Program; registries and mortality rates are for the entire United States. Rates are age-adjusted to the year 2000 standard. The y-axis rates are expressed as a percentage of the 1990 incidence and mortality rates. The *red lines* represent incidence rates, and the *blue lines* represent mortality rates. The *straight dotted green lines* represent the linear trend that would need to be followed to achieve a 50% mortality reduction between 1990 and 2015. (Data from National Cancer Institute. Surveillance, Epidemiology, and End Results Program Web site. http://seer.cancer.gov, and Centers for Disease Control and Prevention. U.S. mortality data. http://wonder.cdc.gov/ucd-icd10.html)

The antiestrogens tamoxifen and raloxifene have both been shown to reduce the risk of incident breast cancer.[24] The safety profile for tamoxifen discourages its widespread use, but there is a more favorable risk/benefit balance of raloxifene. Nonetheless, neither of these drugs is commonly used for breast cancer prevention among postmenopausal women in the United States.

The average decline in breast cancer death rates of 2% per year since 1990 is the combined result of earlier diagnosis and better treatment.[25] Progress in breast cancer treatment is continuing, especially in the development and application of hormone-targeted therapies. Aromatase inhibitors have largely replaced tamoxifen therapy for breast cancer treatment for postmenopausal women. Because all antiestrogens substantially reduce the incidence of second primary cancers in the contralateral breast, they impact both therapy and prevention. In the coming decade, the longer term effects of decreased HRT use, increased antiestrogen use, reversal of the obesity trends, and continued improvements in therapies will likely lead to continued decreases in both the incidence and mortality rates from breast cancer.

Prostate Cancer

The prostate is the leading site for cancer incidence and the second leading site for cancer death among men in the United States.[6] The incidence of prostate cancer has been extremely variable over the period 1990 to 2010. The incidence spike observed in the early 1990s actually began in the late 1980s, coincident with the advent of PSA testing. The reasons for the 2.8% annual downward trend in prostate cancer mortality since 1990 are uncertain, however, because the ongoing PSA screening trials have not yet demonstrated a mortality benefit from screening anywhere as large as the downward mortality

TABLE 12.3

Trends in Age-Adjusted Cancer Mortality Rates in the United States by Cancer Site, 1990–2010[a]

	Men		Women		Both Genders	
	Lung	Prostate	Lung	Breast	Colorectal	All Sites
1990	90.6	38.6	36.8	33.1	24.6	214.9
1991	89.9	39.3	37.6	32.7	24.0	215.1
1992	88.0	39.2	38.7	31.6	23.6	213.5
1993	87.6	39.3	39.3	31.4	23.3	213.4
1994	85.7	38.5	39.6	30.9	22.9	211.7
1995	84.4	37.3	40.3	30.6	22.6	210.0
1996	82.8	36.0	40.4	29.5	21.9	207.0
1997	81.3	34.2	40.8	28.2	21.5	203.6
1998	79.9	32.6	41.0	27.5	21.2	200.8
1999	77.0	31.6	40.2	26.6	20.9	200.7
2000	76.5	30.4	41.1	26.6	20.7	198.8
2001	75.3	29.5	41.0	26.0	20.2	196.3
2002	73.7	28.7	41.6	25.6	19.8	194.4
2003	72.0	27.2	41.3	25.3	19.1	190.9
2004	70.4	26.2	41.0	24.5	18.1	186.8
2005	69.5	25.4	40.7	24.1	17.6	185.2
2006	67.4	24.2	40.3	23.6	17.3	182.0
2007	65.2	24.2	40.1	23.0	16.9	179.3
2008	63.7	23.0	39.1	22.6	16.5	176.3
2009	61.5	22.1	38.6	22.2	15.8	173.4
2010	60.1	21.8	38.0	22.0	15.5	171.8
Average annual % change 1990–2010	−2.0	−2.8	+0.2	−2.0	−2.3	−1.1

[a] Data source is the National Center for Health Statistics national mortality data set. Rates are age-adjusted to the year 2000 population standard. The average percentage change per year is the mean percent change per year across the 20-year period, 1990 to 2010. (From Centers for Disease Control and Prevention. U.S. mortality data. http://wonder.cdc.gov/ucd-icd10.html)

decline observed since 1990.[26,27] In fact, the US trial findings suggest that there was virtually no mortality benefit within the first decade following the initiation of screening.[28] Therefore, it is not possible to know how much of this favorable trend was related to early diagnosis, how much was related to improvements in treatment, or how much might have been related to other factors, such as changes in the way cause of death has been listed on death certificates.

The Prostate Cancer Prevention Trial provided an important proof of principle that antiandrogen therapies can reduce prostate cancer risk.[29] Although the net benefits of finasteride for prevention are not clearly demonstrated from this trial, other agents that interfere with androgen effects on prostate cancer growth could prove to be useful for prostate cancer chemoprevention in the future. Prostate cancer incidence trends will likely continue to be largely driven by rates of PSA screening in the coming decade. Longer term results of a clearer benefit to mortality from either the PLCO trial in the United States or the European PSA trial would help to better specify screening recommendations.

Other Cancers

Even though mortality rates have been declining by about 2% per year from the four most common causes of cancer death (lung, colorectal, breast, and prostate), very little progress has been made in reducing death rates from the other half of all adult cancers in the United States. Continuing progress in tobacco control will have beneficial effects on many other types of cancer linked to tobacco, and stopping the obesity epidemic will have favorable effects on many obesity-related cancers that have been increasing in recent years, such as adenocarcinoma of the esophagus and renal cancer.[30] Melanoma incidence rates have been increasing substantially in recent years, likely the result of the combined effects of previous sun exposure and increased awareness and surveillance for pigmented skin lesions, but recent advances in therapy for metastatic melanoma may foretell future declines in melanoma morality. Declining rates of stomach cancer incidence and mortality over several decades may be related to the combined effects of historic improvements in nutrition and the declining prevalence of chronic infection with *Helicobacter pylori*. Liver cancer incidence has been substantially increasing in recent years, likely resulting from historic trends in chronic infection with hepatitis B and C viruses. As a result, liver cancer will likely continue to rise in the United States over the coming decade.

The incidence of thyroid cancer has been increasing in the United States for the past several decades, but thyroid cancer mortality rates have been stable, a pattern most likely due to increased detection from improved diagnostic techniques. Invasive cervical

cancer is uncommon in the United States because of widespread screening using Pap smears. Although the vaccination for human papillomavirus (HPV) has been shown to be highly effective in protecting against the serotypes that together account for 70% of cervical cancer cases, so far, HPV vaccine coverage has been low among young women in the United States.[6] For many of the other cancers, such as cancers of the pancreas, brain, ovary, and the hematopoietic malignancies, risk factors are poorly understood, and there are no effective early detection methods. For these cancers, the current hope for improvement resides in the development of better methods for early cancer detection and treatment.

PREDICTING FUTURE CANCER TRENDS

In the United States, cancer is now the leading cause of death under age 85 years. Over the first half of the ACS 25-year challenge period, overall cancer incidence rates have declined by about 0.2% per year, and mortality rates have declined by about 1% per year. The trends in both incidence and mortality from the four leading cancer sites are summarized in Figure 12.1. Using simple linear extrapolation, it therefore seems that the ACS challenge goals of reducing cancer incidence by 25% and mortality by 50% over 25 years may be only half achieved.[31,32] Clearly, though, estimating future trends only by linear extrapolation is a crude way to foretell future events. Projecting cancer trends into the more distant future using complex modeling is possible, however, as knowledge about changes in major cancer risk factors can lead to reasonable predictions about the direction and approximate slope of future trends. One method to incorporate knowledge about trends in risk factors into estimates of future cancer trends is to estimate the impact of changes in the attributable risk (also called the *preventable fraction*) in the population for each risk factor. By making assumptions about latency period, then tying changes in factors to changes in cancer incidence and mortality, cancer trends resulting from risk factor changes can be predicted. For example, if there were a factor that explained 30% of a particular cancer, then cutting that exposure in half would eventually lead to a projected 15% reduction in rates (50% of 30%). This method was used to project cancer mortality trends to 2015 and seems to have projected trends that are quite similar to those observed in recent years.[33]

Progress in cancer prevention, early detection, and treatment since 1990 has been persistent, and there are many reasons to be optimistic about the future. Just how much steeper the future downward slope in cancer death rates can be driven will depend on the extent to which we can discover new factors causing cancer, and effectively deploy ways to better act on our current knowledge about how to prevent and control cancer. Especially important will be progress in reversing the epidemics of tobacco use and obesity, and ensuring that the coming improvements to health care access will lead to access to state-of-the-art cancer screening and therapy for all.

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PART III

Cancer Therapeutics

13 Essentials of Radiation Therapy

Meredith A. Morgan, Randall K. Ten Haken, and Theodore S. Lawrence

INTRODUCTION

The beneficial use of radiation was launched by the experiments of Wilhelm Roentgen, who, in 1895, found that x-rays could pass through materials that were impenetrable to light. Emil Grubbe provided one of the early examples of the therapeutic use of radiation by treating an advanced ulcerated breast cancer with x-rays in January 1896. We have made great progress since these early days, which has been strongly influenced by research in radiation chemistry, biology, and physics.

BIOLOGIC ASPECTS OF RADIATION ONCOLOGY

Radiation-Induced DNA Damage

Radiation is administered to cells either in the form of photons (x-rays and gamma rays) or particles (protons, neutrons, and electrons). When photons or particles interact with biologic material, they cause ionizations that can either directly interact with subcellular structures or they can interact with water, the major constituent of cells, and generate free radicals that can then interact with subcellular structures (Fig. 13.1).

The direct effects of radiation are the consequence of the DNA in chromosomes absorbing energy that leads to ionizations. This is the major mechanism of DNA damage induced by charged nuclei (such as a carbon nucleus) and neutrons and is termed *high linear energy transfer* (Fig. 13.2). In contrast, the interaction of photons with other molecules, such as water, results in the production of free radicals, some of which possess a lifetime long enough to be able to diffuse to the nucleus and interact with DNA in the chromosomes. This is the major mechanism of DNA damage induced by x-rays and has been termed *low linear energy transfer*.[1]

A free radical generated through the interaction of photons with other molecules that possess an unpaired electron in their outermost shell (e.g., hydroxyl radicals) can abstract a hydrogen molecule from a macromolecule such as DNA to generate damage. Cells that have increased levels of free radical scavengers, such as glutathione, would have less DNA damage induced by x-rays, but would have similar levels of DNA damage induced by a carbon nucleus that is directly absorbed by chromosomal DNA. Furthermore, a low oxygen environment would also protect cells from x-ray–induced damage because there would be fewer radicals available to induce DNA damage in the absence of oxygen, but this environment would have little impact on DNA damage induced by carbon nuclei.[2]

Cellular Responses to Radiation-Induced DNA Damage

Checkpoint Pathways

The cell cycle must progress in a specific order; checkpoint genes ensure that the initiation of late events is delayed until earlier events are complete. There are three principal places in the cell cycle at which checkpoints induced by DNA damage function: the border between G1 phase and S phase, intra-S phase, and the border between G2 phase and mitosis (Fig. 13.3). Cells with an intact checkpoint function that have sustained DNA damage stop progressing through the cycle and become arrested at the next checkpoint in the cell cycle. For example, cells with damaged DNA in G1 phase avoid replicating that damage by arresting at the G1/S interface. If irradiated cells have already passed the restriction point, a position in G1 phase that is regulated by the phosphorylation of the retinoblastoma tumor suppressor gene (*Rb*) and its dissociation from the E2F family of transcription factors, they will transiently arrest in S phase. The G1/S and intra-S phase checkpoints inhibit the replication of damaged DNA and work in a coordinated manner with the DNA repair machinery to permit the restitution of DNA integrity, thereby increasing cell survival.

The earliest response to radiation is the activation of ataxia-telangiectasia mutated (ATM), which involves a conformational change that results in the activation of its kinase domain and phosphorylation of serine 1981 (see Fig. 13.3).[3] This phosphorylation causes the ATM homodimer to dissociate into active monomers that phosphorylate a wide range of proteins such as 53BP1, the histone variant H2AX, Nbs1 (Nijmegen breakage syndrome; a member of the *MRN complex*, composed of Mre11, Rad50, and Nbs1), BRCA1, and SMC1 (structural maintenance of chromosomes), and these proteins coordinate repair with the cell cycle.[4] In response to DNA damage, H2AX is rapidly phosphorylated by ATM and localizes to sites of DNA double-strand breaks in multiprotein complexes described as foci (Fig. 13.4). Phosphorylation of H2AX by ATM results in the direct recruitment of Mdc1 and forms a complex with H2AX to recruit additional ATM molecules, forming a positive feedback loop.

The G1/S phase checkpoint is the best understood. In response to DNA damage, activated ATM can directly phosphorylate p53 and mdm2, the ubiquitin ligase that targets p53 for degradation. These phosphorylations are important for increasing the stability of the p53 protein. In addition to ATM, checkpoint kinase 2 (Chk2) also phosphorylates p53 and can enhance p53 stability. Activated p53 transcriptionally increases the expression of the $p21^{WAF1/CIP1}$ gene, which results in a sustained inhibition of G1 cyclin/Cdk, and prevents phosphorylation of pRb and progression from G1 into S.[5] Mutations in p53 that are commonly found in solid tumors result in loss of transcriptional activity and compromised checkpoint function.

Control of the S-phase checkpoint is mediated in part by the Cdc25A phosphatase inhibiting Cdk2 activity and the loading of Cdc45 onto chromatin. If Cdc45 fails to bind to chromatin, DNA polymerase α is not recruited to replication origins and replicon initiation fails to occur.[6] A more prominent mechanism for S-phase arrest is signaled through the MRN complex and the cohesin protein SMC1 by ATM.[7] Loss of ATM, MRN components, or SMC1 leads to the loss of the intra-S phase checkpoint function and increased radiosensitivity. Both the CDC45 and ATM pathways represent parallel, but seemingly independent, pathways to protect replication forks from trying to replicate through DNA strand

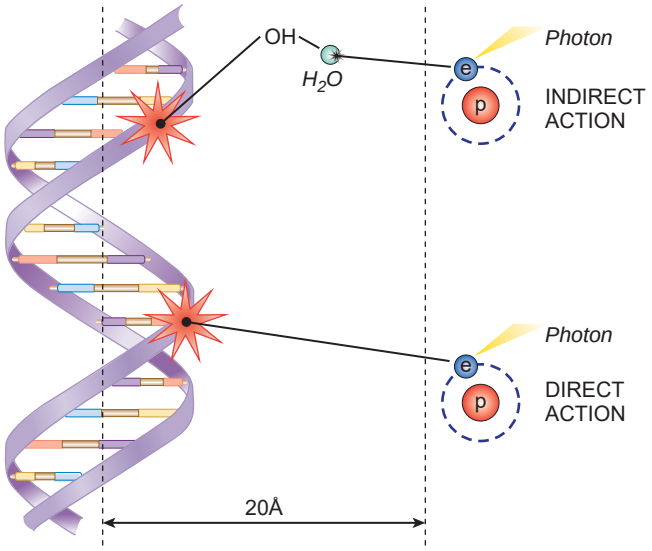

Figure 13.1 The direct and indirect effects of ionizing radiation on DNA. Incident photons transfer part of their energy to free electrons (Compton scattering). These electrons can directly interact with DNA to induce DNA damage, or they can first interact with water to produce hydroxyl radicals that can then induce damage.

breaks. Although ATM has received the lion's share of attention in signaling checkpoint activation in response to ionizing radiation, its family member ATR (ataxia telangiectasia and rad3-related) also plays a role in S-phase checkpoint responses.[8] ATM kinase activity is inducible by radiation, whereas ATR kinase activity is constitutive and does not significantly change with irradiation. (ATR is described in more detail in Chapter 19.) In contrast to Cdc45 and ATM, ATR is probably more important in monitoring

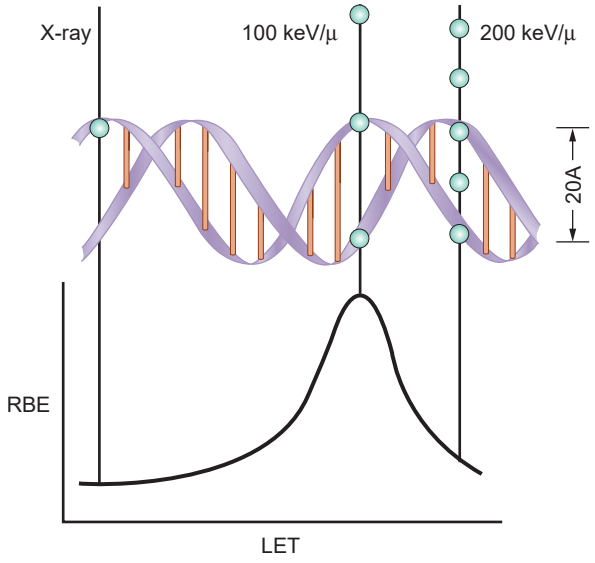

Figure 13.2 Linear energy transfer and DNA damage. Ionizing radiation deposits energy along the track (linear energy transfer [LET]), which causes DNA damage and cell killing. The most biologically potent (highest relative biologic effectiveness [RBE]) LET is 100 keV per μm because the separation between ionizing events is the same as the diameter of the DNA double helix (2 nm). (From Hall EJ, Giaccia AJ. *Radiobiology for the Radiologist*. Philadelphia: Lippincott Williams & Williams; 2012, with permission.)

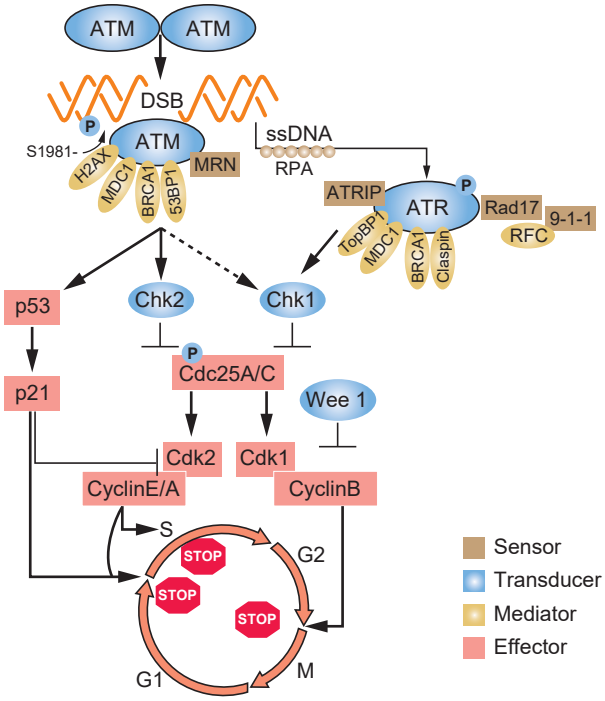

Figure 13.3 In response to DNA damage, the MRN complex—composed of MRE11, Rad50, and NBS1—together with ataxia-telangiectasia mutation (ATM) and H2AX are the earliest proteins recruited to the site of the break. ATM is released from its homodimer complex, activated by transautophosphorylation and, in turn, phosphorylates H2AX. Other members are recruited to the complex such as BRCA1 and 53BP1. As the DNA at the double-strand break (DSB) is resected, single-stranded DNA is formed and bound by replication protein A (RPA), resulting in the activation of the ataxia-telangiectasia and Rad3-related (ATR) pathway. The net result of ATM/ATR activation is the downstream activation of p53, leading to the transcription of the Cdk inhibitor, p21, and the activation of Chk1/Chk2, resulting in the degradation of Cdc25 phosphatases, Cdk-cyclin complex inactivation, and cell cycle arrest at phase G1, intra S, or G2. Note that ATM is also partially activated by changes in chromatin structure induced by DNA double-strand breaks.

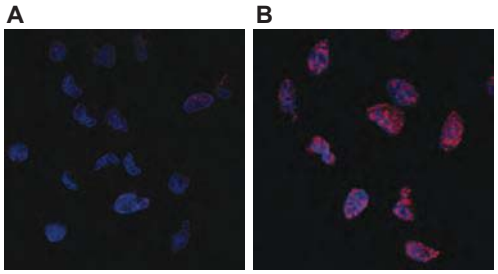

Figure 13.4 Phosphorylated histone variant H2AX as a marker of DNA damage. Phosphorylated histone variant H2AX (also called gamma H2AX) localizes to sites of DNA double-strand breaks, so that its appearance and disappearance correspond with induction and repair of breaks. The cells in panels **A** and **B** have been stained with DAPI (4′,6-diamidino-2-phenylindole) (*blue*) in order to visualize cell nuclei and stained with an antibody, which recognizes *gamma* H2AX (*red*). The cells in **A** are untreated and exhibit little to no gamma H2AX staining, whereas the cells in **B** are treated with 7.5 Gy radiation and exhibit strong gamma H2AX staining at punctate foci in the nuclei, which are thought to correlate with sites of DNA double-strand breaks. (Image provided by Dr. Leslie Parsels, University of Michigan.)

perturbations in replication that are the result of stalled replication forks to prevent the formation of DNA double-strand breaks.

The arrest of cells in the G2 phase following DNA damage is one of the most conserved evolutionary responses to ionizing radiation. It makes sense to have a final checkpoint in the G2 phase to prevent cells from entering into mitosis with damaged DNA that could be transmitted to their progeny. It follows that cells lacking the G2 checkpoint are radiosensitive because they try to divide with damaged chromosomes that cannot be aligned at metaphase to be properly apportioned to daughter cells. At the biochemical level, the regulation of the mitosis-promoting factor cyclin B/Cdk1 is the critical step in the activation of this checkpoint. At the molecular level, ATM and Chk1/2 are activated by DNA damage in the G2 phase and inhibit the activation of Cdc25A and C phosphatases, which are essential for the activation of cyclin B/Cdk1.[9,10] The pololike kinase family (Plk1 and Plk3) also responds to DNA damage and can inhibit Cdc25C activation.[11] A great deal of effort has been focused on the development of small molecules to inhibit checkpoint response proteins, such as Chk1, with the idea that they would inhibit radiation-induced G2 arrest and perhaps repair and thus be used as radiation sensitizers.[12]

DNA Repair

Ionizing radiation causes base damage, single-strand breaks, double-strand breaks, and sugar damage, as well as DNA–DNA, and DNA–protein cross-links. The critical target for ionizing radiation-induced cell inactivation and cell killing is the DNA double-strand break.[13,14] In eukaryotic cells, DNA double-strand breaks can be repaired by two processes: homologous recombination repair (HRR), which requires an undamaged DNA strand as a participant in the repair, and nonhomologous end joining (NHEJ), which mediates end-to-end joining.[15] In lower eukaryotes, such as yeast, HRR is the predominant pathway used for repairing DNA double-strand breaks, whereas mammalian cells use both HHR and non-HHR to repair their DNA. In mammalian cells, the choice of repair is biased by the phase of the cell cycle and by the abundance of repetitive DNA. HRR is used primarily in the late S phase/G2 phases of the cell-cycle, and NHEJ predominates in the G1-phase of the cell cycle (Fig. 13.5). NHEJ and HRR are not mutually exclusive, and both have been found to be active in the late S/G2 phase of the cell cycle, indicating that factors in addition to the cell-cycle phase are important in determining which mechanism will be used to repair DNA strand breaks.

Nonhomologous End Joining. In the G1-phase of the cell cycle, the ligation of DNA double-strand breaks is primarily through NHEJ because a sister chromatid does not exist to provide a template for HRR. The damaged ends of DNA double-strand breaks must first be modified before rejoining. The process of NHEJ can be divided into at least four steps: synapsis, end processing, fill-in synthesis, and ligation (Fig. 13.6).[16] Synapsis is the critical initial step where the Ku heterodimer and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) bind to the ends of the DNA double-strand break. Ku recruits not only DNA-PKcs to

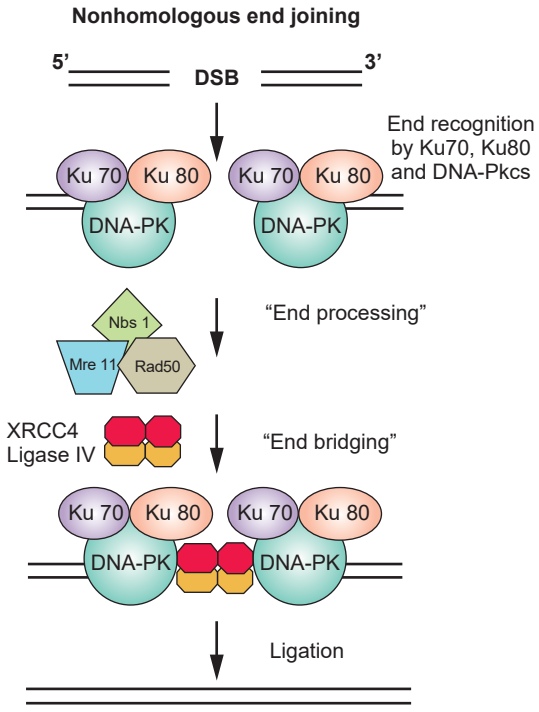

Figure 13.5 Schematic of the critical steps and proteins involved in nonhomologous end joining (NHEJ). The process of NHEJ can be divided into at least four steps: synapsis, end processing, fill-in synthesis, and ligation. DSB, double-strand break.

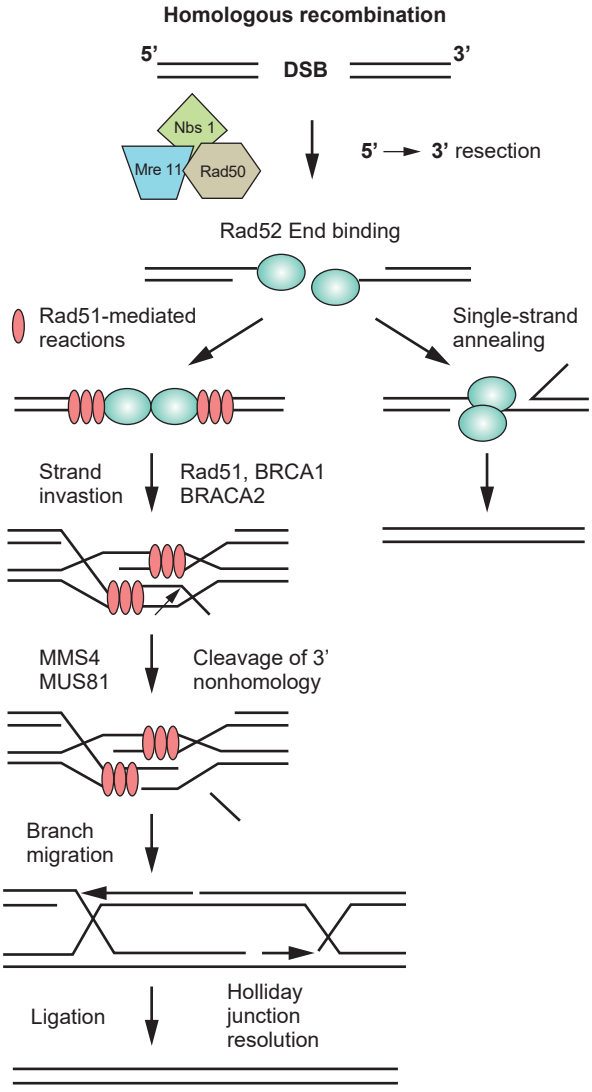

Figure 13.6 Schematic of the critical steps and proteins involved in homologous recombination repair (HRR). The process of HRR can be divided into the following steps: double-strand break (DSB) targeting by H2AX and the MRN complex, recruitment of the ataxia-telangiectasia mutation (ATM) kinase, end processing and protection, strand exchange, single-strand gap filling, and resolution into unique double-stranded molecules.

the DNA ends, but also artemis, a protein that possesses endonuclease activity for 5′ and 3′ overhangs as well as hairpins.[17] DNA-PKcs that is bound to the broken DNA ends phosphorylates artemis and activates its endonuclease activity for end processing. This role of artemis' endonuclease activity in NHEJ may not necessarily be required for the ligation of blunt ends or ends with compatible termini. DNA polymerase μ is associated with the Ku/DNA/XRCC4/DNA ligase IV complex, and is probably the polymerase that is used in the fill-in reaction. The actual rejoining of DNA ends is mediated by a XRCC4/DNA ligase IV complex, which is also probably recruited by the Ku heterodimer.[18,19] Although NHEJ is effective at rejoining DNA double-strand breaks, it is highly error prone. In fact, the main physiologic role of NHEJ is to generate antibodies through V(D)J rejoining, and the error-prone nature of NHEJ is essential for generating antibody diversity.

Homologous Recombination. HRR provides the mammalian genome a high-fidelity pathway of repairing DNA double-strand breaks. In contrast to NHEJ, HRR requires physical contact with an undamaged DNA template, such as a sister chromatid, for repair to occur. In response to a double-strand break, ATM as well as the complex of Mre11, Rad50, and Nbs1 proteins (MRN complex), are recruited to sites of DNA double-strand breaks (Fig. 13.6).[20] The MRN complex is also involved in the recruitment of the breast cancer tumor suppressor gene, *BRCA1*, to the site of the break.[21] In addition to recruiting *BRCA1* to the site of the DNA strand break, Mre11 and as yet unidentified endonucleases resect the DNA, resulting in a 3′ single-strand DNA that serves as a binding site for Rad51. *BRCA2*, which is recruited to the double-strand break by BRCA1, facilitates the loading of the Rad51 protein onto replication protein A (RPA)-coated single-strand overhangs that are produced by endonuclease resection.[22] The Rad51 protein is a homolog of the *Escherichia coli* recombinase RecA, and possesses the ability to form nucleofilaments and catalyze strand exchange with the complementary strand of the undamaged chromatid, an essential step in HRR. Five additional paralogs of Rad51 also bind to the RPA-coated single-stranded region and recruit Rad52, which binds DNA and protects against exonucleolytic degradation.[23] To facilitate repair, the Rad54 protein uses its ATPase activity to unwind the double-stranded molecule. The two invading ends serve as primers for DNA synthesis, resulting in structures known as Holliday junctions. These Holliday junctions are resolved either by noncrossing over, in which case the Holliday junctions disengage and the DNA strands align followed by gap filling, or by crossing over of the Holliday junctions and gap filling. Because inactivation of most of the HRR genes discussed previously results in radiosensitivity and genomic instability, these genes provide a critical link between HRR and chromosome stability.

Chromosome Aberrations Result from Faulty DNA Double-Strand Break Repair

Unfaithful restitution of DNA strand breaks can lead to chromosome aberrations such as acentric fragments (no centromeres) or terminal deletions (uncapped chromosome ends). Radiation-induced DNA double-strand breaks also induce exchange-type aberrations that are the consequence of symmetric translocations between two DNA double-strand breaks in two different chromosomes (Fig. 13.7). Symmetrical chromosome translocations often do not lead to lethality, because genetic information is not lost in subsequent cell divisions. In contrast, when two DNA double-strand breaks in two different chromosomes recombine to form one chromosome with two centromeres and two fragments of chromosomes without centromeres or telomeres, cell death is inevitable. These types of chromosome aberrations are the consequence of asymmetrical chromosome translocations where the genetic material is recombined in what has been termed an *illegitimate* manner (e.g., a chromosome containing an extra centromere).

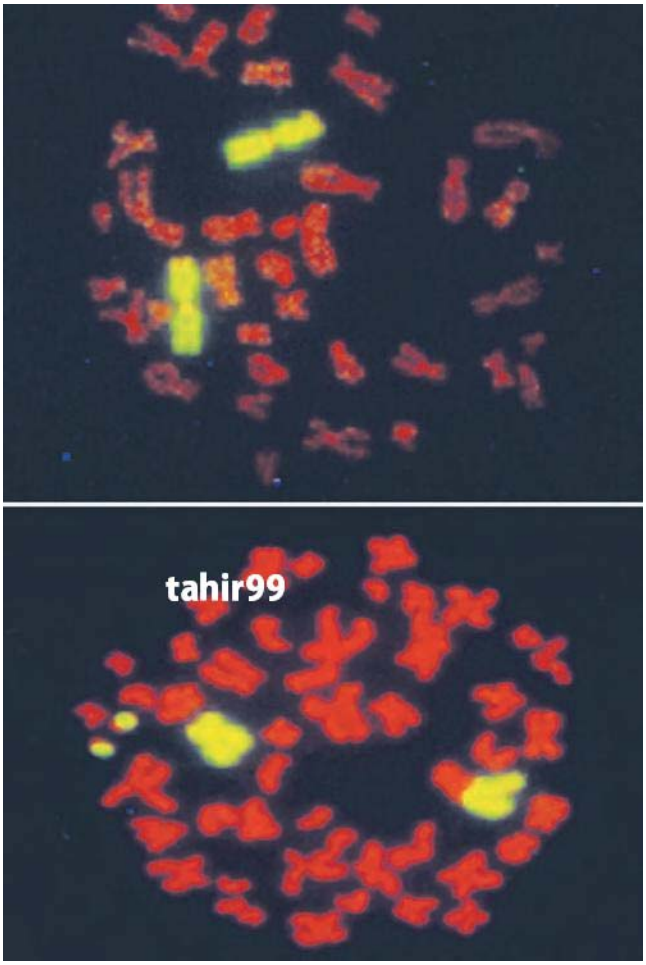

Figure 13.7 Fluorescent in situ hybridization of DNA probes that specifically recognize chromosome 4. In unirradiated cells **(top)**, two chromosome 4s are visualized. In irradiated cells **(bottom)**, one chromosome 4 illegitimately recombined with another chromosome to produce an asymmetrical chromosome aberration, with resulting acentric fragments that will be lost in subsequent cell divisions.

During mitosis, when a cell divides, aberrant chromosomes that have two centromeres, lack a centromere, or are in the shape of a ring have difficulty in separating, resulting in daughter cells with unequal or asymmetric distribution of the parental genetic material. The quantification of asymmetric chromosome aberrations induced by radiation is difficult and has to be performed by the first cell division because these aberrations will be lost during subsequent cell divisions. For this reason, symmetrical chromosome aberrations have been used to assess radiation-induced damage many generations after exposure because they are not lost from the population of exposed cells. In fact, symmetrical chromosome aberrations can be detected in the descendants of survivors of Hiroshima and Nagasaki, indicating that they are stable biomarkers of radiation exposure.[24]

Membrane Signaling

Apart from the direct of effects on DNA, radiation also affects cellular membranes. As part of the cellular stress response, radiation activates membrane receptor signaling pathways such those initiated via epidermal growth factor receptor (EGFR) and transforming growth factor β (TGF-β).[25,26] Activation of these pathways promotes overall survival in response to radiation by promoting DNA damage repair and/or cellular proliferation. In addition,

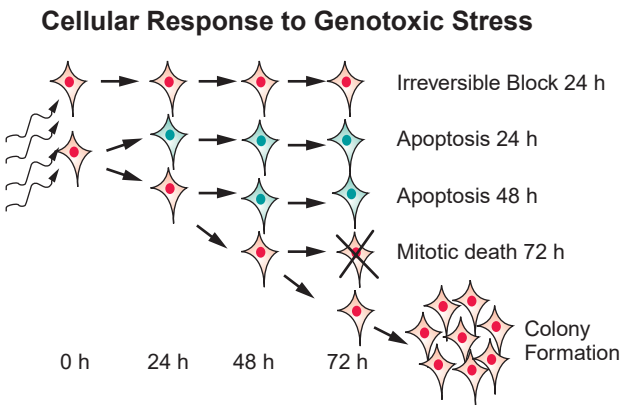

Figure 13.8 Consequences of exposure to ionizing radiation at the cellular level. Cells exposed to ionizing radiation can enter a state of senescence where they are unable to divide, but are still able to secrete growth factors. Alternatively, cells can die through apoptosis, mitotic linked cell death, or they can repair their DNA damage and produce viable progeny.

radiation also induces ceramide production at the membrane via activation of sphingomyelinases, which hydrolyze sphingomyelin to form ceramide. Ceramide production is linked to radiation-induced apoptosis.[27]

The Effect of Radiation on Cell Survival

The major potential consequences of cells exposed to ionizing radiation are normal cell division, DNA damage–induced senescence (reproductively inactive but metabolically active), apoptosis, or mitotic-linked cell death (Fig. 13.8). These manifestations of DNA damage can occur within one or two cell divisions or can manifest at later times after many cell divisions.[28] Effects that occur at later times have been termed *delayed reproductive cell death* and may also be influenced by secreted factors that are induced in response to radiation.[29]

The ability to culture cells derived from both normal and tumor tissues has allowed us to gain insight into how radiosensitivity varies between tissues by analyzing the shape of survival curves. Survival curves of tumor cells often possess a shouldered region at low doses that becomes shallower as the dose increases and eventually becomes exponential. A shoulder on a survival means that these low doses of radiation are less efficient in cell killing, presumably because cells are efficient at repairing DNA strand breaks.[13,14] Killing at low doses of radiation can be described in the form of a linear quadratic equation: $S = e^{-\alpha D - \beta D2}$ (Fig. 13.9).[30] In this equation, S is the fraction of cells that survive a dose (D) of radiation, whereas α and β are constants. Cell killing by the linear and quadratic components are equal when $\alpha D = \beta D^2$ or $D = \alpha/\beta$. Over a larger dose range, the relationship between cell killing and dose is more complex and is described by three different components: an initial slope (D_1), a final slope (D_0), and the width of the shoulder (n, the extrapolation number) or Dq, the quasi-threshold dose (Fig. 13.10). The extrapolation number, *n*, defines the place where the shoulder intersects the ordinate when the dose is extrapolated to zero, and the quasithreshold dose, Dq, defines the width of the shoulder by cutting the dose axis when there is a survival fraction of unity. In contrast to photons, the shoulder on the survival curve disappears when cells are exposed to densely ionizing radiation from particles, indicating that this form of radiation is highly effective at killing cells at both low and high doses.

In Vivo Survival Determination of Normal Tissue Response to Radiation

Although much of our knowledge on the effects of radiation on cell survival has come from cell culture studies, investigators have also devised experimental approaches to assess the clonogenic survival of normal tissues. The earliest example came from McCulloch and Till,[31] who developed an assay to measure the

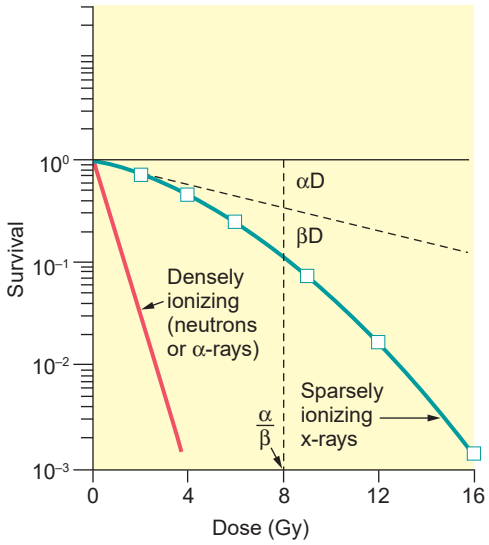

Figure 13.9 An analysis of survival curves for mammalian cells exposed to radiation by the linear quadratic model. The probability of hitting a critical target is proportional to dose (aD): the alpha component. The probability of hitting two critical targets will be the product of those probabilities; therefore, it will be proportional to dose^2 (βD^2): the beta component. The dose at which killing by both the alpha and beta components is equal is defined as $D = \alpha/\beta$. (From Hall EJ, Giaccia AJ. *Radiobiology for the Radiologist*. Philadelphia: Lippincott Williams & Williams; 2012, with permission.)

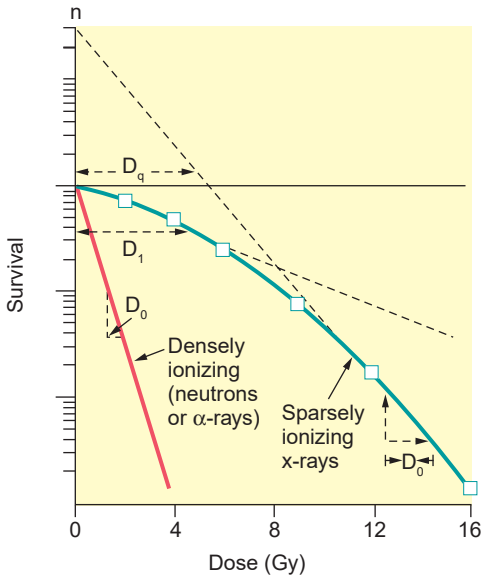

Figure 13.10 An analysis of survival curves for mammalian cells exposed to radiation by the multitarget model. This survival is described by an initial slope (D_1; dose to decreased survival to 37% on initial portion of the curve), a final slope (D_0; dose to decrease survival from starting point to 37% of that point on straight line portion of the curve), an extrapolation number (*n*; an estimate of the width of the shoulder), and a quasithreshold (D_q; a type of threshold dose below which radiation has no effect). (From Hall EJ, Giaccia AJ. *Radiobiology for the Radiologist*. Philadelphia: Lippincott Williams & Williams; 2012, with permission.)

clonogenic survival of bone marrow–derived cells in response to radiation by injecting them into a recipient mouse and quantifying the number of colonies that developed in the spleen. An analysis of these in vivo spleen assays indicated that bone marrow cells are highly radiosensitive (perhaps the most radiosensitive of all mammalian cells) in that their cell survival curve lacked a shoulder. These experiments represent two important firsts in the radiation sciences: They described the first development of an in vivo assay to assess normal tissue survival to radiation, and they demonstrated the first existence of normal tissue stem cells. Soon after, Withers and colleagues[32] developed an assay to assess the survival of skin stem cells, and Withers and Elkind[33] developed an assay to quantify the viability of small intestinal clonogens.

Because these ingenious approaches cannot be applied to all normal tissues, loss of tissue function instead of clonogenic survival has been used as an end point to assess radiation effects. Effects on tissue function can be grouped into the acute or late variety. Desquamation of skin by radiation is an example of an acute loss of function, whereas loss of spinal cord function is an example of a late functional effect. Acutely sensitive tissues such as skin, bone marrow, and intestinal mucosa possess a significant component of tissue cell division, whereas delayed sensitive tissues, such as spinal cord, breast, and bone, do not possess a significant amount of cell division or turnover and manifest radiation effects at later times.

In Vivo Determination of Tumor Response to Radiation

Assays have also been developed to assess the clonogenic survival of tumor cells in animals. Perhaps the most relevant of these assays is the tumor control dose 50% (TCD_{50}) assay,[34] in which the dose of radiation needed to control the growth of 50% of the tumors is determined in large cohorts of tumor-bearing animals. The TCD_{50} assay in animals most closely approximates the clinical situation because tumors are irradiated in animals and the ability to kill all viable tumor cells is assessed. Unlike assays in which tumor cells are irradiated ex vivo, the TCD_{50} assay takes into account the effects of the tumor microenvironment on tumor response. In contrast to the TCD_{50} assay, the tumor growth delay assay reflects the time after irradiation that a transplanted tumor reaches a fixed multiple of the pretreatment volume compared to an unirradiated control. This end point can be achieved by measuring tumor volume through the use of calipers or by a noninvasive measurement of tumor volume using bioluminescent molecules such as luciferase or fluorescent proteins. In the latter approach, all the tumor cells are stably transfected with a bioluminescent marker before implantation, and tumor growth is measured by bioluminescent activity.[35] The advantage of this approach is that tumor cells can be assessed even if they are orthotopically transplanted into their tissue of origin. In another approach, tumors or cells are first irradiated in vivo, the tumor is excised and made into a single-cell suspension, and these cells are then injected into a non–tumor-bearing animal. If the cells are injected subcutaneously under the skin, the end point is tumor formation.[36] If the tumor cells are injected in the tail vein of the mouse, the end point is colony formation in the lungs.[37] The major advantage of these assays is that the actual number of viable cells can be determined.

FACTORS THAT AFFECT RADIATION RESPONSE

The Fundamental Principles of Radiobiology

Studies on split-dose repair (SDR) by Elkind et al.[38] uncovered three of what we now recognize as the most fundamental principles of fractionated radiotherapy: repair, reassortment, and repopulation (Fig. 13.11). (Reoxygenation, described in the following paragraphs, is the fourth). SDR describes the increased survival

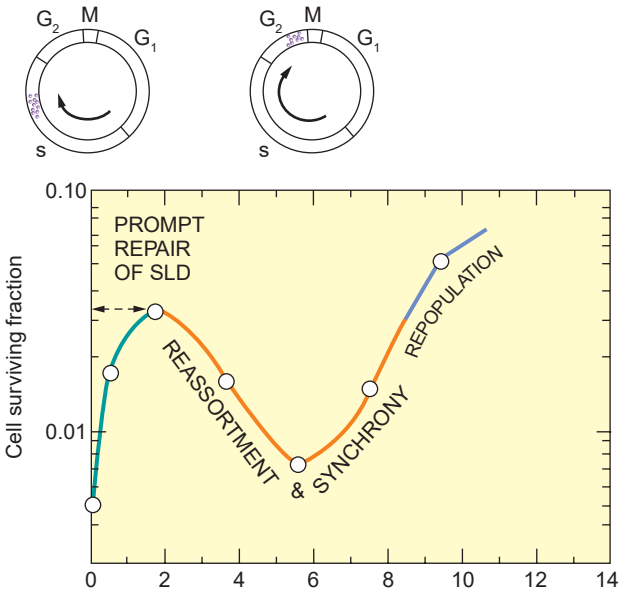

Figure 13.11 Idealized survival curve of rodent cells exposed to two fractions of x-rays. This figure illustrates how the time interval between doses alters the sensitivity of cells when exposed to multiple fractions. In this case, cells move from a resistant phase of the cell cycle (late S phase) to a sensitive phase of the cell cycle (G2 phase). This is known as *reassortment*. If longer periods of time occur between fractions of radiation, cells will undergo division. This latter process is called *repopulation*. SLD, sublethal damage. (From Hall EJ, Giaccia AJ. *Radiobiology for the Radiologist*. Philadelphia: Lippincott Williams & Williams; 2012, with permission.)

or tumor growth delay found if a dose of radiation is split into two fractions compared to the same dose administered in one fraction. This repair is likely due to DNA double-strand break rejoining. Elkind et al. found that the survival of cells increased with an increase in time between doses for up to a maximum of about 6 hours. This finding is consistent with the clinical observation that a separation of radiation treatments by 6 hours produces similar normal tissue injury as a 24-hour separation. The shoulder of a survival curve is strongly influenced by SDR: The broader the shoulder, the more SDR and the smaller α/β ratio.

Similar to repair, reassortment and repopulation are also dependent on the interval of time between radiation fractions. If cells are given short time intervals between doses, they can progress from a resistant portion of the cell cycle (e.g., S phase) to a sensitive portion of the cell cycle (e.g., G2 phase). This transit between resistant and sensitive phases of the cell cycle is termed *reassortment*. If irradiated cells are provided even longer intervals of time between doses, the survival of the population of irradiated cells will increase. This increase in split-dose survival after longer periods of time is the result of cell division and has been termed *repopulation*. Reassortment and repopulation appear to have more protracted kinetics in normal tissues than rapidly proliferating tumor cells, and thereby enhance the tumor response to fractionated radiotherapy compared to normal tissues.

Dose-Rate Effects

For sparsely ionizing radiation, dose rate plays a critical factor in cell killing. Lowering the dose rate, and thereby increasing exposure time, reduces the effectiveness of killing by x-rays because of increased SDR. A further reduction in dose rate results in more SDR and reduces the shoulder of the survival curve. Thus, if one plots the survival for individual doses in a multifraction experiment so that there is sufficient time for SDR to occur, the resulting survival curve would have little shoulder and appear almost linear.[39]

In some cell types, there is a threshold to the lowering of dose rate, and in fact, one paradoxically finds an increase, instead of a decrease, in cell killing. This increase in cell killing under these conditions of protracted dose rate is due to the accumulation of cells in a radiosensitive portion of the cell cycle. In summary, the magnitude of the dose rate effect varies between cell types because of SDR, the redistribution of cells through the cell cycle, and the time for cell division to occur.

Cell Cycle

The phase of the cell cycle at the time of radiation influences the cell's inherent sensitivity to radiation. Cells synchronized in late G1/early S and G2/M phases are most sensitive, whereas cells in G1 and mid to late S phase are more resistant to radiation.[1] These differences in sensitivity during the cell cycle are exploited by the concept of reassortment during fractioned radiotherapy as well by the use of chemotherapeutic agents, which reassort cells into more sensitive phases of the cell cycle in combination with radiation.

Tumor Oxygenation

The major microenvironmental influence on tumor response to radiation is molecular oxygen.[40] Decreased levels of oxygen (hypoxia) in tissue culture result in decreased killing after radiation, which can be expressed as an *oxygen enhancement ratio* (OER). Operationally, OER is defined as the ratio of doses to give the same killing under hypoxic and normoxic conditions. At high doses of radiation, the OER is approximately 3, whereas at low doses, it is closer to 2.[41] Oxygen must be present within 10 μs of irradiation to achieve its radiosensitizing effect. Under hypoxic conditions, damage to DNA can be repaired more readily than under oxic conditions, where damage to DNA is "fixed" because of the interaction of oxygen with free radicals generated by radiation. These changes in radiation sensitivity are detectable at oxygen ranges below 30 mm Hg. Most tumor cells exhibit a survival difference halfway between fully aerobic and fully anoxic cells when exposed to a partial pressure of oxygen between 3 and 10 mm Hg.[1] The presence of hypoxia has greater significance for single-dose fractions used in the treatment of certain primary tumors and metastases and is less important for fractionated radiotherapy, where reoxygenation occurs between fractions. Furthermore, most hypoxic cells are not actively undergoing cell division, thus impeding the efficacy of conventional chemotherapeutic agents that are targeted to actively dividing cells.

Although normal tissue and tumors vary in their oxygen concentrations, only tumors possess levels of oxygen low enough to influence the effectiveness of radiation killing. Although the variations in normal tissue oxygenation are in large part due to physiology governing acute changes in oxygen consumption, the variations in tumor oxygen can be directly attributed to abnormal vasculature that results in a more chronic condition. Thomlinson and Gray[42] observed that variations in tumor oxygen occur because there is insufficient vasculature to provide oxygen to all tumor cells. They hypothesized that oxygen is unable to reach tumor cells beyond 10 to 12 cell diameters from the lumen of a tumor blood vessel because of metabolic consumption by respiring tumor cells. This form of hypoxia caused by metabolic consumption of oxygen has been termed *chronic* or *diffusion-mediated hypoxia*. In contrast, changes in blood flow due either to interstitial pressure changes in tumor blood vessels that lack a smooth muscle component or red blood cell fluxes can cause transient occlusion of blood vessels resulting in *acute* or *transient hypoxia*. Chronically, hypoxic cells will only become reoxygenated when their distance from the lumen of a blood vessel decreases, such as during fractionated radiotherapy when tumor cords shrink. In contrast, tumor cells that are acutely hypoxic because of changes in blood flow or interstitial pressure often cycle in an unpredictable manner between oxic and hypoxic states as blood flow changes.

Based on studies demonstrating that hypoxia can alter radiation sensitivity and decrease tumor control by radiotherapy, strategies have been developed to increase tumor oxygenation. Most importantly, it appears that tumor oxygen levels increase during a course of fractionated radiation. This may be one of the most important benefits of fractionated radiation and is termed *reoxygenation* (the fourth of the four Rs of radiobiology). Tumor reoxygenation during a course of fractionated radiation may also offer an explanation for the general lack of clinical efficacy of hypoxic cell sensitizers despite the clear evidence that hypoxia causes radioresistance.

Aside from using fractionated radiation, the most direct approach to increasing tumor oxygenation is to expose patients receiving radiotherapy to hyperbaric oxygen therapy. The underlying concept is that increasing the amount of oxygen in the bloodstream should result in more oxygen being available for diffusion to the hypoxic regions of tumors. Experimentally, hyperbaric oxygen therapy increases the sensitivity of transplanted tumors to radiation. The results of clinical studies with hyperbaric oxygen therapy, when combined with radiotherapy, showed improvement for two sites—head and neck cancers, and cervix cancers—but failed to show an improvement with other sites, thus calling into question its general usefulness in radiotherapy.[43] In a related approach, erythropoietin (EPO), a hormone released by the kidney that increases red blood cell production, should also increase tumor oxygenation by increasing the delivery of hemoglobin-bound oxygen molecules. EPO has been effective at correcting anemia, but has not been successful in combination with radiation to control head and neck cancer and may, in fact, stimulate tumor growth.[44, 45]

Another strategy to increase tumor oxygenation has been the combined use of nicotinamide, which increases tissue perfusion and carbogen (95% O_2 and 5% CO_2) breathing (accelerated radiotherapy with carbogen and nicotinamide [ARCON] therapy). Recently, a randomized phase III clinical trial demonstrated improved regional but not local tumor control in larynx cancer patients treated with nicotinamide, carbogen, and radiation versus radiation alone.[46] Biologics such as antivascular endothelial growth factor (anti-VEGF) therapy have also been demonstrated to increase tumor oxygenation.[47] Anti-VEGF therapy may increase tumor oxygenation by eliminating abnormal vessels that are inadequate in perfusing tumor cells—the so-called *vascular normalization hypothesis*. Although there is solid experimental evidence to support this hypothesis, there appears to be only a short window of time in which it could be effectively combined with radiotherapy.

Because the presence of hypoxia has both prognostic and potential therapeutic implications, a substantial effort has been invested in trying to image hypoxia.[48] The goal of using imaging to "paint" radiation doses to different regions of tumors, although technically possible (as described in the next section, Radiation Physics), faces the problem that changes in oxygenation are dynamic.[49] In the future, hypoxia-directed treatment may evolve from the use of hypoxic cell cytotoxins to targeted drugs that exploit cellular signaling changes induced by hypoxia such as hypoxia-inducible factor 1α (HIF-1α). However, despite the strong rationale supporting their use, at this time, there are no agents used in the clinic that target hypoxia.

Immune Response

The abscopal effects of radiation (i.e., tumor cell killing outside of the radiation field) have been attributed to the activation of antigen and cytokine release by radiation, which subsequently activates a systemic immune response against tumor cells.[50,51] This response begins with the transfer of tumor cell antigens to dendritic cells and, subsequently, the activation of tumor-specific T cells and *immunogenic tumor cell death*. It is likely that radiation dose and fractionation influence the optimal immune

response with higher doses and fewer fractions of radiation than those used in conventional fractionation schemes appearing superior in experimental models. Unfortunately, abscopal effects are uncommon because immune system evasion is an inherent characteristic of cancer cells that often dominates, even in the presence of a radiation-induced immune response. Strategies to amplify radiation-induced immune responses, and thus to overcome tumor cell evasion of the immune system, are under investigation. The combination of radiation with immune *checkpoint* modulators such as ipilimumab, an antibody against cytotoxic T-lymphocyte antigen 4 (CTLA-4), have shown promising, albeit anecdotal, clinical effects.

DRUGS THAT AFFECT RADIATION SENSITIVITY

For over 30 years now, chemotherapy and radiotherapy have been administered concurrently. In order to maximize the efficacy of radiochemotherapy, it is necessary to understand the biologic mechanisms underlying radiosensitization by chemotherapeutic agents. The several classes of standard chemotherapeutic agents as well as novel molecularly targeted agents that possess radiosensitizing properties will be discussed in this section.

Antimetabolites

5-fluorouracil is among the most commonly used chemotherapeutic radiation sensitizers. Given in combination with radiation, it has led to clinical improvements in a variety of cancers, including those of the head and neck, the esophagus, the stomach, the pancreas, the rectum, the anus, and the cervix. The combination of 5-fluorouracil with radiation is now a standard therapy for cancers of the stomach (adjuvant), the pancreas (unresectable), and the rectum. For other cancers such as head and neck, esophagus, or anal, 5-fluorouracil and radiation are combined with cisplatin or mitomycin C, respectively. Being an analog of uracil, 5-flourouracil is misincorporated into RNA and DNA. However, the ability of 5-fluorouracil to radiosensitize is related to its ability to inhibit thymidylate synthase, which leads to the depletion of thymidine triphosphate (dTTP) and the inhibition of DNA synthesis. This slowed, inappropriate progression through S phase in response to 5-fluorouracil is thought to be the mechanism underlying radiosensitization.[52] Similar to 5-fluorouracil, the oral thymidylate synthase inhibitor, capecitabine, is also being increasingly used in combination with radiation.

Gemcitabine (2′, 2′-deoxyfluorocytdine [dFdCyd]) is another potent antimetabolite radiosensitizer. Preclinical studies have demonstrated that radiosensitization by gemcitabine involves the depletion of deoxyadenosine triphosphate (dATP) (related to the ability of gemcitabine diphosphate (dFdCDP) to inhibit ribonucleotide reductase) as well as the redistribution of cells into the early S phase of the cell cycle.[53] The combination of gemcitabine with radiation in clinical trials has suggested improved clinical outcomes for patients with cancers of the lung, pancreas, and bladder. Gemcitabine-based chemoradiation has developed into a standard therapy for locally advanced pancreatic cancer. However, in some clinical trials, such as those in lung and head and neck cancers, the combination of gemcitabine with radiation has led to increased mucositis and esophagitis.[54] Thus, it should be emphasized that in the presence of gemcitabine, radiation fields must be defined with great caution. Such is the case with pancreatic cancer, where the combination of full-dose gemcitabine with radiation to the gross tumor can be safely administered if clinically uninvolved lymph nodes are excluded.[55] Conversely, the inclusion of the regional lymphatics in the treatment field in combination with full-dose gemcitabine produces unacceptable toxicities.[56]

Platinums and Temozolomide

Cisplatin is likely the most commonly used chemotherapeutic agent in combination with radiation. Although cisplatin was the prototype for several other platinum analogs, carboplatin is also frequently used in combination with radiation. Cisplatin, in combination with radiation, and sometimes in conjunction with a second chemotherapeutic agent, is indicated for cancers of the head and neck, esophagus (with 5-fluorouracil), the lung, the cervix, and the anus. Radiosensitization by cisplatin is related to its ability to cause inter- and intra-strand DNA cross-links. Removal of these cross-links during the repair process results in DNA strand breaks. Although there are multiple theories to explain the mechanism(s) of radiosensitization by cisplatin, two plausible explanations are that cisplatin inhibits the repair (both homologous and nonhomologous) of radiation-induced DNA double-strand breaks and/or increases the number of lethal radiation-induced double-strand breaks.[57]

Temozolomide in combination with radiation is standard therapy for glioblastoma. Temozolomide is an alkylating agent, which forms methyl adducts at the O^6 position of guanine (as well as at N^7 and N^3-guanine) that are subsequently improperly repaired by the mismatch repair pathway. Radiosensitization by temozolomide involves the inhibition of DNA repair and/or an increase in radiation-induced DNA double-strand breaks due to radiation-induced single-strand breaks in proximity to O^6 methyl adducts. Like cisplatin, temozolomide-mediated radiosensitization does not seem to require cell cycle redistribution.

Taxanes

The taxanes, paclitaxel and docetaxel, act to stabilize microtubules resulting in the accumulation of cells in G2/M, the most radiation-sensitive phase of the cell cycle. The radiosensitizing properties of the taxanes are thought to be attributable to the redistribution of cells into G2/M. Paclitaxel, in combination with radiation (and carboplatin), has demonstrated a clinical benefit in the treatment of resectable lung carcinoma.[58]

Molecularly Targeted Agents

Molecularly targeted agents are especially appealing in the context of radiosensitization because they are generally less toxic than standard chemotherapeutic agents and need to be given in multimodality regimens (given their often inadequate efficacy as single agents). The EGFR has been intensely pursued as a target; both antibody and small molecule EGFR inhibitors, such as cetuximab and erlotinib, respectively, have been developed. The head and neck seem to be the most promising tumor sites for the combination of EGFR inhibitors with radiation therapy. Preclinical data have demonstrated that the schedule of administration of EGFR inhibitors with radiation is important; EGFR inhibition before chemoradiation may produce antagonism.[59] In a randomized phase III trial, cetuximab plus radiation produced a significant survival advantage over radiation alone in patients with locally advanced head and neck cancer.[60] In a subsequent trial, however, cetuximab in combination with concurrent, cisplatin-based chemoradiation failed to produce a survival benefit in head and neck cancer patients.[61] The combination of EGFR inhibitor with cisplatin-radiation requires further preclinical investigation.

Although EGFR inhibition, concurrent with radiation, is by far the best established combination of a molecularly targeted agent with radiation, other exciting molecularly targeted agents are being developed as radiation sensitizers. Targeting DNA damage response pathways is one approach to radiosensitization. Recently, agents that abrogate radiation-induced cell cycle checkpoints, such as Wee1 and Chk1 inhibitors, have been shown to radiosensitize

tumor cells and are currently in clinical development in combination with chemotherapy, with clinical trials planned in combination with radiation.[62,63] In addition, poly(ADP-ribose) polymerase (PARP) inhibitors have been demonstrated to preclinically induce radiosensitization, and several clinical trials combining PARP inhibitors with radiation therapy are underway.[64]

Other Agents

Although the most common clinically used agents in combination with radiation have been shown to produce significant clinical benefit, as described previously, other agents with different mechanisms of action have been used as radiation sensitizers as well as radiation protectors. The vinca alkaloids, such as vincristine, possess radiosensitizing properties due to their ability to block mitotic spindle assembly and, thus, arrest cells in M phase. Although vincristine is used in combination with radiation to treat medulloblastoma, rhabdomyosarcoma, and brain stem glioma, its use is principally based on its lack of myelosuppressive side effects, which are dose limiting for radiation in these types of tumors, rather than its potential radiosensitizing properties.

Also worth mention in a discussion of modulators of radiation sensitivity are agents designed to radioprotect normal tissues. One such type of drug, amifostine, is a free radical scavenger with some selectivity toward normal tissues that express more alkaline phosphatase than tumor cells, the enzyme of which converts amifostine to a free thiol metabolite. Clinical trials in head and neck as well as lung cancers have shown a reduction in radiation-related toxicities such as xerostomia, mucositis, esophagitis, and pneumonitis, respectively.[65,66] However, further clinical investigations are necessary to conclusively demonstrate a lack of tumor protection and safety in combination with chemoradiotherapy regimens.

RADIATION PHYSICS

Physics of Photon Interactions

Tumors requiring radiation can be found at depths ranging from zero to 10s of centimeters below the skin. The goal of treatment is to deliver sufficient ionizing radiation to the tumor site, which can result in an absorbed dose. This involves both the availability of treatment beams and delivery techniques, and the methods to plan the treatments and ensure their safe delivery. This section will establish the general physical basis for the use of ionizing radiation in the treatment of tumors, briefly describe some of the treatment equipment, indicate physical qualities of the treatment beams themselves, and summarize the treatment planning process. Those who desire more in-depth details are referred to textbooks and other resources dedicated to medical physics and the technologic aspects of radiation oncology.[67] Most patients who are treated with radiation receive high-energy, external-beam photon therapy. Here, *external* indicates that the treatment beam is generated and delivered from outside of the body. High-energy (6 to 20 MV) photon beams (electromagnetic radiation) penetrate tissue, enabling the treatment of deep-seated tumors. Modern equipment generates these beams with sufficient fluence to ensure delivery of therapeutic fractions of dose in short treatment sessions. Other types of particles and beams also exist for use in treating tumors both externally and internally. They are mentioned briefly later. However, as external photon beams dominate the practice (and as common basic physics principles related to delivered dose exist among the modalities), the focus here will be on photon beam generation and interactions in tissue.

As mentioned earlier, ionizing radiation kills cells via both direct and indirect mechanisms. Radiation therapy aims to instigate those ionizations and events in the tumor cells. Photons are massless, uncharged packets of energy that primarily interact with matter via electromagnetic processes. As a consequence of those interactions, an incident photon can become either entirely absorbed (giving up its energy to the ejection of an atomic electron [photoelectric effect]), or create an energetic electron-positron pair (pair production), or scatter off an electron with a reduction in energy and a change in direction and subsequent transfer of parts of its energy to the free electron (Compton scattering). The secondary electrons generated as a consequence of these interactions have residual energy, mass, and, most importantly, electric charge. They slow down in matter through multiple interactions with (primarily) the electrons of atoms, leading to excitation and ionization of those atoms. These ionizations (hence the term *ionizing radiation*) lead to a local absorption of energy (i.e., dose = energy absorbed per unit mass) and the direct and indirect cell killing effects necessary to treat tumors.

Thus, the use of external photon beams for cancer therapy involves a two-step process: interaction (scattering) of the photons, with subsequent dose deposition via the secondary electrons. The probability of photon interactions is energy dependent. Photoelectric interactions dominate at lower photon energies. Whereas these beams are ideal for diagnostic procedures (for their preferential absorption by tissues of differing atomic number, leading to good subject contrast), they are attenuated too quickly in tissues to supply enough interactions to be useful for therapy for any but the most superficial tumors. Pair production interactions dominate at higher photon energies; however, the probability of interacting in tissues for those high-energy photons is so low as to preclude them from general use as well. In the 10s to 100s of kiloelectron volt (keV) to the few megaelectron volt (MeV) photon energy range, Compton scattering dominates. As will be shown, these beams have sufficient penetration and can be generated with sufficient intensity to be useful for tumor treatments, especially when combined in treatment plans that comprise multiple beams entering the patient from different directions but overlapping at the tumor.

It is useful to point out physical scales of reference for external photon beam therapy. A typical megavoltage photon beam may have an average photon energy near 2 MeV. Those photons primarily undergo Compton scattering with a mean free path in tissue of approximately 20 cm. An average Compton interaction results in a secondary electron with a mean energy near 0.5 MeV (and a Compton scattered photon near 1.5 MeV, which likely escapes or scatters elsewhere in the patient). A typical secondary electron of approximately 0.5 MeV will cause excitations and ionizations of atoms as it dissipates its energy over a path length of approximately 2 mm. This could be expected to lead to approximately 10,000 ionizations, or about 5 ionizations per micron of tissue. As can be seen, therapeutic damage to the DNA of cancer cells (2 nm; see Fig. 13.2) will require very many Compton scatterings with statistical interaction among the ionizations resulting from the slowing down of the secondary electrons.

Photon Beam Generation and Treatment Delivery

As previously mentioned, effective external-beam photon treatments require higher energy beams capable of reaching deep-seated tumors with sufficient fluence to make it likely that the dose deposition will kill the tumor cells. To spare normal tissues and maximize targeting, beams are arranged to enter the patient from several directions and to intersect at the center of the tumor (treatment isocenter). Although machines containing collimated beams from high-intensity radioactive sources (primarily cobalt 60 [^{60}Co]) are still in use, today's modern treatment machine accelerates electrons to high (MeV) energy and impinges them onto an x-ray production target, leading to the generation of intense beams of Bremsstrahlung x-rays. A typical photon beam treatment machine[68,69] (Fig. 13.12) consists of a high-energy (6 to 20 MeV) linear electron accelerator, electromagnetic beam steering and

Figure 13.12 A shadow view of a C-arm linear accelerator. The electron beam (originating at upper right) is accelerated through a linear accelerator wave guide, selected for correct energy in a bending magnet, and then impinges on an x-ray production target. The x-ray beam (originating at target upper left) is flattened and collimated before leaving the treatment head. Also illustrated (downstream from the beam) is an electric portal imager that is used to measure (image) the beam exiting a patient. (From Varian Medical Systems, Palo Alto, CA, with permission.)

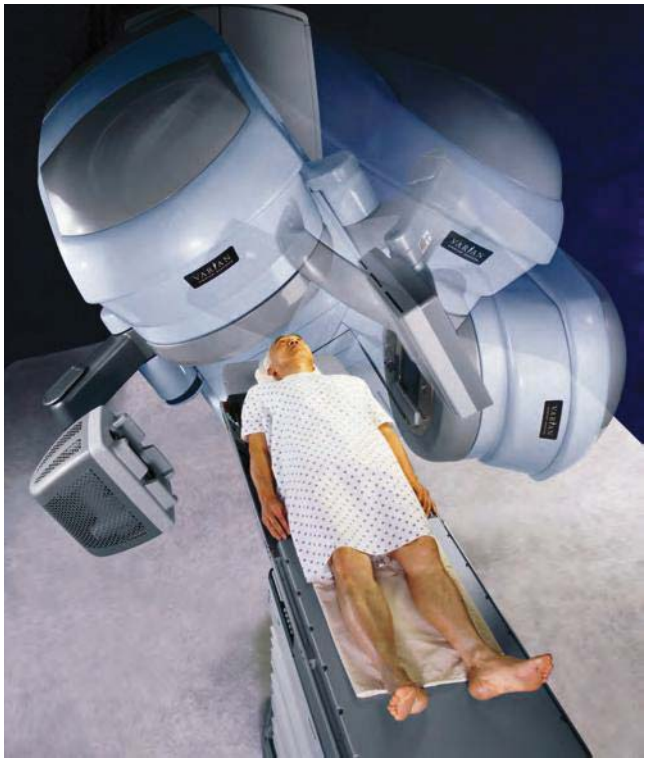

Figure 13.13 Model in treatment position on the patient support table. The treatment delivery head on the gantry's C-arm rotates about the patient, enabling the delivery of beams throughout 360 degrees of rotation. (From Varian Medical Systems, Palo Alto, CA, with permission.)

monitoring systems, x-ray generation targets, high-density treatment field-shaping devices (collimators), and up to a ton of radiation shielding on a mechanical C-arm gantry that can rotate precisely around a treatment couch (Fig. 13.13). These treatment-delivery machines routinely maintain mechanical isocenters for patient treatments to within a sphere of 1 mm radius. The development of *stereotactic radiotherapy*, which will be described in the section titled Clinical Application of Types of Radiation, depends on this level of machine precision.

X-ray production by monoenergetic high-energy electrons results in an x-ray (photon) beam that contains a continuous spectrum of energies with maximum photon energy near that of the incident electron beam. Lower energy photons appear with a much greater probability than do the highest energy ones, but they also become preferentially filtered out of the beam through the absorption in the target and the attenuation in the flattening filter. This generally results in a treatment beam energy spectrum with a mean photon energy of approximately one-third of the initial electron beam energy. In this energy range, the resulting photon beam exits the production target with a narrow angular spread focused primarily in the forward direction. These forward-peaked intensity distributions generally need to be modulated (flattened) to produce a large (up to 40 cm diameter at the patient) photon beam with uniform intensity across the beam. All modern treatment units take advantage of extensive computer control, monitoring, and feedback to produce highly stable and reproducible treatment beams.

The resulting photon beam requires beam shaping for conformal dose delivery. Some combination of primary, high-density field blocks (collimators) together with additional edge blocks generally provide the required shaping and shielding. Modern machines use computer-controlled multileaf collimators (Fig. 13.14)

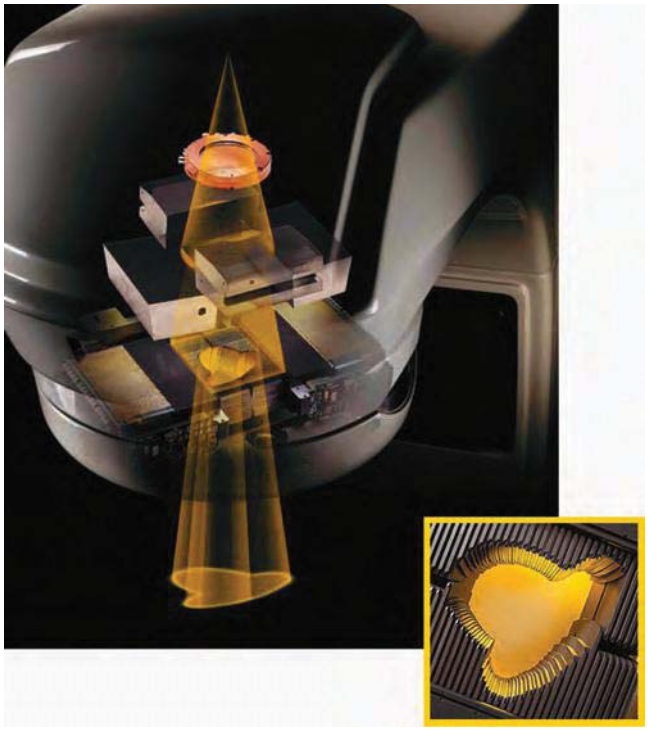

Figure 13.14 Multileaf collimator shaping of an x-ray treatment beam from a linear accelerator. *Inset* shows a view of the multileaf collimator. (From Varian Medical Systems, Palo Alto, CA, with permission.)

Figure 13.15 **(A)** A shadow view of linear accelerator, x-ray beam production system, and x-ray fan beam for helical tomotherapy treatment delivery. The beam production system rotates within its enclosed gantry. **(B)** The model patient on treatment table slides into the treatment unit. During treatment, the table moves as the collimated fan beam rotates about the patient, creating a modulated helical dose delivery pattern. (From TomoTherapy, Inc., Madison, WI, with permission.)

for the edge sculpting subsequent to setting the primary collimators for maximal shielding. This computer control provides high precision and reproducibility in the definition of field edges. Additionally, automation allows for a precise reshaping of the treatment beam for each angle of incidence, allowing not only conformation of irradiation to target volumes, but also modulation of the beam intensity patterns across the field (intensity-modulated radiation therapy [IMRT]).

Variations on the standard linear accelerator (linac) plus C-arm scenario that are being used for external-beam radiation treatments throughout the body include helical tomotherapy and nonisocentric miniature linac robotic delivery systems.[70] In helical tomotherapy, the accelerator, photon-production target, and collimation system are mounted on a ring gantry (similar to those found on diagnostic computed tomography [CT] scanners) (Fig. 13.15). It produces a fan beam of photons, and the intensity of each part of the fan being modulated by a binary collimator. As the gantry rotates, the patient simultaneously slides through the bore of the machine (again analogous to modern x-ray/CT imagers), which allows for the continuous delivery of intensity-modulated radiation in a helical pattern from all angles around a patient. Another delivery system uses an industrial robot to hold a miniature accelerator plus photon beam-production system (Fig. 13.16). The bulk of the system is reduced by keeping the field sizes small (spotlike). However, computer control of the robot provides flexibility in irradiating tumors from nearly any position external to the patient. The same control allows for the selection and use of many differing beam angles to build up the dose at the tumor location.

To take advantage of the precision of modern beam delivery, it is crucial to localize the patient's tumor and normal tissue.[71] This process can be divided into patient immobilization (i.e., limiting the motion of the patient) and localization (i.e., knowing the tumor and normal tissue location precisely in space). Although these concepts of immobilization and localization are related, they are not identical. Patients can be held reasonably comfortable in their treatment pose with the aid of foam molds and meshes (i.e., immobilization devices). Traditionally, localization has been achieved by indexing the immobilization device to the computer-controlled treatment couch and by using low-power laser beams aligned to skin marks. These techniques make it possible to reproducibly couple the surface of each patient with the treatment machine isocenter.

However, what is truly needed is to localize the tumor and normal tissues. The development of in-room, online x-ray, ultrasound, and infrared imaging equipment can now be used to ensure that the intended portions of each patient's internal anatomy are correctly positioned at the time of treatment. In particular, the development of rugged, low-profile, active matrix, flat-panel imaging devices, either attached to the treatment gantry or placed in the vicinity of the treatment couch, together with diagnostic x-ray generators or the patient treatment beam (see Fig. 13.13), allows the digital capture of projection x-ray images of patient anatomy with respect to the isocenter and treatment field borders. These digitized electronic images are immediately available for analysis. Software tools allow for a comparison to reference images and the generation of correction coordinates, which are in turn available for downloading to the treatment couch for automated fine adjustment of the patient's treatment position. Other precise localization systems rely on the identification of the positions of small, implanted radiopaque markers or other types of *smart* position-reporting devices. Careful use of these image-guided radiation

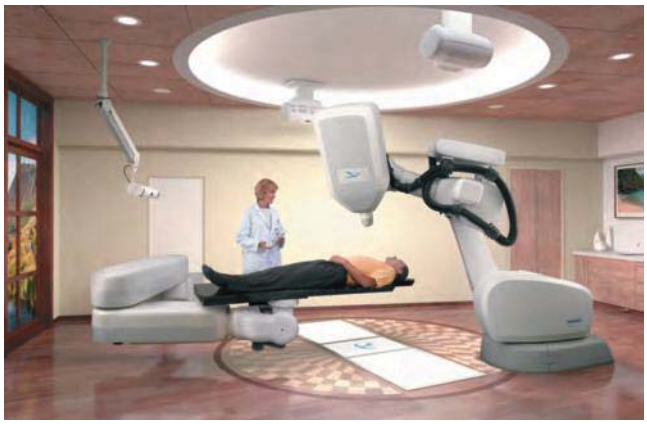

Figure 13.16 A miniature accelerator plus x-ray production system on a robotic delivery arm. Both the treatment table and the treatment head is set by a computer for multiple arbitrary angles of incidence. (From Accuray, Sunnyvale, CA, with permission.)

therapy (IGRT) systems[71, 72] can result in the repeated reducibility of patient position to within a few millimeters over a 5- to 8-week course of treatment.

The final part of external-beam patient treatment is dose delivery. All modern treatment units have computer monitoring (and often control) of all mechanical and dose-delivery components. Treatment-planning information (treatment machine parameters, treatment field configurations, dose per treatment field segment) is downloaded to a work station at the treatment unit that first assists with and then records treatment. This information, together with the readbacks from the treatment machine, are used to reproducibly set up and then verify each patient's treatment parameters, which prevents many of the variations that used to occur when all treatment was performed simply by following instructions written in a treatment chart.

Treatment Beam Characteristics and Dose-Calculation Algorithms

Beyond a basic understanding of the interactions of ionizing radiation with matter lies the requirement of being able to characterize the treatment beams for purposes of planning and verifying treatments. By virtue of a few underlying principles, this generally can be accomplished via a two-step process of absolute calibration of the dose at some reference point in a phantom (i.e., measurement media representative of a patient's tissues), with relative scaling of dose values in other parts of the beam or phantom with respect to that point.

As mentioned earlier, the predominant mode of interaction for therapeutic energy photon beams in tissuelike materials is through Compton scattering. The probability of Compton scattering events is primarily proportional to the relative electron density of the media with which they interact. Because many body tissues are waterlike in composition, it has been possible to make photon beam dosimetric measurements in phantoms consisting mostly of water (water tanks) or tissue-equivalent plastic and to then scale the interactions via relative electron density values (for example, as can be derived from computed x-ray/CT) to other waterlike materials. Thus, the relative fluence of photons in a therapeutic treatment beam is attenuated as it passes through a phantom, primarily via Compton scattering.

It was stated earlier that the photon beam is generated at a small region in the head of the machine. That fluence of photons spreads out through the collimating system before reaching the patient. Thus, without any interactions (e.g., if the beam were in a vacuum), the number of photons crossing any plane perpendicular to the beam direction would remain constant. However, the cross-sectional area of the plane gets larger the farther it is located from the source point. In fact, both the width and length of the cross-sectional area increase in proportion to the distance from the source, and thus the area increases in proportion to the square of the distance. This means that the primary photon fluence per unit area in a plane perpendicular to the beam direction of a pointlike source also decreases as one over the square of the distance, the so-called $1/r^2$ reduction in fluence as a function of distance, r, from the source.

Thus, we have two processes, attenuation and $1/r^2$ reduction, which reduce the photon fluence from an external therapeutic beam as a function of depth in a patient. There is also a process that can increase the photon fluence at a point downstream. Recall that Compton scattering interactions lead not only to secondary electrons (which are responsible for deposition of dose), but also to Compton scattered photons. These photons are scattered from the interaction sites in multiple, predominantly forward-looking directions. Thus, Compton-scattered photons originating from many other places can add to the photon fluence at another point. As the irradiated area (field size) increases, the amount of scattered radiation also increases.

As mentioned earlier, dose *deposition* is a two-step process of photon interaction (proportional to the local fluence of photons) and energy transfer to the medium via the slowing down of secondary electrons. Thus, the point where a photon interacts is not the place where the dose is actually deposited, which happens over the track of the secondary electron. Dose has a very strict definition of energy *absorbed* per unit mass (i.e., due to the slowing down charged particles) and should be distinguished from the energy released at a point, defined as kerma (e.g., energy transfer from the scattering incident photon). Thus, although the photon beam fluence will always be greatest at the entrance to a patient or phantom, the actual *absorbed dose* for a megavoltage photon beam builds up over the first couple of centimeters, reaching a maximum (d-max) at a depth corresponding to the range of the higher energy Compton electrons set in motion. This turns out to be a second desirable characteristic of these beams (beyond their ability to treat deep-seated lesions), because the dose to the skin (a primary dose-limiting structure in earlier times) is greatly reduced.

The relative distributions of dose, normalized to an absolute dose measurement (using a small thimblelike air ionization chamber at a standard depth and for a standard field size according to nationally and internationally accepted protocols), are the major inputs into treatment-planning systems. The major features of these distributions are (1) the initial dose buildup up to a depth of d-max, with a more gradual drop off in dose as a function of depth into the phantom due to the attenuation and $1/r^2$ factors at deeper depths (relative depth dose), and (2) the shape of the dose in the plane perpendicular to the direction of the beams; both as a function of field size. Central axis depth dose curves for typical external photon beams are shown in Figure 13.17 for two beam energies and for both a large and smaller field size. Notice both the expected increase in penetration with increasing beam energy and the increase in dose at a particular depth with increasing field size; the latter effect due to increased numbers of secondary Compton-scattered photons for larger irradiated areas. The change in dose perpendicular to the central axis is less remarkable, because the beams are designed to be uniform across a field as a function of depth.

It is useful to also point out the depth dose characteristics of clinical external treatment beams produced using ionizing

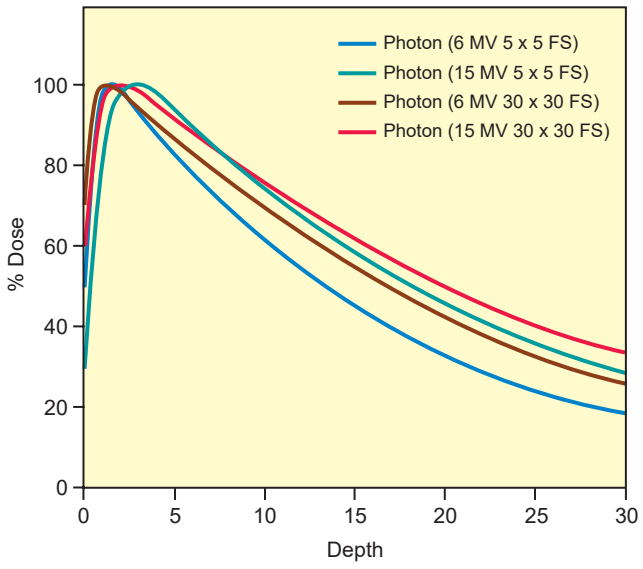

Figure 13.17 Sample depth-dose curves (change in delivered dose as a function of depth) along the central axis of some typical photon treatment beams for low (6 MV) and intermediate (15 MV) energy beams, and large (30×30 cm^2) and smaller (5×5 cm^2) field sizes (FS).

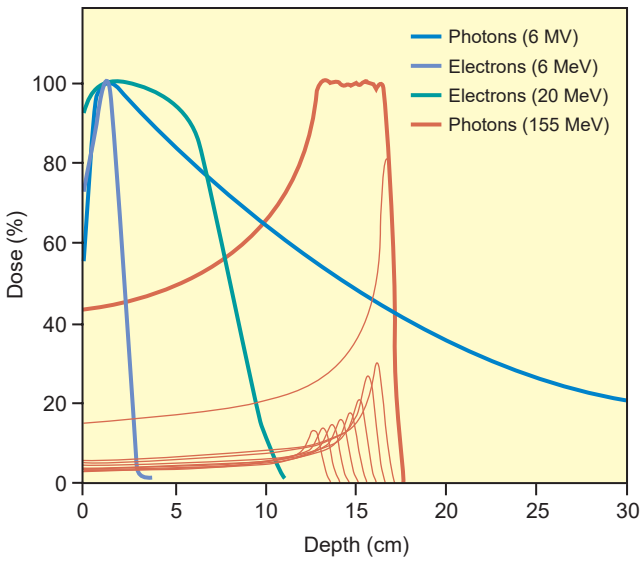

Figure 13.18 Sample depth-dose curves along the central axis of some typical charged particle treatment beams compared with that of a 6-MV proton beam. The spread out Bragg peak at the end of the 155-MeV proton beam (*thick pink curve*) is a composite dose deposition pattern from the addition of the multiple range-shifted proton curves (*thinner pink curves*).

radiations other than photons, primarily through the direct use of charged particles. Those beams (Fig. 13.18) illustrate interesting characteristics, which, when added to the options available for treatment planning (or used by themselves), can produce advantageous results. Relative to the photon beam, the direct use of electron beams leads to deposition of dose over a more localized range, but at the expense of a relative lack of penetration. Thus, electron beams are most widely used for treating, or boosting the treatment of, more superficial tumors and regions (see the section titled Clinical Application of Types of Radiation). The heavier charged particle beams (protons and carbon ions) appear to exhibit even more interesting *depth-dose* characteristics, with the advantage of both (when necessary) being highly penetrating and also lacking a significant dose beyond a certain depth (a depth that can be controlled and purposefully placed, for example, at the distal edge of a target volume).

The results of measurements such as these have been modeled so as to develop dose-calculation algorithms used in treatment-planning systems. These models all use measured beam data to set or adjust parameters used by those algorithms in their dose-distribution computations. Because most of the input data used for beam fitting come from measurements in water phantoms (or waterlike plastic phantoms), patient-specific adjustments are needed for the water phantom data to account for both geometry and tissue properties. It is the task of the dose-calculation algorithms to take those changes into account. The accuracy and precision actually realized for all dose-calculation algorithms generally need to be traded off against the time required to complete the calculation. Although the availability of ever more powerful computers has made calculation time less of a concern for broad, open-beam treatment planning, issues still remain for more specialized planning exercises that use many small beams or parts of beams such as IMRT (discussed later). Typically, relative dose distributions can be computed within patients on the scale of a few millimeters with a precision of better than a few percentage points.

An important area of research is the development of treatment-planning systems that calculate dose based on the principles of how radiation interacts with tissues, rather than simply by fitting data. These approaches use Monte Carlo techniques,[71,73] which build a dose distribution by summing the calculated paths of thousands of photons and scattered electrons. This approach is more accurate than beam-fitting algorithms in regions of differing tissue densities, such as the lung, and therefore, will ultimately replace the current generation of treatment-planning systems, particularly for complex conditions. However, the time to perform these calculations is still prohibitive for a clinic, and it is anticipated that Monte Carlo calculations will be introduced over a period of years by balancing the need for accuracy in a particular clinical situation with the need to initiate patient treatment.

TREATMENT PLANNING

As discussed in the previous section, single-treatment beams usually deposit more of the dose closer to where they enter the patient than they do at depths corresponding to where a deep-seated tumor might be located. The use of multiple beams entering the patient from different directions that overlap at the target produces more dose per unit volume throughout the tumor volume than is received by normal tissues. In fact, as noted earlier, the treatment-delivery machines are designed to make this easy to accomplish. Planning patient treatments under these circumstances should be a somewhat trivial matter of first selecting a sufficient number of beam angles to realize the desired buildup of the dose in the overlap region relative to the doses in the upstream parts of each beam, and then second, designing beam apertures that shape the edges of the beams to match the target. However, dose-limiting normal tissues often also lie in the paths of one or more of the beams. These normal tissues are often more sensitive to radiation damage than the tumor, and regardless, it is best practice to minimize the dose in any case as a general principle. Computerized treatment-planning systems function to develop patient-specific anatomic or geometric models and then use these models together with the beam-specific dose deposition properties (derived from phantom measurements, as previously described) to select beam angles, shapes, and intensities that meet an overall prescribed objective. That is, modern radiation oncology dose prescriptions contain both tumor and normal tissue objectives, and the modern computerized treatment-planning systems make it possible to design treatments that meet these objectives.

The development and use of three-dimensional (3D) models of each patient's anatomy, treatment geometry, and dose distribution led to a paradigm shift in radiation therapy treatment planning. Computerized radiation treatment planning began in the 1980s as a mainly x-ray/CT–based reconstruction of 3D geometries from information manually contoured on multiple two-dimensional (2D) transverse CT images. Today, these models often incorporate imaging data from multiple sources. Geometrically accurate anatomic information from an x-ray/CT scan still anchors these studies (as well as provides tissue density information necessary for dose calculations). However, it is now quite common to also register the CT data set with other studies such as magnetic resonance imaging (MRI), which may add anatomic detail for soft tissues, or functional MRI or positron emission tomography (PET) studies,[74,75] which provide physiologic or molecular information about tumors and normal tissues. Once registered with each other, the unique or complementary information from each data set can be fused for inspection and incorporated into the design of each patient's target and normal tissue volumes (Fig. 13.19). Beyond the ability to more fully define the extent of the primary target volume (for instance, as the encompassing envelope of disease appreciated on all the imaging studies) lies the ability to define subvolumes of the tumor volume that might be appropriate for simultaneous treatment to higher dose. For example, it should soon become possible to define different biologic components of the tumor that could potentially be targeted and then monitored for response using these same imaging techniques.[76]

Current treatment planning makes the tacit assumption that the planning image yields "the truth" about the location and condition

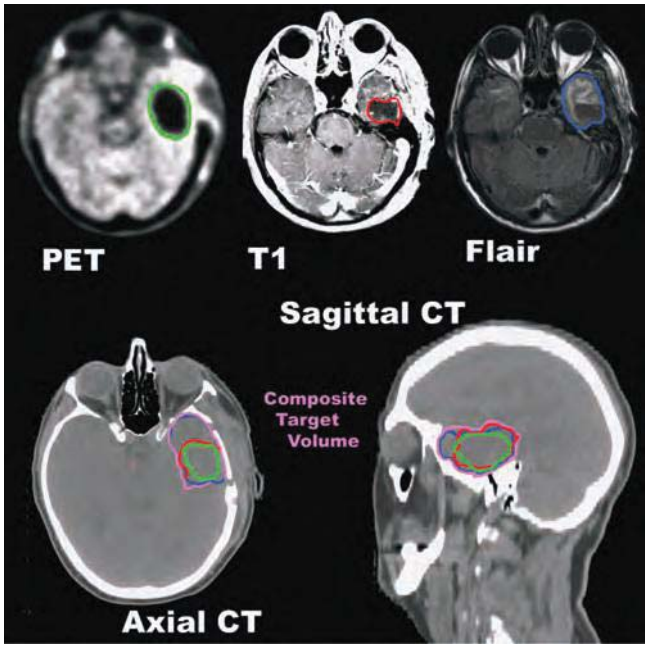

Figure 13.19 An illustration of the brain tumor target volume delineated on coregistered nuclear medicine and magnetic resonance imaging studies fused with computed tomography (CT) data for treatment planning. PET, positron emission tomography.

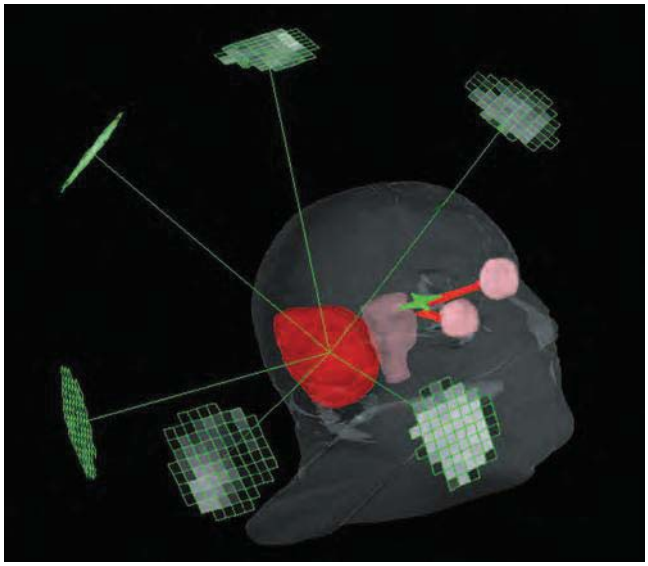

Figure 13.20 Six intensity-modulated treatment ports planned for treatment of a brain tumor (*large object in red*). Differing intensities of the 5 × 5 mm *beamlets* in each port illustrated by gray scale (brighter beamlet = higher intensity). The computer optimization of the beamlet intensities is designed to generate a delivered dose distribution that will conform to the tumor region, yet avoid critical normal tissues such as the brain stem (*dark pink*), optic chiasm (*green*), and optic nerves (*red tubular structures*).

of tumors and normal tissues throughout the course of treatment. However, this ignores the complexity inherent in attempting to build accurate 3D models from multimodality imaging for purposes of planning patient treatments. First, patients breathe and undergo other physiologic processes during a single treatment, changes that require dynamic modeling or other methods of accounting for the changes. Furthermore, the patient's condition may change over time (and hence their model). Thus, a complete design and assessment of a patient undergoing high-precision treatment requires the construction of four-dimensional (4D) patient models. Indeed, the recent ready availability of multidetector CT scanners with subsecond gantry rotations, and even more recently, the availability of cone-beam CT capabilities on the radiation therapy treatment simulators and treatment machines themselves, now makes it possible to construct 4D patient models. A very active area of physics research[72,75] deals with IGRT, including the formation of 4D patient models (including distortions and changes in anatomy) of the motion over time and the determination of the accumulated dose received by a moving tumor as well as the surrounding normal tissues such as uninvolved lung.

Complementary to the availability of these patient and dose models has come a much better understanding of the doses safely tolerated by normal tissues adjacent to a tumor volume (e.g., spinal cord) or surrounding it (e.g., brain, lung, liver).[77] Indeed, not only has knowledge of whole organ tolerances to irradiation been obtained, but it has also become possible to characterize in some detail the complex dependence of the probability of incurring a complication with respect to the highly (intentionally) inhomogeneous dose distributions these normal tissues receive as part of the planning process designed to avoid treating them. Modeling partial organ tolerances to irradiation is of great use in planning patient treatments because it enables[78] integration and manipulation of variable dose and volume distributions with respect to possible clinical outcomes.

Making the vast amount of tumor and normal tissue information useful for planning treatments requires equally sophisticated new ways of planning and delivering dose, potentially preferentially targeting subvolumes of the tumor regions or specifically avoiding selected portions of adjacent organs at risk. As mentioned earlier, modern treatment machines are capable of either varying the intensity of the radiation across each treatment port or projecting many small beams at a targeted region. This modulation of beam intensities (IMRT) from a given beam direction, together with the use of multiple beams (or parts of beams) from different directions, gives many degrees of freedom to create highly sculpted dose distributions, given that a system for designing the intensity modulation is available. Much computer programming and computational analysis has gone into the design of treatment-planning optimization systems to perform these functions.[79,80]

In IMRT, as most often applied, each treatment beam portal is broken down into simple basic components called beamlets, typically 0.5 to 1 cm × 1 cm in size, evenly distributed on a grid over the cross-section of each beam. Optimization begins with precomputation of the relative dose contribution that each of these beamlets gives to every subportion of tumor and normal tissue that the beamlet traverses as it goes through the patient model. Sophisticated optimization engines and search routines then iteratively alter the relative intensities of each beamlet in all the beams to minimize a cost function associated with target and normal tissue treatment goals. These, often hundreds of beamlets (each with its own intensity) (Fig. 13.20), provide the necessary flexibility and degrees of freedom to create dose distributions that can preferentially irradiate subportions of targets and also produce sharp dose gradients to avoid nearby organs at risk (Fig. 13.21). The cost-function approach also facilitates the ability to include factors such as the normal tissue and tumor-response models, mentioned previously in the optimization process, thus integrating the overall effects of the complex dose distributions across whole organ systems or target volumes within the planning process.

OTHER TREATMENT MODALITIES

Other types of external-beam radiation treatments use atomic or nuclear particles rather than photons. Beams of fast neutrons have been used for some cancers,[81] primarily because of the

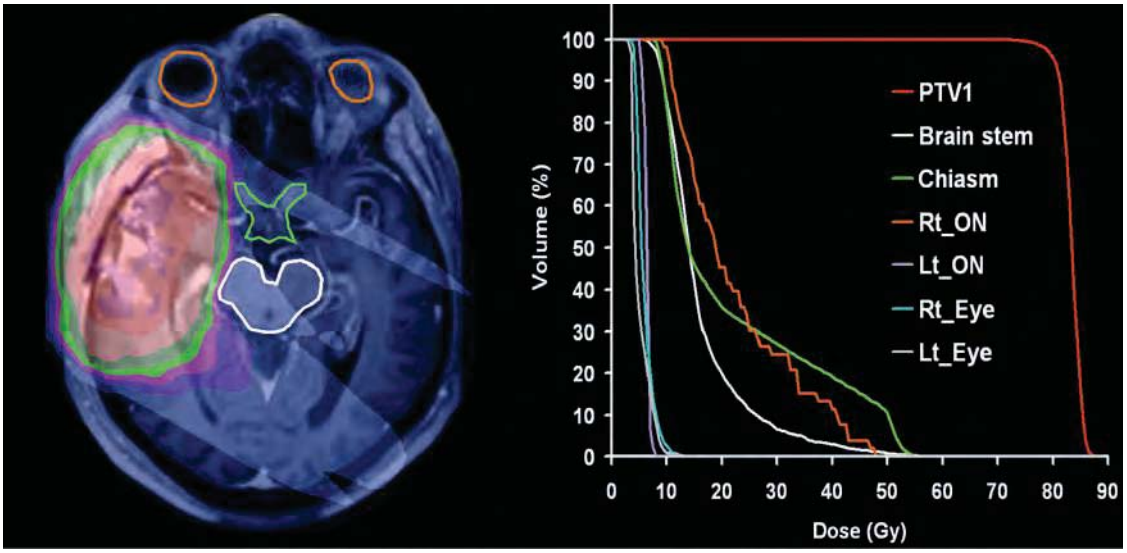

Figure 13.21 Resulting isodose distribution for an optimized intensity-modulated brain treatment. Dose-intensity pattern in the *left panel* is overlaid on the patient's magnetic resonance images used in planning. Also contoured are the optic chiasm (*green*), the brain stem (*white*), and the eyes (*orange*). In the *right panel,* the dose distribution throughout all slices of the patient's anatomy is summarized via cumulative dose-volume histograms for the various tissues and volumes that have been previously segmented. Each location on each curve represents the fraction of the volume of that tissue (%) that receives greater than or the same as the corresponding dose level.

dense ionization patterns they produce as they slow down in tissue (making cell killing less dependent on the indirect effect previously discussed). Being uncharged particles, neutron beams of therapeutic energy penetrate in tissue (have depth-dose characteristics) similar to photon beams, but with denser dose deposition in the cellular scale. Most other external-beam treatments use charged particles, primarily either electrons[82] (produced on the same machines used for photon beam treatments) or protons or heavier particles such as carbon ions.[83,84] The latter beams have desirable dose-deposition properties (see Fig. 13.18), because they can spare tissues downstream from the target volume and generally give less overall dose to normal tissue. There can also be some radiobiologic advantage to the heavier charged particle beams, similar to neutrons. The generation and delivery of proton beams and heavier charged particle beams generally requires an accelerator (in its own vault) plus a beam transport system and some sort of treatment nozzle, often located on an isocentric gantry. The cost of the accelerator is generally leveraged by having it supply beams to multiple treatment rooms, but these units still cost many times that of a standard linear accelerator.

Brachytherapy[85] is a form of treatment that uses direct placement of radioactive sources or materials within tumors (interstitial brachytherapy) or within body or surgical cavities (intracavitary brachytherapy), either permanently (allowing for full decay of short-lived radioactive materials) or temporarily (either in one extended application or over several shorter term applications). The ability to irradiate tumors from close range (even from the inside out) can lead to conformal treatments with low normal tissue doses. The radioactive isotopes most generally used for these treatments are contained within small tubelike or seedlike sealed source enclosures (which prevents direct contamination). They emit photons (gamma and x-rays) during their decay, which penetrate the source cover and interact with tissue via the same physical processes as described for external-beam treatments. The treatments have the advantage of providing a high fluence (and dose) very near each source that drops in intensity as 1 over the square of the distance from the source ($1/r^2$). Radioactive sources decay in an exponential fashion characterized by their individual half-lives. After each half-life ($T_{1/2}$) the strength of each source decreases by half. Brachytherapy treatments are further generally classified into the two broad categories of low–dose-rate and high–dose-rate treatments. Low–dose-rate treatments attempt to deliver tumoricidal doses via continuous irradiation from implanted sources over a period of several days. High–dose-rate treatments use one or more higher activity sources (stored external to the patient) together with a remote applicator or source transfer system to give one or more higher dose treatments on time scales and schedules more like external-beam treatments.

Isotopes for brachytherapy treatments are selected on the basis of a combination of specific activity (i.e., how much activity can be achieved per unit mass [i.e., to keep the source sizes small]), the penetrating ability of the decay photons (together with the $1/r^2$ fall off determines how many sources or source location will be required for treatment), and the half-life of the radioactive material (which must be accounted for in computation of dose, but also determines how often reusable sources will need to be replaced). Table 13.1 lists those isotopes most commonly used, along with some of their primary applications.

The dose-deposition patterns surrounding each type of source can be measured or computed. These data (or the parameterization

TABLE 13.1

Common Isotopes for Brachytherapy Treatment

Isotope	Form	Primary Applications
^{125}I	Implantable sealed seed	LDR: Permanent prostate implants, brain implants, tumor bed implants, eye plaques
^{192}Ir	Implantable sealed seed	LDR: Interstitial solid tumor treatments
^{192}Ir	High activity sealed source on a remote transfer wire	HDR: Intracavitary GYN treatments, intraluminal irradiations
^{137}Cs	Sealed source tubes	LDR: Intracavitary GYN treatments

LDR, low-dose rate; HDR, high-dose rate; GYN, gynecologic; ^{137}Cs, caesium-137.

of same) can be stored within a computerized treatment-planning system. Planning a brachytherapy treatment-delivery scheme (desirable source strengths and arrangements) proceeds within the planning system by distributing the sources throughout the treatment area and having the computer add up the contributions of each source to designated tumor and normal tissue locations (e.g., obtained from a CT scan). Source strengths or spacing can be adjusted until an acceptable result is obtained. Indeed, optimization systems are now routinely used to fine tune this process.

Other types of therapeutic treatments with internal sources of ionizing radiation, generally classified as systemic targeted radionuclide therapy (STaRT), use antibodies or other conjugates or carriers such as microspheres to selectively deliver radionuclides to cancer cells.[86] Computing the effective dose to tumors and normal tissues via these techniques requires information on how much of the injected activity reaches the targets (biodistribution) as well as the energy and decay properties of the radionuclide being delivered. Imaging techniques and computer models are aiding in these computations.

CLINICAL APPLICATIONS OF RADIATION THERAPY

In contrast to surgical oncology and medical oncology, which focus on early- or late-stage disease, respectively, the field of radiation oncology encompasses the1p8.49 entire spectrum of oncology. Board certification requires 5 years of postdoctoral training, typically beginning with an internship in internal medicine or surgery, followed by 4 years of radiation oncology residency. Education, as defined by leaders in the field,[87] begins with a thorough knowledge of the biology, physics, and clinical applications of radiation. It also includes training in the theoretical and practical aspects of the administration of radiation protectors and anticancer agents used as radiation sensitizers and the management of toxicities resulting from those treatments. In addition, residents receive education in palliative care, supportive care, and symptom and pain management. This training is in preparation for a practice that, in a given week, might include patients with a 2-mm vocal cord lesion or a 20-cm soft tissue sarcoma, both of whom can be treated with curative intent, as well as a patient with widely metastatic disease who needs palliative radiation, medical care for pain and depression, and discussion of end-of-life issues. More than 50% of (nonskin) cancer patients receive radiation therapy during the course of their illness.[88]

Clinical Application of Types of Radiation

Electrons are now the most widely used form of radiation for superficial treatments. Because the depth of penetration can be well controlled by the energy of the beam, it is possible to treat, for instance, skin cancer, a small part of the breast while sparing the underlying lung, or the cervical lymph nodes but not the spinal cord, which lies several centimeters more deeply. Superficial tumors, such as of skin cancers, can also be treated very effectively with low-energy (kilovoltage) photons, but their use has decreased because a separate machine is required for their production.

The main form of treatment for deep tumors is photons. As described in the Radiation Physics section, photons spare the skin and deposit dose along their entire path until the beam leaves the body. The use of multiple beams that intersect on the tumor permit high doses to be delivered to the tumor with a relative sparing of normal tissue. The pinnacle of this concept is IMRT, which uses hundreds of beams and can treat concave shapes with relative sparing of the central region (see Figs. 13.20 and 13.21). However, as each beam continues on its path beyond the tumor, this use of multiple beams means that a significant volume of normal tissue receives a low dose. There has been considerable debate concerning the magnitude of the risk of second cancers produced by radiating large volumes with low doses of radiation.[89] Charged particle beams (proton and carbon, in this discussion) differ from photons in that they interact only modestly with tissue until they reach the end of their path, where they then deposit the majority of their energy and stop (the Bragg peak; see Fig. 13.18). This ability to stop at a chosen depth decreases the region of low dose. The chief form of charged particle used today is the proton. In the decade from 1980 to 1990, proton therapy could deliver higher doses of radiation to the target than photon therapy because protons could produce a more rapid fall off of dose between the target and the critical normal tissue (e.g., tumor and brain stem). Therefore, initially, their main application was in the treatment uveal melanomas, base-of-skull chondrosarcomas, and chordomas. In contrast, today's IMRT photons are more conformal in the high-dose region than protons due to the range uncertainty of the latter.[90] Thus, it seems unlikely that protons will permit a higher target dose to be delivered than photons. In contrast, protons have the potential to decrease regions of low dose. This would be of particular advantage in the treatment of pediatric malignancies, where low doses of radiation would tend to increase the chance of second cancers and could affect neurocognitive function in the treatment of brain tumors.

A carbon ion beam has an additional potential biologic advantage over protons. As discussed in the section Biologic Aspects of Radiation Oncology, hypoxic cells, which are found in many tumors, are up to 3 times more resistant to photon or proton radiation than well-oxygenated cells. In contrast, hypoxia does not cause resistance to a carbon beam. Whether hypoxia is a cause of clinical resistance to fractionated radiation is still debated.[91] A carbon beam is available at a few sites in Europe and Japan.

Two major issues have affected the widespread acceptance of protons. The most widely recognized is cost. Proton (approximately $120 million) and carbon beam facilities (in excess of $200 million) are substantially more expensive than a similar-sized photon facility (approximately $25 million). The operating costs appear to be significantly higher as well. Although the majority of patients who have received proton therapy have prostate cancer, there is no evidence that protons produce superior results to those obtained with IMRT planned photons.[92,93] The lack of solid evidence that protons are superior to photons for any disease site and the magnitude of these costs are of societal importance.[94] Although less expensive single gantry proton units are under construction, there are no functioning units at the time of this writing. A second, less well-appreciated issue concerns the need to develop full integration of charged particle beams with IGRT, as has already been accomplished with photons, although this feature is being incorporated into second-generation proton units.

Neutron therapy attracted significant interest in the 1980s, based on the principle that it would be more effective than photons against hypoxic cells that some have thought are responsible for radiation resistance of tumors. The effectiveness of neutron therapy has been limited by initial difficulties with collimation and targeting, although there is evidence that they have a role in the treatment of refractory parotid gland tumors.[95]

Brachytherapy refers to the placement of radioactive sources next to or inside the tumor. The chief sites where brachytherapy plays a role are in prostate and cervical cancer, although it has applications in head and neck cancers, soft tissue sarcomas, and other sites. In the case of prostate cancer, most experience is with low–dose-rate permanent implants using iodine-125 (^{125}I) or, more recently, palladium-103 (^{103}Pd). Over the last 5 years, there has been an increasing emphasis on improving the accuracy of seed placement, guided by ultrasound and confirmed by CT or MRI, and in skilled hands, outstanding results can be achieved.[96] In the case of cervical cancer, high–dose-rate treatment, which can be performed in an outpatient setting, has essentially replaced low–dose-rate treatment, which typically requires general anesthesia and a 2-day hospital stay. The results from both techniques appear to be approximately equivalent.

Yttrium microspheres represent a distinct form of brachytherapy. These spheres carry yttrium-90 (^{90}Y), a pure beta emitter with a range of about 1 cm. These have been used to treat both primary hepatocellular cancer and colorectal cancer metastatic to the liver (hepatic arterial or systemic chemotherapy) by administration through the hepatic artery.

TREATMENT INTENT

Radiation doses are chosen so as to maximize the chance of tumor control without producing unacceptable toxicity. The dose of radiation required depends on the tumor type, the volume of disease (number of tumor cells), and the use of radiation-modifying agents (such as chemotherapeutic drugs used as radiation sensitizers). Except for a subset of tumors that are exquisitely sensitive to radiation (e.g., seminoma, lymphoma), doses that are required are often close to the tolerance of the normal tissue. A key fact driving the choice of dose is that a 1-cm^3 tumor contains approximately 1 billion cells. It follows that the reduction of a tumor that is 3 cm in diameter to 3 mm, which would be called a complete response by CT scan, would still leave 1 million tumor cells. Because each radiation fraction appears to kill a fixed fraction of the tumor, the dose to cure occult disease needs to be more similar to the dose for gross disease than one might otherwise expect. Thus, radiation doses (using the standard fractionation) of 45 to 54 Gy are typically used in the adjuvant setting when there is moderate suspicion for occult disease, 60 to 65 Gy for positive margins or when there is a high suspicion for occult disease, and 70 Gy or more for gross disease.

It is common during the course of radiation to give higher doses of radiation to regions that have a higher tumor burden. For example, regions that are suspected of harboring occult disease may be targeted to receive (in once daily 2-Gy fractions) 54 Gy, whereas, to control the gross tumor, the goal may be to administer a total dose of 70 Gy. Because the gross tumor will invariably reside within the region at risk for occult disease, it has become standard practice to deliver 50 Gy to the entire region, and then an additional *boost* dose of 20 Gy to the tumor. This sequence is called the *shrinking field technique*. With the development of IMRT, it has become possible to treat both regions with a different dose each day and achieve both goals simultaneously. For example, on each of the 35 days of treatment, the gross tumor might receive 2 Gy, and the region of occult disease 1.7 Gy, for a total dose of 59.5 Gy, which is of approximately equal biologic effectiveness to 54 Gy in 1.8-Gy fractions because of the lower dose per fraction (see the section Biologic Aspects of Radiation Oncology).

Radiation therapy alone is often used with curative intent for localized tumors. The decision to use surgery or radiation therapy involves factors determined by the tumor (e.g., is it resectable without a serious compromise in function?) and the patient (e.g., is the patient a good operative candidate?). The most common tumor in this group is prostate cancer, but patients with early-stage larynx cancer often receive radiation for voice preservation, and there are many patients with early-stage lung cancer who are not operative candidates. Control rates for these early-stage lesions are in excess of 70% (and as high as 90% for early-stage larynx cancer) and are usually a function of tumor size.

Stereotactic body radiation therapy (SBRT; sometimes called *stereotactic ablative radiation*) uses many (typically more than eight) cross-firing beams and provides an improved method of curing early-stage lung cancer[97] and liver metastases.[98] This approach uses precise localization and image guidance to deliver a small number (less than five) of high doses of radiation, with the concept of ablating the tumor, rather than using fractionation to achieve a therapeutic index (see the section title Fractionation). SBRT can provide long-term, local control rates of >90% for tumors less than 4 to 5 cm with minimal side effects.

Locally advanced or aggressive cancers can be cured with radiation alone or with a combination of radiation and chemotherapy or a molecularly targeted therapy. The most common examples here are locally advanced lung cancer, head and neck, esophageal, and cervix cancers, with cure rates in the 15% to 40% range, and are discussed in detail in their own chapters. A general principle that has emerged during the last decade is that combination chemoradiation has increased the cure rates of locally advanced cancers by 5% to 10% at the cost of increased toxicity.

An important consideration in the use of radiation (with or without chemotherapy) with curative intent is the concept of organ preservation. Perhaps the best example of achieving organ preservation in the face of gross disease involves the use of chemotherapy and radiation to replace laryngectomy in the treatment of advanced larynx cancer. Combined radiation and chemotherapy does not improve overall survival compared with radical surgery; however, the organ-conservation approach permits voice preservation in approximately two-thirds of patients with advanced larynx cancer.[99] The treatment of anal cancer with chemoradiation can also be viewed in this light, with chemoradiotherapy producing organ conservation and cure rates superior to radical surgery used decades ago.[100] Multiple randomized trials have demonstrated that lumpectomy plus radiation for breast cancer produces survival rates equal to that of modified radical mastectomy, while allowing for the preservation of the breast.

In the last decade, it has become clear that some patients with metastatic disease can be cured with radiation (with or without chemotherapy). The concept underlying this approach was established by the surgical practice of resecting a limited number of liver or lung metastases. A significant fraction of patients have a limited number of liver metastases that cannot be resected because of location, but are able to undergo high-dose radiation (often combined with chemotherapy). This radical approach to *oligometastases*[101] can produce 5-year survivals in the range of 20% in selected patients.[102] Patients with a limited number of lung metastases from colorectal cancer or soft tissue sarcomas are now being approached with stereotactic body radiation with a similar concept as has been used to justify surgical resection.[102] In addition to the direct effect of radiation on metastatic tumor, there is now anecdotal but provocative evidence that radiation can stimulate the immune system so that tumors distant from the irradiated tumor can respond. Distant (abscopal) responses have been reported in patients who receive immune checkpoint inhibitors such as ipilimumab.[103]

Radiation therapy can also contribute to the cure of patients when used in an adjuvant setting. If the risk of recurrence after surgery is low or if a recurrence could be easily addressed by a second resection, adjuvant radiation therapy is not usually given. However, when a gross total resection of the tumor is still associated with a high risk of residual occult disease or if local recurrence is morbid, adjuvant treatment is often recommended. A general finding across many disease sites is that adjuvant radiation can reduce local failure rates to below 10%, even in high-risk patients, if a gross total resection is achieved. If gross disease or positive margins remain, higher doses and/or larger volumes may be required, which may be less well tolerated and are less successful in achieving tumor control.

Adjuvant therapy can be delivered before or after definitive surgery. There are some advantages to giving radiation therapy after surgery. The details of the tumor location are known and, with the surgeon's cooperation, clips can be placed in the tumor bed, permitting increased treatment accuracy. In addition, compared with preoperative therapy, postoperative therapy is associated with fewer wound complications. However, in some cases, it is preferable to deliver preoperative radiation. Radiation can shrink the tumor, diminishing the extent of the resection, or making an unresectable tumor resectable. In the case of rectal cancer, the response to treatment may carry more prognostic information than the initial TNM staging.[104] In patients who will undergo significant surgeries (particularly a Whipple procedure or an esophageal resection), preoperative (sometimes called neoadjuvant) therapy can be more reliably administered than postoperative therapy. Most importantly, after resection of abdominal or pelvic tumors (such as

rectal cancers or retroperitoneal sarcomas), the small bowel may become fixed by adhesions in the region requiring treatment, thus increasing the morbidity of postoperative treatment. A randomized trial has shown that preoperative therapy produces fewer gastrointestinal side effects and has at least as good efficacy as postoperative adjuvant therapy for locally advanced rectal cancer.[105] Taken together, there appears to be a trend toward preoperative or neoadjuvant therapy in cancers of the gastrointestinal track (esophagus, stomach, pancreas, rectum), postoperative radiation seems to be favored in head and neck, lung, and breast cancer, and soft tissue sarcoma seems equally split.

The effectiveness of adjuvant therapy in decreasing local recurrence has been demonstrated in randomized trials in lung, rectal, and breast cancers. More recently, randomized trials have shown that postmastectomy radiation improved the survival for women with breast cancer and four or more positive lymph nodes, all of whom also received adjuvant chemotherapy. A fascinating analysis has revealed that, across many treatment conditions, each 4% increase in 5-year local control is associated with a 1% increase in 5-year survival.[106] It has been proposed that the long-term survival benefit of radiation in these more recent studies was revealed by the introduction of effective chemotherapy, which prevented such a high fraction of women from dying early with metastatic disease.[107] This concept has been developed into a hypothesis that the effect of adjuvant radiation on survival will depend on the effectiveness of adjuvant chemotherapy. If chemotherapy is either ineffective or very effective, adjuvant radiation may have little influence on the survival in a disease in which systemic relapse dominates survival. Radiation will have its greatest impact on survival when chemotherapy is moderately effective.[108]

In addition to these curative roles, radiation plays an important part in palliative treatment. Perhaps most importantly, emergency irradiation can begin to reverse the devastating effects of spinal cord compression and of superior vena cava syndrome. A single 8-Gy fraction is highly effective for many patients with bone pain from a metastatic lesion. There is increasing evidence of the effectiveness of body stereotactic radiation to treat vertebral body metastases in patients who have a long projected survival or who need retreatment after previous radiation.[109] Stereotactic treatment can relieve symptoms from a small number of brain metastasis, and fractionated whole-brain radiation can mitigate the effects of multiple metastases. Bronchial obstruction can often be relieved by a brief course of treatment as can duodenal obstruction from pancreatic cancer. Palliative treatment is usually delivered in a smaller number of larger radiation fractions (see the section titled Fractionation) because the desire to simplify the treatment for a patient with limited life expectancy outweighs the somewhat increased potential for late side effects.

FRACTIONATION

Two crucial features that influence the effectiveness of a physical dose of radiation are the dose given in each radiation treatment (i.e., the fraction) and the total amount of time required to complete the course of radiation. Standard fractionation for radiation therapy is defined as the delivery of one treatment of 1.8 to 2.25 Gy per day. This approach produces a fairly well-understood chance of tumor control and risk of normal tissue damage (as a function of volume). By altering the fractionation schemes, one may be able to improve the outcome for patients undergoing curative treatment or to simplify the treatment for patients receiving palliative therapy.

Two forms of altered fractionation have been tested for patients undergoing curative treatment: accelerated fractionation and hyperfractionation. Accelerated fractionation emerged from analyses of the control of head and neck cancer as a function of dose administered and total treatment time. It was found that with an increasing dose there was increasing local control, but that protraction of treatment was associated with a loss of local control that was equivalent to about 0.75 Gy per day.[110] The data were best modeled by assuming that, approximately 2 weeks into treatment, tumor cells began to proliferate more rapidly than they were proliferating early in treatment (called *accelerated repopulation*).[111] In accelerated fractionation, the goal is to complete radiation before the accelerated tumor cell proliferation occurs. The most common method of achieving accelerated fractionation is to give a standard fraction to the entire field in the morning and to give a second treatment to the boost field in the afternoon (called *concomitant boost*). As in standard radiation, the boost would be given by extending the length of the treatment course; this concomitant boost approach can shorten treatment from 7 weeks to 5 weeks in head and neck cancer.

The second approach to altering fractionation is called *hyperfractionation*. Hyperfractionation is defined as the use of more than one fraction per day separated by more than 6 hours (see the section titled Biologic Aspects of Radiation Oncology), with a dose per fraction that is less than standard. Hyperfractionation is expected to produce fewer late complications for the same acute effects against both rapidly dividing normal tissues and tumors. Pure hyperfractionation might give 1 Gy twice a day, so that the total dose per day would be 2 Gy, and thus be equal to standard fractionation. In practice, hyperfractionated treatments are usually in the range of 1.2 Gy, which means that, compared with a standard fractionation, a somewhat higher dose is administered during the same period of time (so that most hyperfractionation also includes modest acceleration). The overall effect is to increase the acute toxicity (which resolves) and tumor response, while not increasing the (dose-limiting) late toxicity, which can improve cure rate. Both accelerated fractionation and hyperfractionation have been demonstrated in a meta-analysis to be superior to standard fractionation in the treatment of head and neck cancer with radiation alone.[112] However, a recent randomized trial has shown that there is no increase in control or survival, but there is an increase toxicity using chemotherapy with hyperfractionation compared to standard chemoradiation; therefore, the use of altered fractionation schemes has decreased dramatically during the last few years.[113]

Hypofractionation refers to the administration of a smaller number of larger fractions than is standard. Hypofractionation might be expected to cause more late toxicity for the same antitumor effect than standard or hyperfractionation. In the past, this approach was reserved for palliative cases, with the sense that a modest potential for increased late toxicity was not a major concern in patients with limited life expectancy. However, more recently, it has been proposed that the ability to better exclude normal tissue by using IGRT may permit hypofractionation to be used safely and that, in the specific case of prostate cancer, hypofractionation may have beneficial effects.[114]

ADVERSE EFFECTS

Radiation produces adverse effects in normal tissues. Although these are discussed in detail in later chapters as part of comprehensive discussions of organ toxicity, it is worth making some general comments here from the perspective of how radiation biology relates to the clinical toxicities. The term *radiation toxicity* is used to describe the adverse effects caused by radiation alone and radiation plus chemotherapy. Although this latter toxicity would be better labeled as *combined modality toxicity*, the pattern typically resembles a more severe form of the toxicity produced by radiation alone. Adverse effects from radiation can be divided into acute, subacute, and chronic (or late) effects. Acute effects are common, rarely serious, and usually self-limiting. Acute effects tend to occur in organs that depend on rapid self-renewal, most commonly the skin or mucosal surfaces (oropharynx, esophagus, small intestine, rectum, and bladder). This is due to radiation-induced cell death that occurs during mitosis, so that cells that divide rapidly show the most rapid cell loss. In the treatment of head and neck cancer,

mucositis becomes worse during the first 3 to 4 weeks of therapy, but then will often stabilize as the normal mucosa cell proliferation increases in response to mucosal cell loss. It seems likely that normal tissue stem cells are relatively resistant to radiation compared with the more differentiated cells, because these stem cells survive to permit the normal mucosa to reepithelialize. Acute side effects typically resolve within 1 to 2 weeks of treatment completion, although occasionally these effects are so severe that they lead to consequential late effects, as described later.

Because lymphocytes are exquisitely sensitive to radiation, there has been considerable investigation into the effects of radiation on immune function. In contrast to mucosal cell killing, which requires mitosis, radiation kills lymphocytes in all phases of the cell cycle by apoptosis, so that lymphocyte counts decrease within days of initiating treatment. These effects do not tend to put patients at risk for infection, because granulocytes, which are chiefly responsible for combating infections, are relatively unaffected.

Two acute side effects of radiation do not fit neatly into these models relating to cell kill: nausea[115,116] and fatigue.[117,118] The origin of radiation-induced nausea is not related to acute cell loss, because it can occur within hours of the first treatment. Nausea is usually associated with radiation of the stomach, but it can sometimes occur during brain irradiation or from large-volume irradiation that involves neither the brain nor the stomach. Irradiation typically produces fatigue, even if relatively small volumes are irradiated. It seems likely that the origins of both of these *abscopal* effects of radiation (i.e., effects that occur systemically or at a distance for the site of irradiation) are related to the release of cytokines, but little is known.

Radiation can also produce subacute toxicities in the form of radiation pneumonitis and radiation-induced liver disease. These typically occur 2 weeks to 3 months after radiation is completed. The risk of radiation pneumonitis and radiation-induced liver disease is proportional to the mean dose delivered.[119,120] Thus, the 3D tools that permit the calculation of dose-volume histograms (described in the physics section) are currently used to determine the maximum safe treatment that can be delivered in terms of dose and volume. These toxicities appear to be initiated subclinically during the course of radiation as a cascade of cytokines in which TGF-β, tumor necrosis factor α, interleukin 6, and other cytokines play a role.[121] High TGF-β plasma levels during a course of treatment have been found to be associated with a greater risk of radiation pneumonitis.[122] Thus, in the future, we might look toward a combination of physical dose delivery, measured by the dose-volume histogram, the functional imaging of normal tissue damage, and the detection of biomarkers of toxicity, such as TGF-β, to improve the ability to individualize therapy. Attempts to determine the genomic basis of radiation sensitivity, beyond the known rare genetic defects such as ataxia telangiectasia, have not yet been successful.[123]

Late effects, which are typically seen 6 or more months after a course of radiation, include fibrosis, fistula formation, or long-term organ damage. Two theories for the origin of late effects have been put forth: late damage to the microvasculature and direct damage to the parenchyma. Although the vascular damage theory is attractive, it does not account for the differing sensitivities of organs to radiation. Perhaps the microvasculature is unique in each organ.[124] Regardless of the mechanism of toxicity, the tolerance of whole-organ radiation is now fairly well established (Table 13.2). Late complications can also be divided into two categories: consequential and true late effects. The best example of a consequential late effect is fibrosis and dysphagia after high-dose chemoradiation for head and neck cancer. Here, late fibrosis or ulceration appears to be the result of the mucosa becoming denuded for a prolonged time period. Late consequential effects are distinct from true late effects, which can follow a normal treatment course of self-limited toxicity and a 6-month or more symptom-free period. Examples of true late effects are radiation myelitis, radiation brain necrosis, and radiation-induced bowel obstruction. In the past, radiation fibrosis was thought to be an irreversible condition. Therefore, an exciting recent development is that severe radiation-induced breast fibrosis is an active process that can be reversed by drug therapy (pentoxifylline and vitamin E).[125] Radiation therapy also causes second cancers, which is addressed in detail in Chapter 143.

TABLE 13.2

Radiation Tolerance Doses for Normal Tissues

Site	TD 5/5 (Gy)[a] Portion of Organ Irradiated			TD 50/5 (Gy)[b] Portion of Organ Irradiated			Complication End Point(s)
	1/3	2/3	3/3	1/3	2/3	3/3	
Kidney	50	30	23	—	40	28	Nephritis
Rain	60	50	45	75	65	60	Necrosis, infarct
Brain stem	60	53	50	—	—	65	Necrosis, infarct
Spinal cord	50 (5–10 cm)	—	47 (20 cm)	70 (5–10 cm)	—	—	Myelitis, necrosis
Lung	45	30	17.5	65	40	24.5	Radiation pneumonitis
Heart	60	45	40	70	55	50	Pericarditis
Esophagus	60	58	55	72	70	68	Stricture, perforation
Stomach	60	55	50	70	67	65	Ulceration, perforation
Small intestine	50	—	40	60	—	55	Obstruction, perforation, fistula
Colon	55	—	45	65	—	55	Obstruction, perforation, fistula, ulceration
Rectum	(100 cm^3 volume)		60	(100 cm^3 volume)		80	Severe proctitis, necrosis, fistula
Liver	50	35	30	55	45	40	Liver failure

[a] TD 5/5, the average dose that results in a 5% complication risk within 5 years.
[b] TD 50/5, the average dose that results in a 50% complication risk within 5 years.
Adapted from Emami B, Lyman J, Brown A, et al. Tolerance of normal tissue to therapeutic irradiation. *Int J Radiat Oncol Biol Phys* 1991;21:109–122.

PRINCIPLES OF COMBINING ANTICANCER AGENTS WITH RADIATION THERAPY

Combining chemotherapy with radiation therapy has produced important improvements in treatment outcome. Randomized clinical trials show improved local control and survival through the use of concurrent chemotherapy and radiation therapy for patients with high-grade gliomas and locally advanced cancers of the head and neck, lung, esophagus, stomach, rectum, prostate, and anus. There are least two proposed reasons why chemoradiotherapy might be successful. The first is radiosensitization. In the laboratory, radiosensitization is defined as a synergistic relationship, using mathematical approaches such as isobologram or median effect analysis.[126,127] The underlying concept is that the observed effect of using chemotherapy and radiation concurrently is greater than simply adding the two together. A second proposed reason to combine radiation and chemotherapy is to realize the benefit of improved local control radiation along with the systemic effect of chemotherapy, a concept called *spatial additivity*.[128]

Clinical results show that both radiosensitization and spatial additivity contribute to varying extents in different clinical settings. In the case of head and neck cancer, radiosensitization predominates. This conclusion is supported by the meta-analysis of head and neck cancer: sequential chemotherapy and radiotherapy produces little if any improvement in survival, whereas concurrent chemoradiation produces a significant increase in survival.[129] Furthermore, in the early positive studies using concurrent chemoradiation, systemic metastases were unaffected even though survival was improved. Radiosensitization may also predominate in the success of chemoradiotherapy for locally advanced lung cancer. For instance, although initial studies indicated that sequential chemotherapy and radiation had some benefit for lung cancer,[130] more recent work indicates that concurrent therapy is superior, and it is now the standard treatment.[131] However, there are also examples of spatial additivity. For example, both radiosensitization and spatial additivity is provided by the use of chemoradiation for locally advanced cervical cancer in that both local and systemic relapses are decreased by combined therapy.[132]

By targeting the aberrant growth factor or proangiogenic pathways that are specific to cancer cells rather than all rapidly proliferating cells, molecularly targeted therapies offer the potential to improve outcome without increasing toxicity. Even a selective cytostatic effect against the tumor would be predicted to act synergistically with radiation (Fig. 13.22). Although preclinical studies (summarized in the previous biology section) have highlighted the potential therapeutic gains that could be achieved by adding EGFR inhibitors to radiation, the best validation of this combination has been from the results of clinical trials in head and neck cancer. A phase III clinical trial demonstrated that, in a cohort of 424 patients with local–regionally advanced squamous cell carcinoma of the head and neck, the addition of cetuximab nearly doubled the median survival of patients (compared to radiotherapy alone), from 28 to 54 months. This study represents the first major success

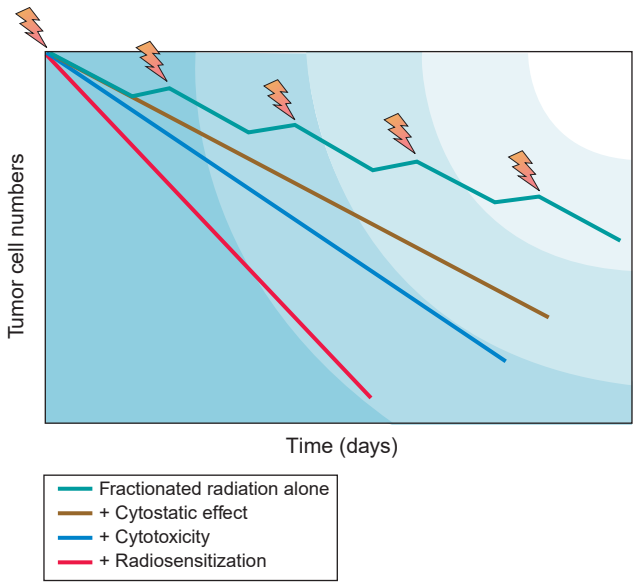

Figure 13.22 Potential mechanisms of synergy between epidermal growth factor receptor (EGFR) inhibitors and radiation. Although each daily radiation treatment kills a fraction of the cells, some cells grow back by the next day, which attenuates the effectiveness of radiation. If an EGFR inhibitor has only a selective cytostatic effect and blocks regrowth between fractions, the result would be a dramatic increase in radiation efficacy. The benefit of the inhibitor would be even greater if it caused tumor cell cytotoxicity or radiosensitization.

achieved by the addition of an EGFR antagonist to radiotherapy. This improvement was achieved without enhanced toxicity. Notably, the rates of pharyngitis and weight loss were identical in the two arms.[60] Local control was improved rather than the development of metastases, suggesting synergy rather than spatial additivity. Thus, the principle that can be derived from this study is that in tumors expressing high EGFR levels and that are likely to depend on aberrant EGF signaling, combining a true cytotoxic agent such as radiation with a cytostatic agent such as cetuximab has considerable promise.

Because of the success of chemoradiotherapy, the natural tendency has not been to substitute molecularly targeted agents such as cetuximab for chemotherapy, but to add cetuximab to chemoradiotherapy. Thus, the combination of cisplatin, cetuximab, and radiation was recently found to have the same control rate as cisplatin and radiation for patients with locally advanced head and neck cancer, but the cetuximab arm had greater toxicity. Unfortunately, the triple therapy was never evaluated preclinically, and it has been shown preclinically that when EGFR inhibitors are given prior to chemotherapy, they can produce antagonism.[133] The principles of adding molecularly targeted therapy to chemoradiation are still evolving.[63]

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14 Cancer Immunotherapy

Steven A. Rosenberg, Paul F. Robbins, Giao Q. Phan,
Steven A. Feldman, and James N. Kochenderfer

INTRODUCTION

Progress in understanding basic aspects of cellular immunology and tumor–host immune interactions have led to the development of immune-based therapies capable of mediating the rejection of metastatic cancer in humans. Early studies of allografts and transplanted syngeneic tumors in mice demonstrated that it was the cellular arm of the immune response rather than the action of antibodies (humoral immunity) that was responsible for tissue rejection. Thus, studies of immunotherapy have focused on enhancing antitumor immune responses of T cells that recognize cancer antigens. Antibodies that recognize growth factors on the surface of tumors can contribute to tumor regression, primarily by interfering with growth signals rather than by the direct destruction of tumor cells. The use of monoclonal antibodies in cancer treatment will be considered in Chapter 29.

Evidence for specific tumor recognition by cells of the immune system was obtained in experiments first conducted in the 1940s using murine tumors generated or induced by the mutagen methylcholanthrene (MCA). Mice that received a surgical resection of previously inoculated tumors could be protected against a subsequent tumor challenge with the immunizing tumor but not generally protected against challenge with additional MCA tumors. The observation that CD8+ cytotoxic T cells were primarily responsible for mediating the rejection of MCA-induced tumors in mice led to the identification of genes that encoded tumor rejection antigens expressed on murine tumors as well as the subsequent identification of antigens recognized by human tumor-reactive T cells. The identification of widely shared nonmutated tumor antigens led to the expectation that effective vaccine therapies could be developed for the treatment of cancer patients; however, the response rates in clinical cancer vaccine trials targeting these antigens have, to this point, been disappointingly low. Vaccination with viruslike particles expressing human papilloma virus (HPV) proteins are successful in preventing the establishment of cervical cancer and immunization with peptides derived from the oncogenic HPV E6 and E7 proteins can mediate tumor regression in woman with high vulvar neoplasia.[1] Immune-based therapies have, however, been identified that mediate the regression of large, established tumor metastases. Nonspecific immune stimulation with interleukin-2 (IL-2) administration can lead to objective clinical responses in patients with melanoma and renal cancer,[2] and inhibition of regulatory pathways mediated by CTLA-4[3] or PD-1[4] can lead to tumor regression in patients with metastatic melanoma and lung cancer. The adoptive transfer of melanoma reactive T cells can mediate objective clinical responses in 50% to 70% of patients with melanoma,[5] and the ability to genetically modify antitumor lymphocytes is expanding this cell transfer therapy approach to the treatment of patients with other cancer histologies.[6] Studies aimed at identifying potent tumor rejection antigens, as well as mechanisms that regulate immune responses to cancer, are being actively pursued.

HUMAN TUMOR ANTIGENS

To be recognized by immune lymphocytes, intracellular proteins must be digested and the resulting peptides transported to the cell surface and bound to Class I or II main histocompatibility molecules (Fig. 14.1). A variety of approaches have been used to identify the antigens that are naturally processed and presented on tumor cells. These include evaluating the ability of cells transfected with tumor cDNA library pools along with genes encoding autologous major histocompatibility complex (MHC) molecules, as well as the ability of target cells pulsed with peptides eluted from tumor cell surface MHC molecules for their ability to stimulate tumor reactive T cells. Reverse immunology approaches that involve either repeated in vitro T cell sensitization or in vivo immunization with candidate peptides or proteins have also lead to the identification of tumor antigens. Candidate epitopes identified on the basis of their ability to bind to a particular MHC molecule, however, may not necessarily be naturally processed and presented on the tumor cell surface, and there are conflicting reports on the ability of T cells generated using some candidate epitopes to recognize unmanipulated tumor targets, as discussed further.

Additional tumor antigens have been identified using antisera from cancer patients to screen tumor cell cDNA libraries, a method that has been termed serological analysis of recombinant cDNA expression (SEREX).[7] Although some of the proteins identified using this technique are expressed in a tumor-specific manner, many of these antigens are simply expressed at higher levels in tumor cells than in normal cells. This may occur due to the release of normal self-proteins from necrotic and apoptotic tumor cells leading to the generation of antibodies against intracellular proteins that are normally sequestered from the immune system.

Finally, the use of recently described approaches involving whole exomic sequencing of tumor cells has led to the identification of mutated tumor antigens. These studies will be discussed further in the section devoted to mutated tumor antigens

Cancer/Germ-Line Antigens

The first antigen identified as a target of human tumor reactive T cells was isolated by screening a melanoma genomic DNA library with an autologous cytotoxic T lymphocyte (CTL) clone.[8] The gene that was isolated, termed *MAGE-1*, was found to be a nonmutated gene that was a member of a large, previously unidentified gene family, many of whose members encode antigens recognized by tumor reactive T cells.[9] Members of this family of antigens are expressed in the testes and placenta, both of which lack an expression of MHC molecules, but often not in other normal tissues, which has led to their designation as cancer germ-line (CG) antigens. Members of the MAGE gene family are expressed in a variety of tumor types, including melanoma, breast, prostate, and esophageal cancers. The expression patterns of three

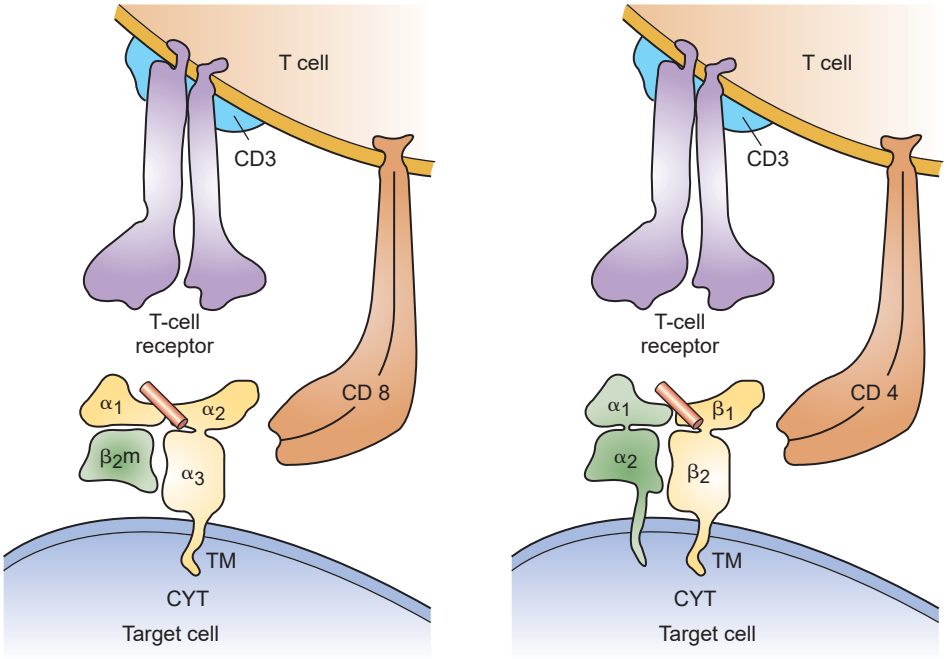


Figure 14.1 CD8 and CD4 cells use different molecules that interact with major histocompatibility complex (MHC) class I and II molecules respectively on the cell surface and serve to potentiate immune reactions.

different cancer/testes antigens in multiple tumor types is shown in Figure 14.2. The NY-ESO-1 antigen—a CG antigen that is unrelated to the MAGE family of genes—is expressed in approximately 30% of breast, prostate, and melanoma tumors, as well as between 70% and 80% of synovial cell sarcomas.[10]

Clinical adoptive immunotherapy trials targeting CG antigens have now been conducted in patients with melanoma as well as other tumor types. In a recent trial, objective clinical responses were seen in approximately 50% of patients with melanoma and 80% of patients with synovial cell sarcoma receiving autologous peripheral blood mononuclear cell (PBMC) transduced with a T-cell receptor directed against an HLA-A*02:01 restricted NY-ESO-1 epitope.[6] A trial targeting a MAGEA3 epitopes was recently carried out using a T-cell receptor (TCR) isolated from an HLA-A*02:01$^+$ transgenic mouse immunized with the MAGEA3:112–120 peptide.[11] Objective clinical responses were observed in five of nine melanoma patients receiving the adoptively transferred PBMC that were transduced with the MAGEA3-reactive TCR.[12] Unexpectedly, neural toxicity was observed in three of the patients treated in this trial, two of whom lapsed into a coma and subsequently died. Autopsy samples of patients' brains revealed that *MAGEA12*, which encodes a cross-reactive epitope recognized by the MAGEA3 TCR, was expressed at low levels in patients' brains, which may have been responsible for the observed neurologic toxicities. In a recent trial carried out using an affinity-enhanced human TCR directed against the

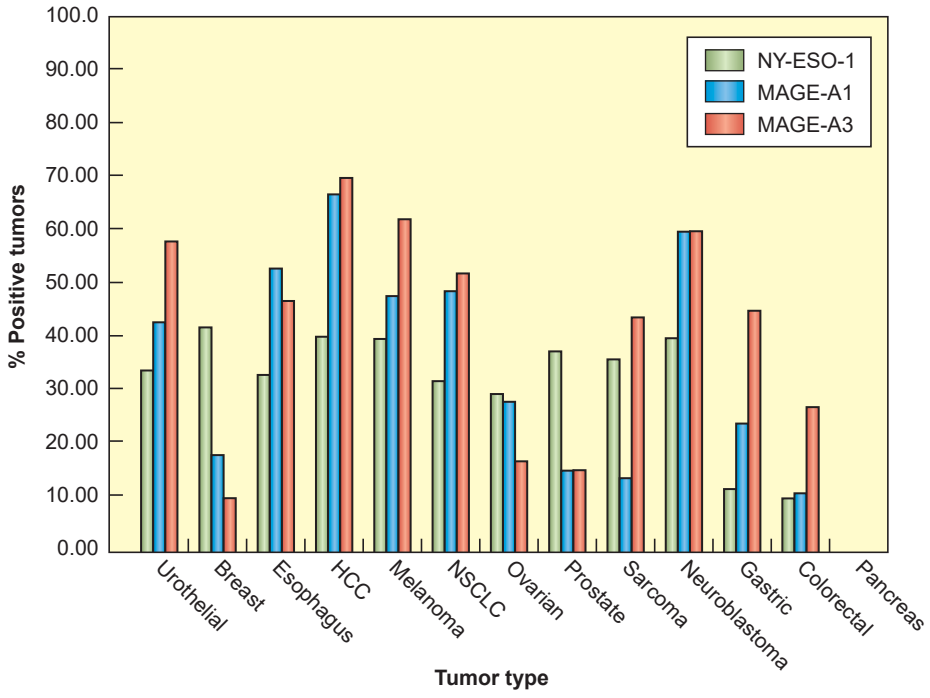


Figure 14.2 Expression of three different cancer/testes antigens in many different tumor types is shown. These data reflect reverse transcription–polymerase chain reaction measurements and is more sensitive than results obtained by immunohistochemistry. NSCLC, non–small-cell lung cancer. (Data compiled by Dr. J. Wargo. Massachusetts General Hospital.)

HLA-A*01:01-restricted, MAGEA3:168-176 epitope, the first two patients receiving TCR-transduced autologous PBMC died of cardiac arrest 4 to 5 days following infusion, which was attributed to cross-reactivity with titin, a protein expressed at high levels in cardimyocytes.[13] Taken together, these findings demonstrate the need for caution in evaluating cross-reactivity of high affinity TCRs recognizing tumor antigens.

Melanocyte Differentiation Antigens

Melanoma-reactive T cells have been frequently found to recognize gene products, termed melanocyte differentiation antigens (MDA), that are expressed in melanomas as well as in normal melanocytes present in the skin, eye, and ear but not in other normal tissues or tumor types. These include epitopes derived from gp100,[14,15] tyrosinase,[16] TRP-1,[17] and TRP-2,[18] proteins that had previously been found to play important roles in melanin synthesis. The screening of melanoma cDNA libraries with an HLA-A2–restricted tumor reactive T cells lead to the isolation of a previously unidentified gene, termed MART-1[19] or Melan-A.[20] The MART-1 antigen, which is expressed in 80% to 90% of fresh melanomas and cultured melanoma cell lines as well as normal melanocytes, represents an MDA of unknown function. The majority of melanoma reactive, HLA-A2–restricted tumor-infiltrating lymphocytes (TIL) recognize a single MART-1 epitope.[21] Studies carried out using a variety of approaches have also resulted in the identification of human leukocyte antigen (HLA) class II restricted epitopes of tyrosinase, TRP-1, TRP-2, and gp100.[9]

Overexpressed Gene Products

Gene products that are expressed at low levels in a variety of normal tissues but are overexpressed in a variety of tumor types have also been shown to be recognized by T cells. Screening of an autologous renal carcinoma cDNA library with a tumor reactive, HLA-A3–restricted T-cell clone resulted in the isolation of FGF5,[22] a protein that was expressed only at low levels in normal tissues but upregulated in multiple renal carcinomas as well as prostate and breast carcinomas. The peptide epitope recognized by FGF5-reactive T cells was generated by protein splicing, a process in which distant protein regions are joined together in the proteasome that had previously only been described in plants[23] and unicellular organisms.[24] Subsequent studies have led to the identification of multiple epitopes that result from protein splicing, suggesting that this represents a general mechanism for generating T-cell epitopes.[25–28] Screening of an autologous cDNA library led to the identification of a previously unknown gene that was termed *PRAME*.[29] This gene product was expressed in relatively high levels in melanomas as well as in additional tumor types but was also expressed at lower levels in a variety of normal tissues that included the testis, endometrium, ovary, and adrenals. The HLA-A24–restricted PRAME reactive T-cell clone, however, expressed the natural killer (NK) inhibitory receptor p58.2, and tumor cell recognition was dependent on the loss of expression of the HLA C*07 allele that represented the ligand for the inhibitory receptor, which may explain the lack of recognition of normal tissues that express relatively high levels of this HLA gene product.

Attempts have also been made to generate T cells directed against overexpressed candidate antigens by repeatedly stimulating PBMC in vitro with peptides that were identified as high binders for particular MHC molecules either using direct binding assays or *in silico* analysis carried out using peptide/MHC binding algorithms.[30,31] Using this approach, candidate epitopes have been identified from a variety of proteins that include prostate-specific antigen (PSA)[32] and prostate-specific membrane antigen (PSMA),[33] as well as Her-2/neu, a protein that is frequently overexpressed in a variety of tumor types, including breast carcinomas. Initial studies indicated that T cells derived by in vitro stimulation with a peptide that was predicted to bind with high affinity to HLA-A*02:01, Her-2/neu:369–377, recognized the appropriate natural tumor targets.[34] In one study, T cells generated following two in vitro stimulations of postvaccination PBMC from three of the four patients who were tested efficiently recognized peptide-pulsed targets but failed to recognize appropriate tumor targets.[35] Similarly, although stimulation with a peptide corresponding to amino acids 540 through 548 of the human telomerase reverse transcriptase (hTERT) catalytic subunit was initially reported to generate tumor-reactive T cells,[36] additional observations indicated that T cells generated using this peptide failed to recognize tumor targets.[37] These factors responsible for these discrepancies remain unresolved, although the in vitro stimulation of T cells with target cells pulsed with relatively high peptide concentrations could have led to the generation of low-avidity T cells that were incapable of recognizing naturally processed antigens.

Alternative screening approaches employed for tumor antigen discovery that may help to address these issues include the use of tandem mass spectrometry to sequence peptides that have been eluted from tumor cell surface MHC molecules. Use of this technique, coupled with microarray gene expression profiling, resulted in the identification of peptides derived from proteins that appeared to be overexpressed in tumor cells.[38] Peptides identified using this approach may, in many cases, not be immunogenic due to the fact that their expression in normal tissues, although lower than in tumor cells, may be high enough to lead to central or peripheral tolerance. Nevertheless, one of the peptides that were identified in this study also appeared to be recognized by human tumor reactive T cells. Recently, a similar approach was used to identify candidate peptides presented on cell surface MHC molecules that appeared to be derived from proteins that were overexpressed on glioblastomas.[39] In a clinical trial involving vaccination of patients with pools of the identified peptides, overall survival was associated with the number of peptides in the vaccine pool that elicited immune response[40]; however, this may simply reflect the fact that T cells from healthier patients can more readily generate peptide-specific responses.

Transgenic mice that express human HLA molecules have also been immunized with candidate antigens in an attempt to identify high avidity tumor-reactive T cells. Immunization of transgenic mice expressing HLA-A*0201 with the native human p53:264–272 peptide that differed from the corresponding murine p53 sequence at a single position lead to the generation of T cells that recognized tumor cells expressing high levels of p53.[41] Human T cells transduced with a murine p53 TCR isolated from an immunized mouse recognized a variety of human tumor cells; however, transduced T cells also recognized normal cells expressing lower p53 levels, indicating the dangers of targeting a normal self-protein whose expression is not strictly limited to tumor cells.[42] Similarly, a TCR that was highly reactive with HLA-A*02:01$^+$ tumor cells expressing the human carcinoembryonic antigen (CEA), a protein that is overexpressed in colon and breast carcinomas, was isolated by immunizing HLA-A*02:01$^+$ transgenic mice with the CEA:691–699 peptide.[43] The adoptive transfer of human PBMC transduced with the CEA-reactive TCR lead to an objective clinical response in one of the three treated patients; however, severe colitis was observed in all three of the treated patients.[44] In general, immunotherapies that target antigens present even in small amounts on normal tissues have led to normal tissue destruction and must be applied with caution.

Mutated Gene Products Recognized by CD8+ and CD4+ T Cells

A variety of mutated antigens have also been identified as targets of tumor reactive T cells. The majority of mutated antigens identified using these approaches appear to be unique or only expressed in a relatively small percentage of cancers, and so do not

represent targets that are broadly applicable to the treatment of multiple patients. Nevertheless, these studies have in some cases provided insights into mechanisms involved with tumor development, as the mutations may represent drivers of the transformed phenotype. The CDK4 gene product that was cloned using a CTL clone contained a point mutation that enhanced the binding to the HLA-A2 restriction element.[45] This mutation, which was identified in 1 of an additional 28 melanomas that were analyzed, led to the inhibition of binding to the cell cycle inhibitory protein $p16^{INK4a}$ and may have played a role in the loss of growth control in this tumor cell. A point-mutated product of the β-catenin gene, containing a substitution of phenylalanine for serine at position 37, was isolated by screening a cDNA library with an HLA-24–restricted, melanoma reactive TIL.[46] This mutation was found to stabilize the β-catenin gene product by altering a critical serine phosphorylation site, and 2 of 24 additional melanoma cell lines were found to express transcripts with identical mutations.[47]

The observation that immunization against individual murine tumors did not generally cross-protect against challenge with additional syngeneic murine tumors has provided support for the hypothesis that mutant T-cell epitopes represent the predominant antigens responsible for tumor rejection.[48] Mutated epitopes also represent a foreign antigen, which may render them more immunogenic than the majority of normal self-antigens. Although many of the mutations are specific for individual tumors, T cells have been generated by carrying out in vitro sensitization with peptides encoded at mutational hot spots present in *driver* genes.[49]

Recently, novel approaches have been developed that involve the sequencing of tumor cell DNA to identify potential mutated epitopes. In one study, whole exome sequencing of the murine B16 melanoma led to the identification of mutated epitopes that elicited a T cell that appeared to specifically recognize the mutated but not the corresponding wild-type peptides.[50] In a second study, a mutated antigen was identified by screening candidate epitopes that were expressed by tumors derived from immunodeficient mice that regressed in immune-competent mice.[51] More recently, melanomas from three patients who responded to adoptive immunotherapy were subjected to whole exome sequencing, followed by *in silico* analysis using peptide/MHC binding algorithms to identify candidate epitopes that were predicted to bind to the patients' MHC molecules.[52] Using this approach, a total of seven peptides were identified as targets of the TIL that were administered to these patients. Two mutated epitopes were recently identified by whole exome sequencing of a melanoma from a patient who demonstrated a partial response to treatment with the anti–CTLA-4 antibody ipilimumab, followed by a screening of a panel of mutated candidate peptide/MHC tetramers that were predicted to bind to the patient's HLA-A and B alleles.[53] In addition, a mutated epitope expressed by a bile duct cancer was identified by screening tandem minigenes encoding all mutated epitopes that were identified by whole exome sequencing.[54] The adoptive transfer of T cells directed against this mutation-mediated regression of the patient's cancer. Mutations unique to each cancer represent ideal targets for immunotherapy and can potentially lead to the development of personalized therapies directed against these unique targets.

Antigens Identified in Viral-Associated Cancers

Viruses do not appear to play a role in the development of the majority of human cancers; however, an infection with HPV, a group of double-stranded DNA viruses that infect squamous epithelium, is highly associated with the development of a variety of genital lesions that range from warts to carcinomas, as well as the majority of oropharyngeal carcinomas. Recombinant vaccines have been produced by the generation of viruslike particles (VLP), self-assembling particles that form following the expression of the HPV L1 protein in recombinant viral and yeast systems that were initially found to be protective in animal models. The results of a phase II trial in which 2,392 women between 16 and 23 years of age were immunized with HPV-16 VLPs indicated that 100% of those who were vaccinated were protected against infection with HPV-16.[55,56] Although vaccination with VLP does not lead to the regression of established disease, some success has been seen in therapeutic vaccination trials that target the oncogenic viral proteins E6 and E7. In a trial involving the vaccination of women with HPV-16–positive high-grade vulvar intraepithelial neoplasia with synthetic long peptides that encompass both HLA class I and class II restricted epitopes from the oncogenic HPV proteins E6 and E, clinical responses were observed in 15 of the 19 vaccinated patients, and complete regression of all lesions were seen in 9 of the 19 patients in this trial.[1]

Targeting foreign antigens thus may represent a strategy that can lead to more effective immunotherapies. These include viral epitopes as well as mutated epitopes that are also foreign to the host and therefore may represent more effective targets for these therapies than normal self-antigen.

HUMAN CANCER IMMUNOTHERAPIES

A wide variety of therapies have been evaluated in model systems and are now being developed for the treatment of patients with cancer. These include nonspecific approaches, those that involve direct immunization of patients with a variety of immunogens and approaches that involve the adoptive transfer of activated effector cells (Table 14.1). Much confusion related to the effectiveness of cancer immunotherapy has resulted from the lack of proper evaluation of the results of therapy using standard, accepted oncologic criteria such as the World Health Organization or the Response Evaluation Criteria in Solid Tumors (RECIST). Many clinical trials reported a positive use of *soft* criteria such as lymphoid infiltration or tumor necrosis that can occur in the natural course of cancer growth. Because of the delayed responses seen with some immunotherapy approaches, including tumor regression after initial tumor growth, guidelines have been published suggesting the use of an alternate set of immune-related response criteria for the evaluation of immune-based cancer treatments.[57,58] Other confusion has arisen from the use of inappropriate animal models. Although animal model systems have provided important clues that may lead to improved therapies, model systems that employ artificially introduced foreign antigens or that evaluate protection from tumor challenge do not appear to be relevant to the treatment of patients with bulky metastases. Short-term lung metastasis models involve the treatment of relatively small, nonvascularized tumors and also may not be directly relevant to the majority of tumors that are the targets of current clinical trials.

TABLE 14.1

Three Main Approaches to Cancer Immunotherapy

1. Nonspecific stimulation of immune reactions
   a) Stimulate effector cells
      IL-2 (melanoma and renal cancer)
   b) Inhibit regulatory factors
      Anti-CTLA4 (melanoma)
      Anti–PD-1 (melanoma, lung cancer)
2. Active immunization to enhance antitumor reactions (cancer vaccines)
3. Passively transfer activated immune cells with antitumor activity (adoptive immunotherapy)

Nonspecific Approaches to Cancer Immunotherapy

Progress has surged in the past 10 years in the understanding and utilization of nonspecific immune stimulation for the treatment of metastatic cancers. These agents aim to activate quiescent tumor-reactive immune cells or to remove inhibitory mechanisms to allow immunosuppressed cells to function to their full capacity. Although IL-2 and ipilimumab are currently the only immune stimulants approved by the U.S. Food and Drug Administration (FDA) for the treatment of metastatic renal cell carcinoma (IL-2) and melanoma (IL-2 and ipilimumab), new immune checkpoint inhibitors such as anti–programmed cell death 1 (anti–PD-1) have shown impressive results in recent clinical trials for patients with melanoma, renal cell cancer, and also non–small-cell lung cancer (NSCLC), and will likely be approved in the near future. As expected with nonspecific immunostimulation, systemic and bystander immune-related adverse events such as colitis has been reported with all agents in varying degrees, although most side effects are controllable and reversible if addressed aggressively and promptly by experienced clinicians. Importantly, antitumor responses seen with these immune-based modalities appear to be durable for some patients and may even be potentially curative. As with many therapies for metastatic solid tumors, preliminary trials using combination therapies have suggested better than expected response rates and survival, and confirmatory trials are in process to validate and ensure that toxicities from combining agents would not be prohibitive. Overall, patients with metastatic solid tumors may soon have wider armamentarium of off-the-shelf immunotherapy options.

Interleukin-2

Morgan et al.[59] showed that a *factor* produced in the medium from stimulated normal human blood lymphocytes can allow ex vivo growth and expansion of human T lymphocytes. The identification of this soluble T-cell growth factor (IL-2)[60,61] allowed the ability to culture T cells in vitro. IL-2 is a 15-kd glycoprotein produced in minute amounts by activated peripheral blood lymphocytes, and even with using T-cell hybridomas, minimal quantities could be purified; thus, research using IL-2 was impeded by the limited amounts of purified IL-2 available. The isolation of the cDNA clone in 1983[62] enabled the development in 1984 of recombinant IL-2,[63] which permitted the ability to mass manufacture IL-2. Although murine studies demonstrated the ability of IL-2 to mediate tumor regression,[64] early phase I clinical trials did not show any antitumor response,[65] but was instructive in showing pharmacokinetics and toxicities, which led to more effective regimens. Subsequently, IL-2 was given in higher doses (up to 720,000 IU/kg intravenously every 8 hours) in a landmark trial involving 25 patients, along with nonspecific lymphokine-activated natural killer (LAK) cells, which are non-T and non-B lymphocytes.[66] This report was the first to document the regression of advanced solid cancers (melanoma, renal cell, lung, and colon) using immunotherapy in humans.[66] A follow-up trial randomizing 181 patients to either high-dose IL-2 alone (720,000 IU/kg intravenously every 8 hours) or high-dose IL-2 and LAK cells showed that the tumor response was due to IL-2 alone and not to the nonspecific LAK cells.[67] This study also narrowed the IL-2–sensitive histologies to melanoma and renal cell cancer, which had more consistent responses.

IL-2 Therapy for Metastatic Renal Cell Cancer

Subsequent to the studies discussed previously, high-dose IL-2 was tested by additional centers and in combination with other agents for renal cell cancer. A randomized phase II trial involving 99 kidney cancer patients showed no increase in antitumor responses with the addition of interferon alfa-2b (IFNα-2b). Responses were seen for 12 (17%) of 71 patients who received high-dose IL-2 alone, with 4 complete regressions.[68] A summary report of 227 patients with metastatic renal cell cancer treated with high-dose IL-2 (defined as 600,000 IU/kg or 720,000 IU/kg given intravenously every 8 hours as tolerated up to 15 doses) from 1985 to 1996 at the Surgery Branch of the National Cancer Institute (NCI) documented a total response rate of 19%, with 10% partial and 9% complete; the longest duration of a complete response was over 10 years ongoing (134+ months).[69] Another summary report from seven phase II clinical trials from multiple institutions involving 255 patients with metastatic renal cell cancer receiving high-dose IL-2 showed the overall response rate was 14%, with 9% partial and 5% complete, and responses occurred in all sites of disease, including primary kidney tumors, bone metastases, and bulking visceral tumor burdens.[70] Although the response rates were modest, the durability of the responses was remarkable, with many responses lasting over 5 years ongoing (see Fig. 14.2). Because of the striking durability of the antitumor responses, IL-2 received FDA approval for the treatment of metastatic renal cell cancer in 1992. A follow-up report in 2000 showing the response rates of the 255 renal cell patients in the seven phase II studies to be the same, with complete responses lasting over 10 years ongoing (131+ months for the longest responder), suggesting a potential cure.[71]

To ascertain whether lower doses and/or different administration routes, which would decrease toxicity and obviate the need for inpatient hospitalization for IL-2 therapy, a trial randomizing 400 patients with metastatic renal cell cancer to either standard high-dose intravenous IL-2, low-dose intravenous IL-2 (at 72,000 IU/kg), or low-dose subcutaneous IL-2 (250,000 U/kg per dose daily Monday through Friday in the first week and then 125,000 U/kg per dose daily during the next 5 weeks).[72] Although responses were seen with all three regimens, including complete responses in the low-dose subcutaneous regimen, standard high-dose IL-2 had higher overall response rates (21%) versus low-dose intravenous IL-2 (13%; $p = 0.048$) and low-dose subcutaneous IL-2 (10%; $p = 0.033$), suggesting the superiority of the high-dose intravenous regimen.[72]

The administration of IL-2 represents the only known curative treatment for patients with metastatic renal cell cancer and should be considered as front-line therapy for suitable patients.

IL-2 Therapy for Metastatic Melanoma

Between 1985 and 1993, 270 patients with metastatic melanoma enrolled into eight clinical trials in multiple centers using high-dose IL-2 (defined as 600,000 IU/kg or 720,000 IU/kg given intravenously every 8 hours as tolerated up to 15 doses). Atkins et al.[73] reported overall response rates of 16% (43 patients), with 10% partial and 6% complete; responses occurred at all tumor sites and regardless of initial tumor burden. With median follow-up at that time of 62 months, 20 responders (47%) were still alive, with 15 surviving over 5 years.[73] A follow-up report on those patients in 2000 showed that the response rates were unchanged; with the longest response duration of >12 years ongoing, disease progression was not observed in any patient responding greater than 30 months.[74] As with renal cell cancer, the flat *tail* of the Kaplan-Meier response duration and overall survival curves (Fig. 14.3), showing the potential curative nature of the antitumor responses, was the main compelling reason the FDA approved IL-2 for the treatment of metastatic melanoma in 1998.

Research in subsequent years aimed to increase the response rates of IL-2, led by increasing interests in tumor vaccinations as melanoma-associated antigens were being characterized.[75] Pilot studies suggested that vaccinations using modified melanoma differentiation antigens such as gp100:209–217(210M) could elicit immunologic responses in nearly all patients, and when combined with high-dose IL-2, could elicit potentially higher than expected clinical antitumor responses.[75] A follow-up phase III study[76] randomized 185 patients with HLA*A0201 from 21 centers to either high-dose IL-2 or high-dose IL-2 plus gp100:209–217(210M) concurrent immunization. Although the response rates for the

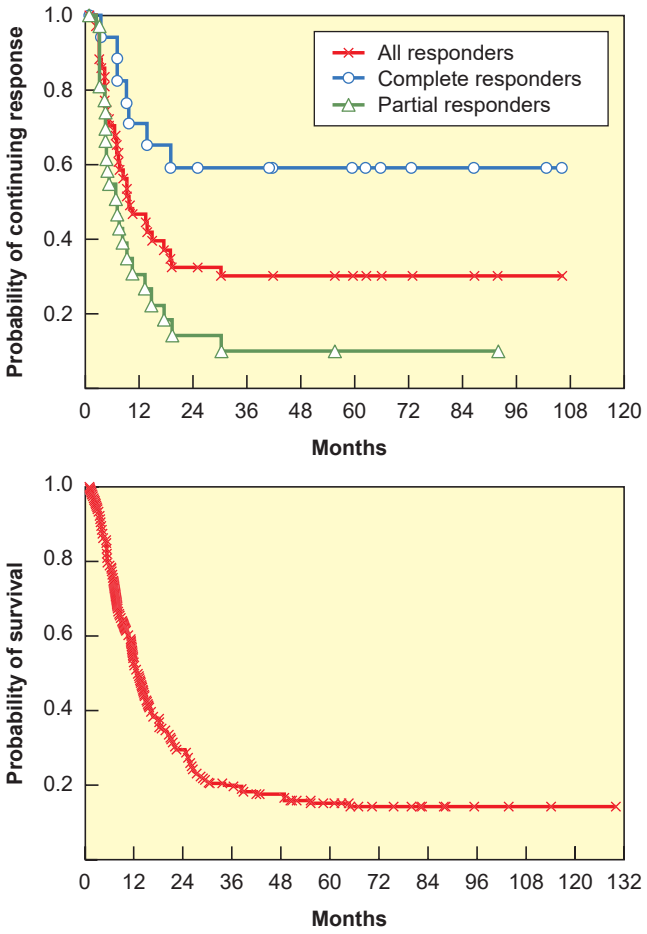


Figure 14.3 Kaplain-Meier plots of response duration (*top*) and overall survival (*bottom*) for 270 patients with metastatic melanoma who were treated with high-dose bolus IL-2 from 1985 to 1993 in eight clinical trials.[73]

IL-2 plus vaccine arm was statistically improved compared to IL-2 alone (16% versus 6%; p = 0.03), the IL-2 alone arm was notable for being much lower than in all prior studies.[76] In addition, a pilot trial of 36 melanoma patients treated high-dose IL-2 concurrently with ipilimumab (an antibody against cytotoxic T lymphocyte–associated antigen 4 discussed in the following section) gave a 25% OR rate, with 17% achieving complete response[77]; however, these data have not been further tested.

Correlative studies suggest that the total doses of IL-2 received during the first treatment course was significantly higher in patients achieving a complete response[69]; however, when limited to patients who were able to complete both cycles of the course, there was no statistical significance, suggesting that patients whose tumors progressed significantly after one cycle (and was not able to complete the second cycle of the course) accounted for some of the difference seen.[78] Responders did have a higher maximal lymphocyte count[69,78] immediately posttherapy and were more likely to develop vitiligo and thyroid dysfunction.[78] There has not been a consistent pretherapy factor that is predictive of response, although one retrospective correlative study involving 374 patients showed that patients with M1a (subcutaneous- and/or cutaneous-only disease) have a response rate of 54% compared with 12% for those with visceral M1b/c (P_2 <0.0001).[78]

Toxicities and Safe Administration of IL-2

High-dose IL-2 has been shown to be associated with adverse events that impact multiple organ systems.[73,79,80] The main component of the toxicities is due to an inflammatory response mediated by the release of cytokines such as IFNγ and tumor necrosis factor alpha (TNF-α)[81] resulting in a capillary-leak syndrome[82] and decreased systemic vascular resistance, which can lead to fever, hypotension, cardiac arrhythmia, lethargy, renal insufficiency, hepatic dysfunction, body edema, pulmonary edema, and confusion; other side effects can also include nausea, diarrhea, rash, anemia, thrombocytopenia, lymphocytosis, and neutrophil chemotactic defect[83] that predispose patients to gram-positive line infections. Since the first clinical trials with IL-2 in 1984, however, much has been learned to permit its safe dosing for appropriately screened patients[82,84,85]; importantly, if patients are appropriately supported, side effects are quickly reversible once IL-2 dosing ceases.[85] Kammula et al.[86] compared the incidences of grade 3/4 toxicities between the 155 patients treated from 1985 to 1986 to 156 patients treated from 1993 through 1997 at the NCI Surgery Branch: grade 3/4 hypotension decreased from 81% to 31%, intubations from 12% to 3%, neuropsychiatric toxicities from 19% to 8%, diarrhea from 92% to 12%, line sepsis from 18% to 4%, cardiac ischemia from 3% to 0%, and mortality from 3% to 0%. In fact, no fatality occurred strictly due to IL-2 therapy since 1989.[86] Overall strategies for the safe administration of high-dose IL-2 include careful screening for appropriately selected patients with adequate cardiopulmonary reserve, having an experienced team of physicians and nurses who are cognizant of the expected toxicities of IL-2, having routine preemptive measures such as prophylactic antibiotics to prevent line infections, and aggressive and prompt management of toxicities.

Checkpoint Modulators

Anti–Cytotoxic T Lymphocyte Antigen 4

CTLA-4 is an immunosuppressive *costimulatory* receptor found on newly activated T cells (and on regulatory T cells) that binds with costimulatory ligands B7-1 and B7-2 on antigen-presenting cells.[87,88] When CTLA-4 is engaged by B7-1 or B7-2, the T cells becomes inhibited,[89,90] suggesting that CTLA-4 likely evolved as a self-protective mechanism to prevent autoimmunity (Fig. 14.4). Thus, overcoming this *checkpoint* molecule was an aim of cancer immunotherapy. After CTLA-4 blockade in murine models led to antitumor immunity,[91,92] anti–CTLA-4 antibodies were tested in clinical trials starting in 2002.

The combination of anti–CTLA-4 blocking antibodies and vaccination worked well in murine models and led to one of the early phase II studies using ipilimumab (a fully human immunoglobulin [IgG_1] monoclonal antibody previously called MDX-010) with two gp100 vaccines, gp100:209–217(210M) and gp100:280–288(288V), in patients with metastatic melanoma.[93] Antitumor regressions were seen (from 11% to 22% overall response rates, with up to 8% complete response rates), along with severe autoimmune toxicities such as colitis, dermatitis, and even hypophysitis,[93–95] as would be expected based on the mechanism of CTLA-4 blockade. In fact, autoimmunity adverse events appeared to correlate with response to ipilimumab.[3] The experience with these early studies led to management strategies to screen aggressively for immune-related adverse events (IRAE), such as routine screening of endocrinopathies, and to treat IRAEs promptly, including high-dose steroids if needed for severe colitis.[96,97] Overall, ipilimumab was in some ways easier to manage for the patients than IL-2 because it was an outpatient infusion given every 3 weeks; IRAEs were unpredictable, however, and can appear suddenly many weeks after receiving a dose.

In 2010, results from a landmark phase III randomized trial comparing three treatment strategies (ipilimumab alone, gp100 peptide vaccine alone, or ipilimumab plus gp100 peptide vaccine) in 676 patients with metastatic melanoma were published showing improvement in median survival in the two arms that received ipilimumab (10 months) compared to the gp100 alone arm (6 months, p <0.001), despite showing a low response rate of 7% (among 540 patients who received ipilimumab).[98] Another

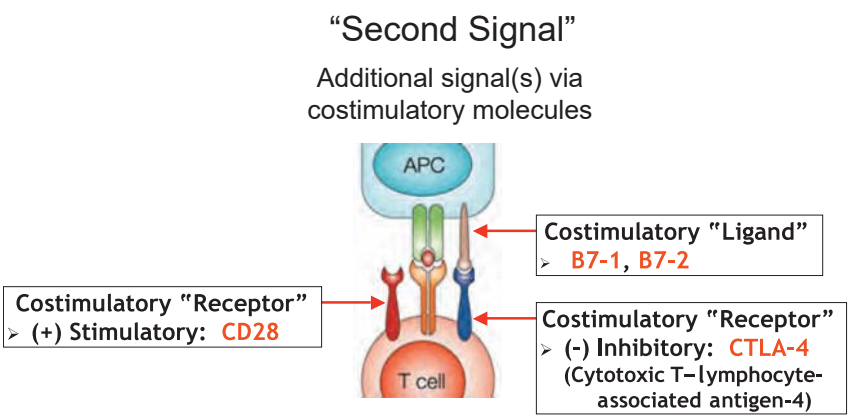


Figure 14.4 Mechanism of action of cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4). When CD28 is engaged on the T cell, reactivity of the T cell is enhanced. When CTLA-4 is engaged on the T cell, reactivity of the T cell is inhibited. Blocking of CTLA-4 with a monoclonal antibody can elicit antitumor immunity but also autoimmunity.

phase III randomized trial comparing dacarbazine plus ipilimumab versus dacarbazine alone again showed improved survival in that arm containing dacarbazine (11.2 months versus 9.1 months; $p < 0.001$).[99] These studies showing survival benefit led to FDA approval of ipilimumab for advanced melanoma in 2011.

The responses seen with ipilimumab appear to be durable.[100] A follow-up study of 177 patients with metastatic melanoma treated on the earliest trials at the NCI Surgery Branch using ipilimumab showed that response duration could last 99+ months ongoing.[77] In fact, 14 out of the 15 complete responders remain disease free 54+ to 99+ months ongoing, suggesting a potential cure for some patients. Interestingly, several patients who were deemed partial responders converted to complete responders several years later, because it look an average of 30 months to have all visible tumor marks on imaging scans to disappear.[77]

Ipilimumab was also tested on other solid tumors, and renal cell cancer again appears to be the only other type beside melanoma that had significant responses. Sixty-one patients with metastatic renal cell cancer were treated, and six developed a response (10%); however, 33% developed grade 3/4 IRAEs.[101] Subsequently, the availability of agents with lower toxicity profiles such as sunitinib and sorafenib prevented further enthusiasm to pursue this drug for renal cell cancer.

Another anti–CTLA-4 antibody, tremelimumab (previously called CP-675,206), has also demonstrated durable responses in melanoma patients.[102,103] A phase III randomized trial randomizing 655 patients with metastatic melanoma to either tremelimumab or physician's choice chemotherapy, however, failed to show a survival difference (despite a significantly different response duration favoring tremelimumab, 35.8 months versus 13.7 months; $p = 0.0011$), possibly due to crossover of chemotherapy patients enrolling into ipilimumab trials and expanded access programs.[103]

Anti–Programmed Death 1 and Anti–Programmed Death Ligand 1

PD-1 is another checkpoint modulator expressed on activated T cells. Although CTLA-4 appears to be involved in the early activation of T cells, PD-1 is involved in the later effector phase of T-cell activation and can function to prevent excessive damage to self by activated T cells in the periphery.[104,105] Interaction with its corresponding ligand, PD-L1 (B7-H1) and PD-L2 (B7-H2) leads to suppressed T-effector function. PD-L1 is expressed on hematopoietic and epithelial cells and is upregulated by cytokines such as IFNγ,[106] whereas PD-L1 is mainly on antigen-presenting cells. Given the clinical results with inhibiting the CTLA-4 checkpoint, recent efforts have focused on inhibiting the PD-1/PD-L1 and PD-1/PD-L2 interactions.

Nivolumab (previously known as BMS-936558, MDX-1106, and ONO-4538) is a fully human anti–PD-1 IgG4 monoclonal antibody that was initially tested in a phase I trial published in 2010 in which 39 patients with advanced solid cancers were treated in escalating doses.[107] Responses were seen in one patient with colon cancer, one with melanoma, and one with renal cell cancer; one patient developed colitis.[108] These hopeful results lead to a larger study in which 236 patients with either NSCLC (74 patients), melanoma (94 patients), or renal cell cancer (33 patients).[4] Objective responses were seen in 18% of patients with NSCLC, 28% with melanoma, and 27% with renal cell cancer.[4] Grade 3/4 adverse events occurred in 14% of patients, including those previously seen with ipilimumab (dermatitis, colitis, hepatitis, thyroiditis, hypophysitis, and pneumonitis). Nine patients developed pneumonitis, six of whom was reversible, and three (1%) with grade 3/4 died despite steroids and infliximab therapy.[4] An update on the status of 107 melanoma patients treated from 2008 to 2012 shows a 31% tumor response rate, with a median response duration of 2 years and a median overall survival of 16.8 months.[109]

Nivolumab was also tested in combination with ipilimumab in melanoma in either concurrent (53 patients) or sequenced (33 patients) regimens. The concurrent group experienced an overall response rate of 40%, whereas the sequenced group had a 20% response rate.[110] The concurrent group also experienced a higher rate of grade 3/4 adverse events (53%), compared to 18% in the sequenced group. Interestingly, 16 of 21 responders in the concurrent group experienced tumor reduction of 80% or greater by 12 weeks,[110] a tempo that is faster than was seen with ipilimumab.

Another anti–PD-1 developed independently, lambrolizumab (previously known as MK-3475, a humanized IgG4κ monoclonal antibody), was tested on 135 patients with metastatic melanoma.[111] The response rate was found to be 38% and was similar between those who had received ipilimumab and those who were ipilimumab naïve,[111] confirming that the antitumor response from lambrolizumab occurs via a different mechanism. Similar to nivolumab, 13% of patients developed grade 3/4 adverse events, with 4% developing pneumonitis, although none developed grade 3/4 pneumonitis.[111]

BMS-936559 is a fully human IgG4 monoclonal antibody that blocks PD-L1 ligation to both PD-1 and CD80. A phase I study was tested in 207 patients (75 with NSCLC, 55 with melanoma, 18 with colon cancer, and 17 with renal cell cancer, 17 with ovarian cancer, 14 with pancreatic cancer, 7 with gastric cancer, and 4 with breast cancer).[112] Among patients who were evaluated for response, objective responses were seen in 16% of melanoma patients, 17% of renal cell cancer patients, 10% of NSCLC patients, and 1 out of 17 ovarian cancer patients. Grade 3/4 toxicities were seen in 9% of patients.[112]

The advent of these checkpoint inhibitors brings additional treatment options to patients with selected advanced cancers, particularly those with histologies deemed previously to be outside the realm of immunotherapy such as NSCLC.[108,113] In addition, a new anti–PD-L1 (MPDL3280A) in clinical trials has also shown some efficacy in melanoma, renal cell cancer, and NSCLC in early reports.

Active Immunization Approaches to Cancer Therapy (Cancer Vaccines)

The molecular characterization of multiple cancer antigens led to a large number of clinical trials that attempted to actively immunize against these antigens with the expectation that cellular immune reactions would be generated capable of inhibiting the growth of established cancers. The results of these efforts have yet to produce significant vaccine efforts of value in the treatment of human cancer. There is a paucity of murine tumor models that suggests that active vaccine approaches can mediate the regression of established vascularized tumors; therefore, it is not surprising that these approaches have, with a few exceptions, shown little efficacy in humans. Enthusiasm about the effectiveness of cancer vaccines has often been grounded in surrogate and subjective end points, rather than reliable objective cancer regressions using standard oncologic criteria. In a review of the world literature, including 107 published cancer vaccine trials involving 2,242 patients, a 3.4% overall objective response rate was observed (Table 14.2).[114,115] In many cases, relatively soft criteria such as stable disease or the regression of individual metastases in the presence of progressive disease at other sites have been reported. A variety of immunizing vectors have been used, including tumor-derived peptides, proteins, whole tumor cells, recombinant viruses, dendritic cells, and heat-shock proteins.[116–122] Although many of these approaches can lead to the development of circulating T cells that can recognize the immunizing tumor antigen, these T cells rarely cause the inhibition of established tumors, a point that has led to much confusion in the field of tumor immunology. The generation of antitumor T cells in vivo is likely a necessary, but certainly not a sufficient criteria for the development of a clinically active immunotherapy. Often, T cells with weak avidity for tumor recognition are generated, and the tolerizing and inhibitory influences that exist in vivo must be overcome for an effective immune response to cause tumor destruction.

A prospective randomized trial of immunization with antigen-presenting cells was carried out by the Dendreon Corporation (Seattle, Washington). This trial used an antigen-presenting cell vaccine loaded with prostatic acid phosphatase linked to GM-CSF compared to placebo in men with hormone-refractory prostate cancer.[123] Of 330 patients who received the vaccine treatment, 1 objective partial response was seen. Only 8 patients experienced a PSA drop of at least 50%. There was no difference in the time to disease progression; however, the vaccine group had a median survival of 25.8 months compared to 21.7 months in the placebo group, and based on this statistically significant survival improvement, this treatment was approved by the FDA (Fig. 14.5).

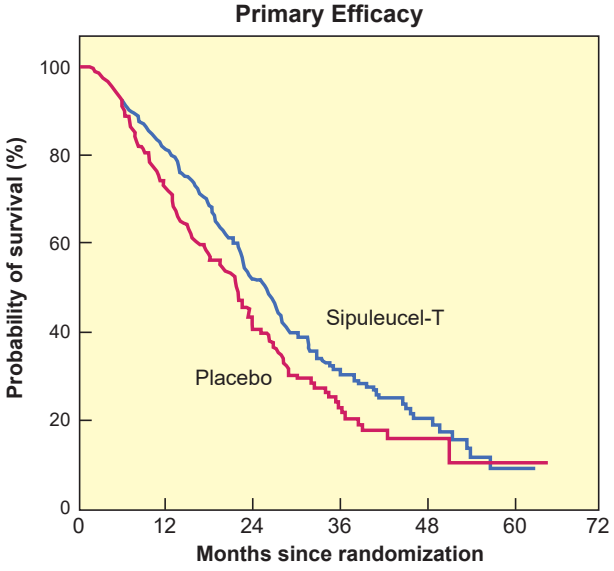
TABLE 14.2
Experience with Therapeutic Cancer Vaccines

	Number of Trials	Number of Patients	Objective Responses
Surgery Branch, National Cancer Institute	25	541	14 (2.6%)
Published before 2005[114]	33	765	29 (4.0%)
Published 2005–2010[115]	49	936	34 (3.7%)
Total	107	2,242	77 (3.4%)

Note: Vaccines include: peptide, protein, dendritic cell, virus, plasmid DNA, and whole tumor cells.

Adoptive Cell Transfer Immunotherapy

Adoptive cellular immunotherapy refers to the transfer to the tumor-bearing host of immune lymphocytes with anticancer activity. The first successful administration of adoptive cell therapy (ACT) involving TIL, in combination with high-dose IL-2 was carried out at the National Cancer Institute Surgery Branch in 1988.[124] Studies that used cell transfer therapy in patients with metastatic melanoma have provided the clearest evidence of the power of the immune system to mediate the regression of advanced metastatic cancers in humans. Adoptive cell therapy has several theoretical as well as practical advantages.[125] Lymphocytes with antitumor activity can be expanded to very large numbers ex vivo for infusion into cancer patients. These cells can be tested in vitro for antitumor activity, and cells with appropriate properties such as high avidity for tumor recognition and a high proliferative potential



Number at risk

Sipuleucel-T	341	274	129	49	14	1
Placebo	171	123	55	19	4	1

Figure 14.5 Kaplan-Meier estimate of the overall survival in patients with metastatic castration-resistant prostate cancer treated with Sipuleucel-T antigen–presenting cell immunotherapy. A modest but statistically significant improvement in survival was seen ($P = 0.03$).

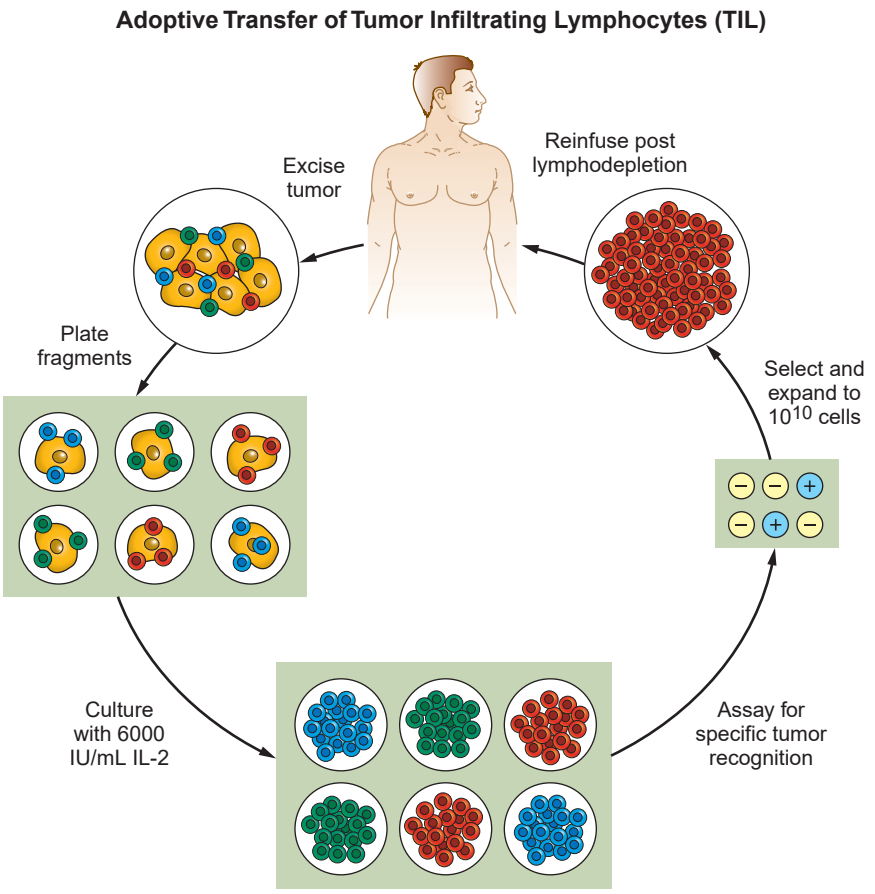


Figure 14.6 Diagram of the adoptive cell therapy of patients with metastatic melanoma. Tumors are resected and individual cultures are grown and tested for antitumor reactivity. Optimal cultures are expanded in vitro and reinfused into the autologous patient who had received a preparative lymphodepleting chemotherapy.

can be identified and selectively expanded for treatment. These cells can be activated in vitro and thus are not subjected to the tolerizing influences that exist in vivo. Perhaps, most important, the host can be manipulated prior to the transfer of the anticancer cells to provide an optimal tumor microenvironment free of in vivo suppressive factors.[125] Studies have shown that the transfer of cultured lymphocytes with antiviral activity can prevent Epstein-Barr virus (EBV) infections as well as the subsequent development of posttransplant lymphoproliferative diseases. Cultured lymphocytes have been used for the treatment of patients with established EBV-induced lymphomas.[126]

The best evidence for the ability of adoptive cell transfer to successfully treat patients with solid tumors comes from the treatment of patients with metastatic melanoma. A diagram that describes the nature of this treatment is shown in Figure 14.6. In patients with metastatic melanoma, TILs can be obtained from resected tumor deposits and individual cultures tested to identify those with optimal anticancer activity.[124,127] These cells are then expanded ex vivo and reinfused along with IL-2, which is the requisite growth factor required for the survival and persistence of these cells. The administration of a preparative lymphodepleting chemotherapy regimen, consisting of cyclophosphamide and fludarabine with or without 2 or 12 Gy total body irradiation, could substantially enhance the survival and persistence of the transferred cells and increase their in vivo antitumor effectiveness.[128,129] In a series of three pilot trials with 93 patients, objective responses were seen in 49% to 72% of patients.[5,130] Of the 93 patients, 20 (22%) experienced a complete regression of all metastatic melanoma. Only 1 of these 20 patients has recurred, and the remaining patients have ongoing complete regressions from 80 to over 104 months (Table 14.3, Fig. 14.7).

TABLE 14.3
Cell Transfer Therapy

Treatment	Total	PR	CR	OR
	Number of Patients (Percentage) (Duration in Months)			
No TBI	43	16 (37%) (84, 36, 29, 28, 14, 12, 11, 7, 7, 7, 7, 7, 4, 4, 2, 2, 2)	5 (12%) (114+, 112+, 111+, 97+, 86+)	21 (49%)
200 TBI	25	8 (32%) (14, 9, 6, 6, 5, 4, 3, 3)	5 (20%) (101+, 98+, 93+, 90+, 70+)	13 (52%)
1,200 TBI	25	8 (32%) (21, 13, 7, 6, 6, 5, 3, 2)	10 (40%) (81+, 78+, 77+, 72+, 72+, 71+, 71+, 70+, 70+, 19)	18 (72%)

Note: 20 complete responses: 19 ongoing at 70 to 114 months.

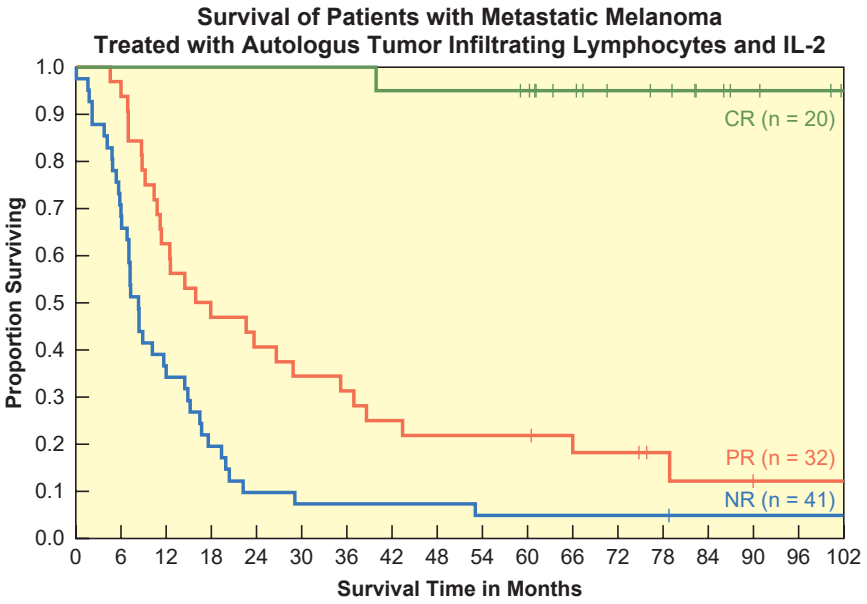


Figure 14.7 Survival curves of 93 patients treated with adoptive cell transfer using autologous TIL. The results of three consecutive trials using different preparative regimens have been combined in this analysis. Of 20 patients who achieved a complete cancer regression, only one has recurred with a median follow-up of over 8 years.[5]

The 5-year survival of these 93 patients was 29% and was similar regardless of the prior treatments that these patients had received.

Extensive genomic studies have shown that TILs that mediate complete cancer regressions recognize mutated epitopes presented by the cancer.[52] The use of exomic sequencing combined with in vitro tests of antitumor activity can be used to select for T-cell populations reactive against the cancer. This approach has now been utilized to identify T cells used to successfully treat a patient with chemotherapy-refractory cholangiocarcinoma and provides a blueprint for the application of cell transfer therapy for a variety of common epithelial cancers.[54]

The difficulty in obtaining TILs with antitumor activity from cancers other than melanoma has also led to the development of approaches using lymphocytes genetically modified using retroviral transduction to insert antitumor T-cell receptors into the normal lymphocytes of patients.[131]

Genetic Modification of Lymphocytes for Use in Adoptive Cell Therapy: Basic Principles and Applications to Solid Tumors

Efforts are in progress to genetically engineer autologous PBMCs through the introduction of exogenous high avidity receptors that specifically recognize tumor antigens (Fig. 14.8). These cells can then be expanded to large numbers in vitro and be readministered back to the patient similar to TILs in order to mediate tumor regression. The use of gene-modified cells for ACT has resulted in objective clinical responses for a variety of cancer histologies including melanoma, synovial sarcoma, and CD19-positive B-cell malignancies.[6,131–133]

There are two key requirements necessary for the use of gene-modified cells for the treatment of solid cancer. The first is the selection of an appropriate gene transfer method in order to achieve high receptor expression levels in the transferred T cells. For this discussion, we will consider both nonviral and viral-based gene delivery platforms. Generally speaking, there are two categories of nonviral gene transfer, chemical and physical. Chemical gene transfer involves the use of positively charged delivery vehicle such as calcium phosphate, cationic lipids, or polymers to form DNA complexes capable of entering a cell through endocytosis.[134] These reagents benefit from their ease of manufacture and ability to form complexes with large DNA sequences; however, low transfection efficiency of human T cells continues to be an issue.

Physical methods for gene delivery may involve direct delivery of DNA into a cell via microinjection or indirect DNA uptake via electroporation.[135] Electroporation of messenger RNA (mRNA) can achieve high levels of protein expression in cells, comparable to many of the viral-mediated gene delivery systems (gammaretroviral or lentiviral).[135,136] High-throughput electroporators should allow one to gene modify large numbers of T cells ex vivo.[137] mRNA electroporation appears to be most suited for this application, because there is significant loss of cell viability following electroporation of large amounts of DNA.[136] The electroporation of mRNA, although gaining traction as a means of redirecting T cell specificity,[137] provides for transient receptor expression because the mRNA will degrade over time. Currently, it is not clear if stable long-term receptor expression is required to mediate tumor regression. However, the main criticism of the non-viral methods described is the lack of stable gene transfer. To overcome this problem, many investigators are now using transposons such as *sleeping beauty* or *piggybac*.[138] Transposons are mobile DNA gene delivery elements encoding a gene of interest (i.e., TCR or chimeric antigen receptors [CAR]) that can randomly integrate into the genome in the presence of the transposase enzyme, thereby allowing for stable gene expression. This technology is currently being used for the ACT of CAR-modified cells targeting B-cell malignancies (see Fig. 14.8B).[139]

Viral-mediated gene delivery is currently the most common method for the genetic modification of immune cells for cancer ACT. Retroviridae is a family of RNA viruses that, upon entry into cells, undergo a process called reverse transcription whereby the viral RNA is converted into DNA as it stably integrates into the host genome. The two most common retroviral vector systems are based on the gammaretrovirus, Moloney murine leukemia virus (MLV), and the lentivirus, HIV type 1 (see Fig. 14.8B). Gammaretroviral vectors have been used in human clinical applications for over 20 years. The only reported toxicity associated with gammaretroviral engineering of human cells involved the retroviral transduction of hematopoietic stem cells for the treatment of children with severe combined immunodeficiency syndrome (X-SCID).[140] There have been no reports of clonal outgrowth following the retroviral transduction of mature T lymphocytes in adults. Highly active vectors have been generated from a variety of murine retroviruses including spleen focus forming virus (SFFV), myeloproliferative sarcoma virus (MPSV), and the murine stem cell virus (MSCV).[141–147] In most cases, these vectors are replication incompetent, but non–self-inactivating in

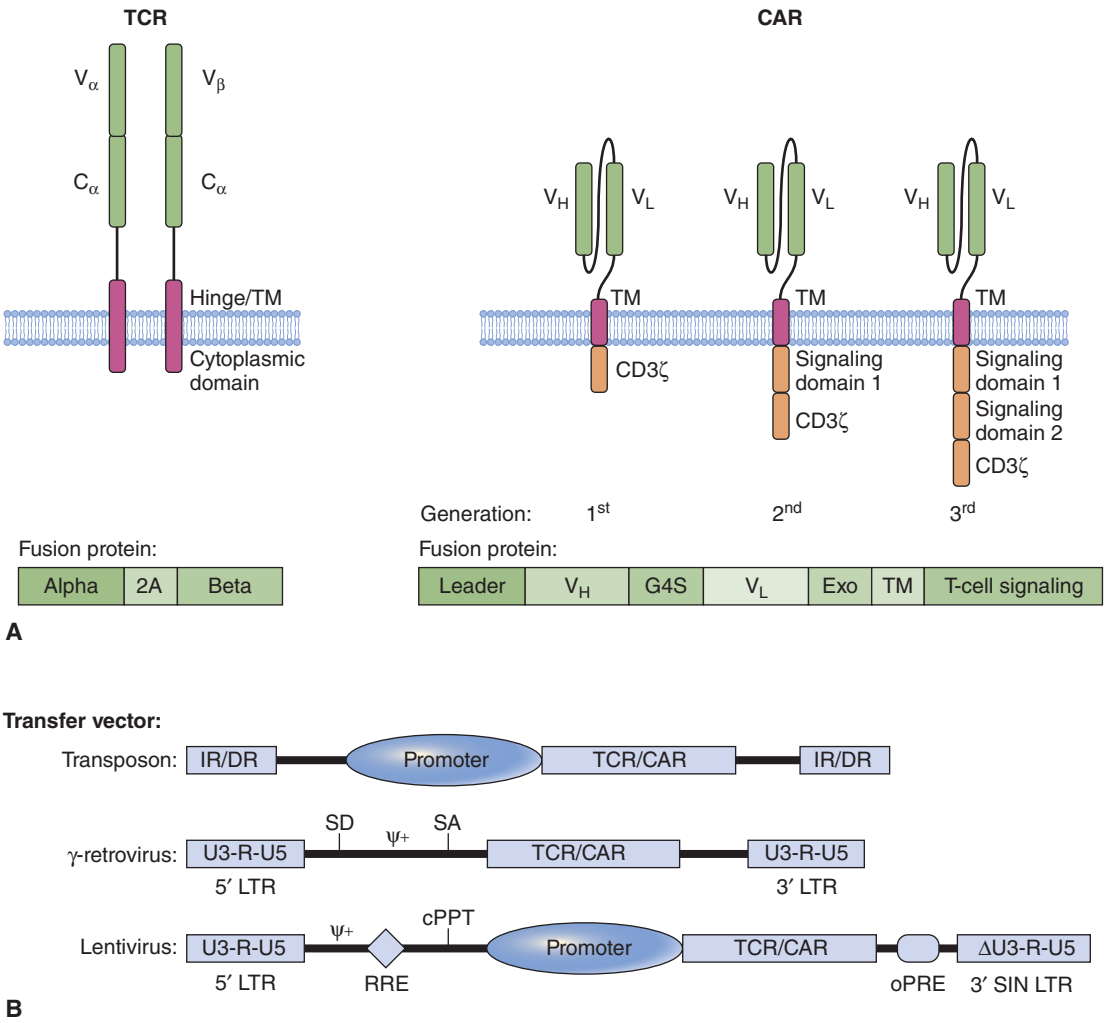


Figure 14.8 Genetic modification of T cells for the treatment of solid cancers. **(A)** In order to gene-modify T cells to confer stable tumor-specific reactivity, one can transduce T cells with an exogenous TCR derived from a naturally occurring or murine T-cell clone or a CAR derived from a tumor-specific monoclonal antibody. The TCR or CAR is synthesized as fusion proteins and inserted into the appropriate gene transfer vector. **(B)** Depending on the transfer vector selected, the T cells are then electroporated (transposon) or transduced (viral vector) to confer tumor specificity. V_α, V_β, and C_α, C_β, TCR alpha and beta chain variable and constant regions, respectively; TM, transmembrane domain; V_H and V_L, immunoglobulin variable regions; 2A and G4S, linker sequences; Exo, extracellular spacer domain; SD, splice donor; SA, splice acceptor; Ψ, packaging signal; LTR, long terminal repeat; U3, unique 3′ region; R, repeat region; U5, unique 5′ region; RRE, rev response element; cPPT, central polypurine tract; wPRE, woodchuck hepatitis virus posttranscriptional regulatory element; ΔU3, truncated unique 3′ region; SIN, self-inactivating.

that the promoter for transgene expression is derived from the viral long terminal repeat (LTR). Self-inactivating (SIN) gammaretroviral vectors have been developed that require an internal promoter to drive transgene expression. The advantage of non-SIN vectors is the ability to use a variety of retroviral packaging cell lines (PG13, Phoenix) engineered to constitutively express gag (capsid protein), pol (reverse transcriptase, integrase, and RNase H enzymes) and env (envelope protein). Transduction of these packaging lines with a non-SIN retroviral vector encoding a transgene allows for the generation of a stable packaging cell line that constitutively releases vector into the medium. This platform is easily scaled up to support large-scale vector production efforts. An alternative to the gammaretroviral vector platform is the lentiviral vector platform. There are some advantages to selecting a lentiviral vector for T-cell engineering in that one can transduce large numbers of minimally stimulated T cells,[148] transfer more complex and larger gene expression cassettes, and yield a potentially safer chromosomal integration profile as compared to gammaretroviruses. However, there has been at least one instance of clonal outgrowth following lentiviral vector transduction of CD34+ stem cells.[149] Therefore, more data will be needed to better understand the risk of insertional mutagenesis associated with the use of lentiviral vectors. The major disadvantage with using lentiviral vectors for ACT is the lack of a robust packaging cell line, which requires transient vector production and is difficult to scale up.

The first successful application ACT involved the use of autologous T cells genetically modified with a conventional αβ TCR targeting MART-1 for the treatment of patients with melanoma.[131] The success of this approach relies on the ability to identify naturally occurring TCRs with sufficiently high avidity for the tumor antigen. For this clinical trial, a tumor-specific TCR was cloned directly from melanoma TIL. Exogenous TCR can also be generated from human PBMC following a variety of in vitro sensitization techniques or immunization of transgenic mice expressing HLA molecules. A T-cell clone expressing a low avidity TCR recognizing MART-1 was isolated and the α and β chains cloned into a gammaretroviral vector. The objective response rate from this trial was 13% (2/15).[131] In

a follow-up trial with a higher avidity TCR that was cloned from the same melanoma TIL, the objective response rate increased to 30% (6/20).[150] However, patients in this trial experienced significant on-target, off-tumor toxicity with the destruction of normal melanocytes in the skin, eye, and ear. These trials showed the potential to use ACT for the treatment of solid cancers, but also highlight the importance of selecting appropriate tumor antigens to target in order to minimize normal tissue toxicities. Perhaps a better class of antigen to target for ACT would be the cancer testes antigens (CTA) that are expressed only on germ cells during fetal development and then reexpressed on cancers but not other normal tissues with the exception of the testes (see Table 14.1). Because the testes do not express class I MHC molecules, they are protected from any adverse immune response.[151] NY-ESO-1 is a CTA overexpressed on melanoma, as well as a variety of solid epithelial cancers.[152–154] A high-avidity TCR was developed targeting NY-ESO-1 and patients with metastatic melanoma or synovial cell sarcoma were treated following adoptive cell transfer using autologous lymphocytes transduced with a gammaretrovirus encoding this receptor.[6] In updated results from this trial, 8 of 17 patients (47%) with melanoma showed objective tumor responses, two of which were complete responses and ongoing at 51 and 48 months after treatment. Nine of 19 patients (47%) with synovial cell sarcoma showed objective tumor response, only one of which is complete and ongoing at 12 months. Of note, no toxicities were observed in any of these trials. Thus, targeting NY-ESO-1 and other CTAs is an attractive strategy for the application of ACT for the treatment of solid cancers (see Table 14.4 for other trials conducted at the National Cancer Institute, Surgery Branch).

Redirection of T-cell specificity using conventional TCR is constrained by HLA restriction, which limits treatment only to patients expressing a particular MHC haplotype. An alternate approach is to use CAR comprised of an monoclonal antibody single chain variable fragment (scFv) fused in frame to T-cell intracellular signaling domains capable of T-cell activation following antigen-specific binding (see Fig. 14.8A).[155] CARs, unlike conventional TCRs, are not MHC restricted but are limited by the requirement for the tumor antigen to be expressed on the cell surface. CARs can also recognize carbohydrate and lipid moieties further expanding their application. To date, there has been limited success using CAR-based ACT for the treatment of solid cancers. In 2008, the first successful CAR trial targeting the disialoganglioside, GD2, for the treatment of neuroblastoma was reported.[156] In this trial, 4 out of 8 patients (50%) with evaluable tumor experienced tumor regression or necrosis with one complete responder. In that same year, a second CAR trial targeting CD20 on non-Hodgkin and mantle cell lymphomas was reported.[157] Of the 7 patients treated, one achieved a partial response. Much greater success has now been achieved using a CAR targeting CD19, a molecule expressed on normal B cells and virtually all B-cell lymphomas. In a trial conducted at the National Cancer Institute, Surgery Branch, Kochenderfer et al.[132] first reported that autologous T cells expressing a CAR targeting CD19 was able to mediate tumor regression in a patient with B-cell lymphoma (hematologic malignancies will be discussed in more detail elsewhere). Successfully expanding CAR-based ACT to other cancer histologies has been limited by the inability to identify suitable tumor antigens to target. At the National Cancer Institute, Surgery Branch, there are active clinical programs with CAR targeting the mutated epidermal growth factor receptor, EGFRvIII, expressed on approximately 40% of glioblastomas as well as head and neck cancers[158]; the vascular endothelial growth factor-2 receptor, VEGFR-2, expressed on tumor vasculature[159]; and mesothelin, expressed on the mesothelial lining of the pleura, peritoneum, and pericardium, but overexpressed on mesothelioma, pancreatic, and ovarian cancers.[160] These trials are currently accruing patients; however, no objective clinical responses have been observed to date. A summary of clinical trials at the National Cancer Institute, Surgery Branch using gene-modified autologous T cells for ACT are shown in Table 14.4. ACT can mediate the regression of large, established tumors in humans. Efforts to identify and specifically target novel tumor antigens are currently underway with the hope that ACT using gene-modified T cells will develop into an effective treatment for patients with a variety of solid cancers.

Genetic Modification of Lymphocytes to Treat Hematologic Malignancies

Immunologic therapies can be useful treatments for some hematologic malignancies as demonstrated by the effectiveness of mono-

TABLE 14.4

Surgery Branch, National Cancer Institute Program for the Application of Cell Transfer Therapy to a Wide Variety of Human Cancers

Receptor	Type	Cancers	Status
MART-1	TCR	Melanoma	Closed
gp100	TCR	Melanoma	Closed
NY-ESO-1	TCR	Epithelial & sarcomas	Accruing
CEA	TCR	Colorectal	Closed
CD19	CAR	Lymphomas	Accruing
VEGFR2	CAR	All cancers	Accruing
2G-1	TCR	Kidney	Accruing
IL-12	Cytokine	Adjuvant for all receptors	Accruing
MAGE-A3[a]	TCR	Epithelial	In development
EGFRvIII	CAR	Glioblastoma	Accruing
SSX-2	TCR	Epithelial	In development
Mesothelin	CAR	Pancreas & mesothelioma	Accruing
HPV16 (E6&7)	TCR	Cervical, oropharyngeal	In development

[a] MAGE-A3 TCRs; restricted by HLA-A2, A1, Cw7, DP4—covers 80% of patients.
EGFR, epidermal growth factor receptor; VEGFR2, vascular endothelial growth factor 2.

clonal antibodies in treating B-cell malignancies and the fact that allogeneic hematopoietic stem cell transplantation (alloHSCT) can cure a variety of hematologic malignancies.[161–167] The results with monoclonal antibodies and alloHSCT clearly prove that immunologic therapies have significant activity against hematologic malignancies, but monoclonal antibodies are not curative as single agents,[162,166] and alloHSCT has a substantial transplant-related mortality rate due to infections and an immunologic attack against normal tissues known as graft versus host disease (GVHD).[163,165] The proven curative potential of alloHSCT and the effectiveness of autologous T-cell transfer therapies for melanoma have encouraged the development of autologous T-cell therapies for hematologic malignancies.[125,129,163,165] Genetically engineering T cells to specifically recognize antigens expressed by malignant cells has emerged as a very promising strategy for cancer immunotherapy.[125,129,168]

T cells can be genetically engineered to express either of two types of receptors, CARs[168–171] or natural TCRs.[6,131,172] T cells expressing either a CAR or TCR gain the ability to specifically recognize an antigen.[171,172] CARs are artificial fusion proteins that incorporate antigen recognition domains and T-cell activation domains.[168,170,172] The antigen recognition domains are most often derived from monoclonal antibodies.[168,170,172] Antigen recognition by TCRs is major histocompatibility complex restricted.[125,128] In contrast to TCRs, recognition of antigens by CARs is not dependent on MHC molecules. An advantage of TCRs over CARs is that TCRs can recognize intracellular antigens, whereas CARs can only recognize cell-surface antigens.

Chimeric Antigen Receptors

CARs targeting hematopoietic antigens have been extensively studied in preclinical experiments and early-stage clinical trials.[168,170,172,173] For a protein to be a promising target for CAR-expressing T cells, it should be uniformly expressed on the malignant cells being targeted but not expressed on essential normal cells. Many cell-surface proteins with restricted normal tissue expression patterns have been identified on malignant hematologic cells, and CARs targeting many of these proteins are under development (Table 14.5).

Many factors can affect CAR T-cell therapies. The types of gene-therapy vectors encoding the DNA of the CAR could be an important factor. The types of vectors currently being used in clinical trials of CAR T cells are gammaretroviruses, lentiviruses, and transposon-based systems.[132,133,174–181] The design of the CAR fusion protein is another important factor. CAR fusion proteins include an antigen-recognition domain that is most often derived from an antibody, costimulatory domains such as CD28 and 4-1BB, and T-cell activation domains that are usually derived from the CD3z molecule.[168,170,171,182] Other factors that could impact the effectiveness of CAR T-cell therapies include the cell culture method used to prepare the cells and administration of chemotherapy or radiation therapy prior to the CAR T-cell infusions.[170,178,179] In mouse models, a profound enhancement of the antimalignancy activity of infused T cells occurs when the T-cell infusions are preceded by lymphocyte-depleting chemotherapy or radiation therapy.[183–185] Because chemotherapy can have a direct antimalignancy effect against hematologic malignancies, the administration of chemotherapy prior to infusions of T cells is a confounding factor that must always be kept in mind when interpreting the results of clinical trials of T-cell therapies.

Anti-CD19 Chimeric Antigen Receptors

CD19 is an appealing target antigen for CARs because CD19 is expressed on almost all malignant B cells, but CD19 is not expressed on normal cells except B cells.[186] The first preclinical studies of anti-CD19 CARs utilized either gammaretrovirus vectors[174] or plasmid electroporation[176] to insert genes encoding anti-CD19 CARs into human T cells. These studies and subsequent preclinical work by other groups showed that T cells expressing anti-CD19

TABLE 14.5

Hematologic Antigens Targeted by Genetically-Modified T-Cells

Antigen	Malignancy Expressing Antigen	Targeted by CAR or TCR	References
CD19	B-cell malignancies	CAR	17, 19, 21, 22, 23, 24, 25, 26, 34, 35, 55, 56
CD20	B-cell malignancies	CAR	36, 37, 38
CD22	B-cell malignancies	CAR	39, 40
CD23	B-cell malignancies	CAR	41
ROR1	B-cell malignancies	CAR	42
Kappa light chain	B-cell malignancies	CAR	43
B-cell maturation antigen (BCMA)	Multiple myeloma	CAR	44
Lewis Y antigen	Multiple myeloma and acute myeloid leukemia (AML)	CAR	45, 46
CD123	AML	CAR	47
CD30	Hodgkin lymphoma	CAR	48, 49
CD70	Hodgkin lymphoma	CAR	50
Wilms tumor-1 (WT1)	AML and acute lymphoid leukemia (ALL)	TCR	51
Aurora kinase-A	AML and chronic myeloid leukemia (CML)	TCR	52
Hyaluronan-mediated motility receptor (HMMR)	AML and ALL	TCR	53

CARs could specifically recognize and kill CD19-expressing malignant B cells in vitro and in vivo.[174–176,187] These preclinical studies compared many different CAR signaling moieties, which led most groups to utilize CARs with T-cell activation domains from the CD3z molecule and costimulatory molecules from either CD28 or 4-1BB (CD137).[165,180,182,187,188] Preclinical studies showed that lymphocyte-depleting radiation therapy administered before anti-CD19 CAR T-cell infusions was critical to the antimalignancy activity of CAR T cells.[183] The addition of lymphocyte-depleting radiation therapy prior to infusions of anti-CD19 CAR T cells increased the percentage of mice cured of lymphoma by the CAR T cells from 0% to 100%.[183] Preclinical experiments with anti-CD19 CARs have led to several early-phase clinical trials.

The first clinical trial to demonstrate in vivo activity of anti-CD19 CAR T cells in humans was conducted in the Surgery Branch of the National Cancer Institute.[132] The gammaretroviral vector used in this trial encoded a CAR with a CD28 costimulatory domain. Patients treated on this clinical trial received cyclophosphamide and fludarabine chemotherapy followed by an infusion of anti-CD19 CAR T cells and a short course of intravenous IL-2.[132,181] Clear antigen-specific activity of the anti-CD19 CAR T cells was demonstrated because blood B cells were selectively eliminated from four of the seven evaluable patients for several months.[181] The duration of B-cell depletion in these patients was much longer than the duration of B-cell depletion caused by the chemotherapy that the patients received.[132,181] This study also generated evidence of an antimalignancy effect by the anti-CD19 CAR T cells because six of seven evaluable patients with advanced B-cell malignancies obtained either complete remissions or partial remissions (Fig. 14.9).[181] One of these remissions is ongoing 45 months after treatment, and another remission is ongoing 31 months after treatment. Significant toxicity, including hypotension and neurologic toxicity, occurred during this clinical trial.[181] The severity of these toxicities correlated with the levels of serum inflammatory cytokines.[181] Except for one patient who died with influenza pneumonia, the toxicities were transient, with all toxicities resolving within 3 weeks of the anti-CD19 CAR T-cell infusions.[181]

Investigators at the Memorial Sloan Kettering Cancer Center treated nine patients with chronic lymphocytic leukemia (CLL) or acute lymphocytic leukemia (ALL) by infusing T cells that expressed a CAR with a CD28 costimulatory domain.[179] The gene therapy vector used in this work was a gammaretrovirus.[179] None of three patients treated with CAR T cells alone experienced a regression of leukemia, and CLL regressed in one of four evaluable patients treated with cyclophosphamide followed by an infusion of CAR T cells. Using the same CAR, the same group went on to treat five patients with ALL.[173] Patients received chemotherapy followed by an infusion of anti-CD19 CAR T cells. Four patients had detectable leukemia prior to their CAR T-cell infusions, and all of these patients became minimal residual disease negative after infusion of CAR T cells. Four of five patients on this trial rapidly underwent allogeneic stem cell transplantation after their CAR T-cell infusions.[173]

Investigators at the Baylor College of Medicine conducted clinical trials of anti-CD19 CAR T cells in which each patient simultaneously received infusions of two types of anti-CD19 CAR T cells.[189] One type of T cell expressed a CAR expressing a CD28 costimulatory domain. The other type of T cell was identical except that the CAR it expressed lacked a CD28 domain. Compared to the T cells lacking a CD28 moiety, the T cells expressing a CAR with a CD28 moiety had higher peak blood levels and longer in vivo persistence.[189] Patients on this trial did not receive chemotherapy, and there were no remissions of malignancy or long-term B-cell depletion.[189]

Investigators at the University of Pennsylvania reported results from three patients with CLL who were treated with chemotherapy followed by infusions of anti-CD19 CAR-expressing T cells.[133,180] The CAR used in this study was encoded by a lentiviral vector and contained a costimulatory domain from the 4-1BB molecule. Two

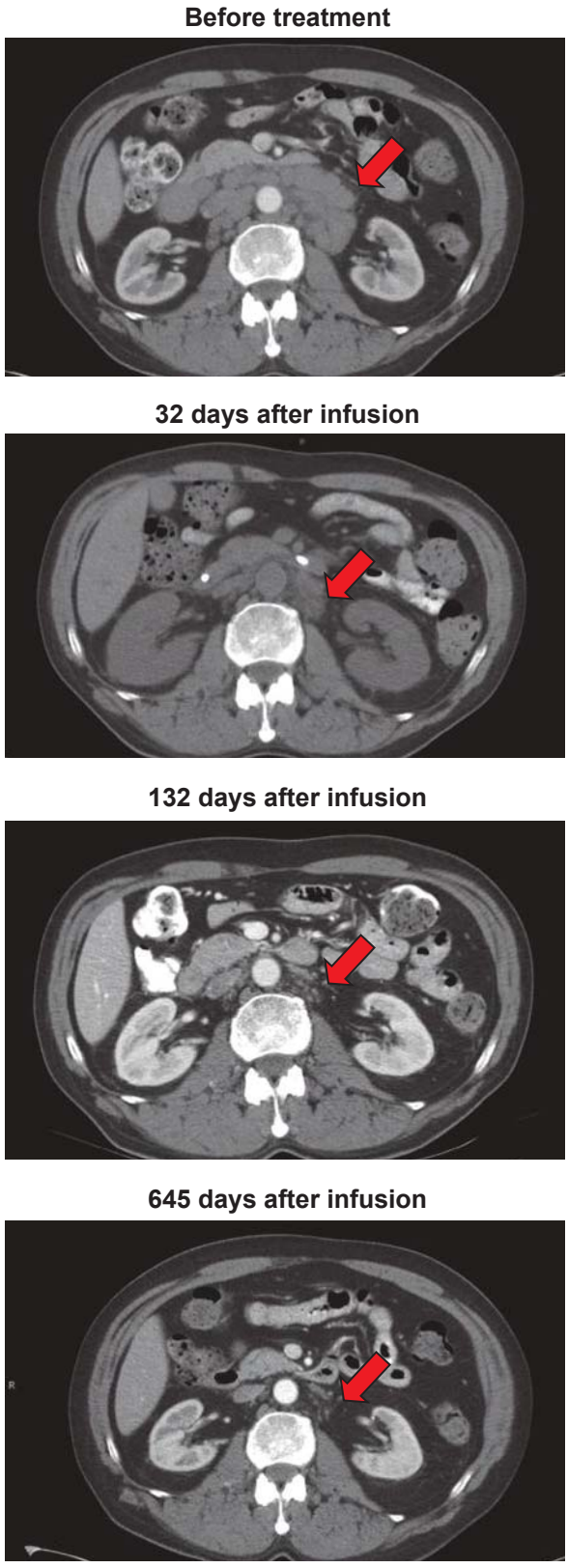


Figure 14.9 Computed tomography (CT) scans show regression of adenopathy in a patient with chronic lymphocytic leukemia (CLL) after treatment with chemotherapy followed by an infusion of autologous anti-CD19 CAR T cells. The time after the cell infusion of each CT scan is indicated. The *arrow* points to a large lymph node mass that resolved completely over time. (Reproduced from Kochenderfer JN, Rosenberg SA. Treating B-Cell cancer with T cells expressing anti-CD19 chimeric antigen receptors. *Nature Rev Clin Oncology* 2013;10:267-276, with permission.)

of the three reported patients obtained prolonged complete remissions.[180] This same CAR design was subsequently evaluated in a clinical trial enrolling patients with ALL.[190] One ALL patient obtained a prolonged complete remission but also experienced significant toxicity that was associated with elevated levels of serum cytokines.[190]

Overall, the early results with anti-CD19 CAR T cells show that this strategy holds great promise to improve the treatment of B-cell malignancies, but anti-CD19 CAR T-cell infusions are also associated with significant toxicity that is usually of short duration. Future progress will require decreasing the toxicity of anti-CD19 CAR T cells while maintaining or enhancing their antimalignancy activity. Parameters that are being studied in an effort to improve anti-CD19 CAR therapy include vector selection, CAR design, cell culture methods, and clinical application.

Chimeric Antigen Receptors and T-Cell Receptors Targeting Hematologic Antigens Other than CD19

CARs and TCRs targeting several hematologic antigens other than CD19 have been evaluated in preclinical or clinical studies. Except for CD19, the B-cell antigen CD20 has been the hematologic antigen most extensively studied as a target of CAR T cells.[191–193] Plasmid electroporation, which is not an optimal method of T-cell genetic modification, was used to transfer the anti-CD20 CAR gene to T cells in these studies. In one trial of anti-CD20 CAR T cells, patients received chemotherapy followed by infusions of T cells expressing a CAR without costimulatory domains.[192] One of seven patients obtained a partial remission that lasted 3 months. In a second trial, patients received chemotherapy followed by anti-CD20 CAR T cells expressing a CAR with both CD28 and 4-1BB costimulatory domains; in this trial, the only evaluable patient obtained a partial remission.[193]

CARs targeting other B-cell antigens including CD22,[157,194] CD23,[195] receptor tyrosine kinase–like orphan receptor-1 (ROR1),[196] and the immunoglobulin kappa light chain[197] have been evaluated in preclinical studies. CARs for treating multiple myeloma are currently being developed. B-cell maturation antigen (BCMA) is expressed on normal and malignant plasma cells, but it is not known to be expressed on other normal cells except for a small subset of mature B cells.[198] CARs targeting BCMA have undergone preclinical testing, and a clinical trial of an anti-BCMA CAR will open soon.[198] Preclinical studies have been performed on CARs targeting the Lewis Y antigen as a treatment for multiple myeloma and acute myeloid leukemia (AML),[199] and activity against AML was recently demonstrated in a phase I clinical trial of a CAR targeting the Lewis Y antigen.[200] CARs targeting the CD123 protein are undergoing preclinical testing for potential use against AML.[201] For Hodgkin lymphoma, CARs have been developed that target the CD30 protein and the CD70 protein, and anti-CD30 CARs are entering early-phase clinical trials.[202–204]

MHC-restricted TCRs targeting some antigens expressed on hematologic malignancies have undergone preclinical testing, but TCRs for treating hematologic malignancies are at a much earlier stage of development than CARs (see Table 14.1). TCRs targeting the Wilms tumor antigen-1 (WT1) are under development to treat ALL and AML.[205] Aurora kinase-A–specific TCRs and hyaluronan-mediated motility receptor (HMMR)-specific TCRs are under preclinical development as leukemia treatments.[206,207]

T-Cell Gene Therapy in the Setting of Allogeneic Hematopoietic Stem Cell Transplantation

A leading cause of death among patients undergoing alloHSCT is relapse of malignancy, and alloHSCT is often complicated by GVHD.[164,165,208] Therefore, a central goal in the field of alloHSCT is to increase the antimalignancy activity of allogeneic T cells without worsening GVHD. One way to accomplish this goal might be to genetically modify T cells to give them the ability to specifically recognize antigens expressed by malignant cells. CARs are well-suited for this task.

Two groups have recently reported promising early results treating B-cell malignancies after alloHSCT with allogeneic donor-derived T cells expressing anti-CD19 CARs.[209,210] Investigators at the National Cancer Institute treated 10 patients with B-cell malignancies that persisted despite alloHSCT and standard donor lymphocyte infusions.[209] Although patients on this trial did not receive chemotherapy before their T-cell infusions, 3 of 10 patients had objective regressions of their malignancies, and 1 patient with CLL remains in CR more than 1 year after treatment.[209] No patient developed GVHD after receiving allogeneic anti-CD19 CAR T cells on this trial.[209] Investigators at the Baylor College of Medicine reported objective antimalignancy responses in two of six patients with relapsed malignancy after infusion of donor-derived allogeneic anti-CD19 CAR T cells that were also specific for viral antigens.[210]

In an effort to improve the safety of infusions of allogeneic lymphocytes by limiting GVHD, investigators have genetically modified T cells to express *suicide genes* that cause death of the T cells containing the suicide gene when certain drugs are administered.[211–214] Suicide gene–expressing T cells are infused to treat malignancy after alloHSCT. This approach has been tested in clinical trials, and rapid abrogation of GVHD has been demonstrated.[211,213,214]

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15 Pharmacokinetics and Pharmacodynamics of Anticancer Drugs

Alex Sparreboom and Sharyn D. Baker

INTRODUCTION

Drug selection and therapy considerations in oncology were originally solely based on observations of the effects produced.[1] To overcome some of the limitations of this empirical approach and to answer questions related to considerations of dose, frequency, and duration of drug treatment, it is necessary to understand the events that follow drug administration. Preclinical in vitro and in vivo studies have shown that the magnitude of antitumor response is a function of the concentration of drug,[2] and this has led to the suggestion that the therapeutic objective can be achieved by maintaining an adequate concentration at the site of action for the duration of therapy.[3] However, drugs are rarely directly administered at their sites of action. Indeed, most anticancer drugs are given intravenously or orally, and yet are expected to act in the brain, lungs, or elsewhere. Drugs must, therefore, move from the site of administration to the site of action and, moreover, distribute to all other tissues including organs that eliminate them from the body, such as the kidneys and liver. To administer drugs optimally, knowledge is needed not only of the mechanisms of drug absorption, distribution, and elimination, but also of the kinetics of these processes.[4]

The treatment of human malignancies involving drugs can be divided into two pharmacologic phases, a *pharmacokinetic* phase in which the dose, dosage form, frequency, and route of administration are related to drug level–time relationships in the body, and a *pharmacodynamic* phase in which the concentration of drug at the site(s) of action is related to the magnitude of the effect(s) produced. Once both of these phases have been defined, a dosage regimen can be designed to achieve the therapeutic objective, although additional factors need to be taken into consideration (Fig. 15.1). The clinical application of this approach allows distinctions between pharmacokinetic and pharmacodynamic causes of an unusual drug response. A basic tenet of pharmacokinetics is that the magnitude of both the desired response and toxicity are functions of the drug concentration at the site(s) of action. Accordingly, therapeutic failure results when either the concentration is too low, resulting in ineffective therapy, or is too high, producing unacceptable toxicity. Between these limits of concentrations lies a region associated with therapeutic success, the so-called *therapeutic window*.[5] Because the concentration of a drug at the site of action can rarely be measured directly, with the exception of certain hematologic malignancies, plasma or blood is commonly measured instead as a more accessible alternative.

PHARMACOKINETIC CONCEPTS

A drug's pharmacokinetic properties can be defined by two fundamental processes affecting drug behavior over time, *absorption* and *disposition*.

Absorption

Historically, most anticancer drugs have been administered intravenously; however, the use of orally administered agents is growing with the development of small-molecule targeted cancer therapeutics, such as tyrosine kinase inhibitors.[6] Moreover, drugs may also be administered regionally, for example into the pleural or peritoneal cavities,[7] the cerebrospinal fluid, or intra-arterially into a vessel leading to a cancerous tissue.[8] The process by which the unchanged drug moves from the site of administration to the site of measurement within the body is referred to as *absorption*. Loss at any site prior to the site of measurement contributes to a decrease in the apparent absorption of a drug. For an orally administered agent, this complex series of events involves disintegration of the pharmaceutical dosage form, dissolution, diffusion through gastrointestinal fluids, permeation of the gut membrane, portal circulation uptake, passage through the liver, and, finally, entry into the systemic circulation. The loss of drug as it passes for the first time through organs of elimination, such as the gastrointestinal membranes and the liver, during the absorption process is known as the *first-pass effect*.[9]

The pharmacokinetic parameter most closely associated with absorption is availability or bioavailability (F), defined as the fraction (or percent) of the administered dose that is absorbed intact. Bioavailability can be estimated by dividing the area under the plasma concentration–time curve (AUC) achieved following extravascular administration by the AUC observed after intravenous administration, and can range from 0 to 1.0 (or 0% to 100%).

Disposition

Disposition is defined as all the processes that occur subsequent to absorption of a drug; by definition, the components of disposition are *distribution* and *elimination*. Distribution is the process of reversible transfer of a drug to and from the site of measurement. Any drug that leaves the site of measurement and does not return has undergone elimination, which occurs by two processes, *excretion* and *metabolism*. Excretion is the irreversible loss of the chemically unchanged drug, whereas metabolism is the conversion of drug to another chemical species.

The extent of drug distribution can be determined by relating the concentration obtained with a known amount of drug in the body and is, in essence, a dilution space. The apparent volume into which a drug distributes in the body at equilibrium in called the volume of distribution (V_d), and may or may not correspond to an actual physiologic compartment.

The rate and extent to which a drug distributes into various tissues depend on a number of factors, including hydrophobicity, tissue permeability, tissue-binding constants, binding to serum proteins, and local organ blood flow.[10] Large apparent volumes of distribution are common for agents with high tissue binding or high lipid solubility,

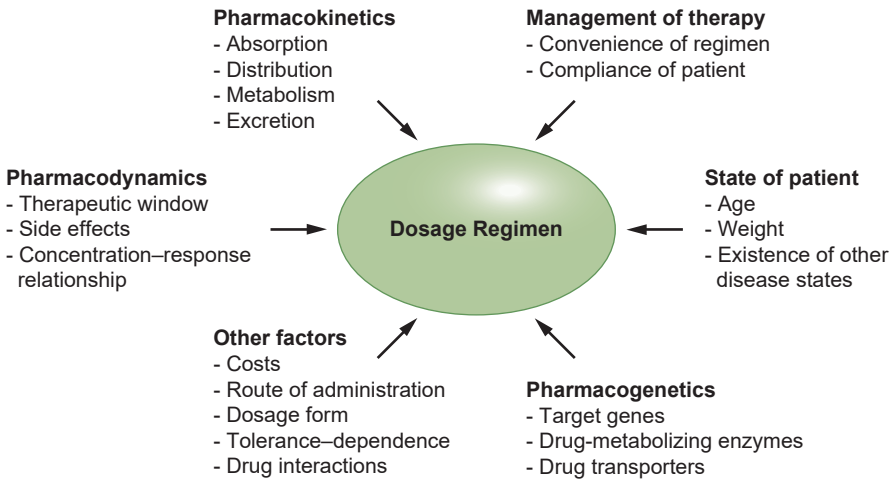


Figure 15.1 Principal determinants of dosage regimen selection for an anticancer drug

although distribution into specific body compartments may be limited by physiologic processes, such as the blood–brain barrier protecting the central nervous system[11,12] or the blood–testes barrier.[13]

Just as V_d is needed as a parameter to relate the concentration to the amount of drug in the body, there is also a need to have a parameter to relate the concentration to the rate of drug elimination, which is known as *clearance* (CL). Of all pharmacokinetic parameters, CL has the most clinical relevance because it defines the key relationship between drug dose and systemic drug exposure (AUC). Derived from V_d and CL is the parameter *elimination rate constant*, which can be regarded as the fractional rate of drug removal. It is, however, more common to refer to the half-life than to the elimination rate constant of a drug. The half-life of a drug is a useful parameter to estimate the time required to reach steady state on a multidose schedule or during a continuous intravenous drug infusion.

Dose Proportionality

When drug concentrations change in strict proportionality to the dose of drug administered, then the condition of dose proportionality (or linear pharmacokinetics) holds. If doubling the dose exactly doubles the plasma concentration or AUC, then pharmacokinetic parameters such V_d, and CL are constant and remain independent of dose and concentration.[14] By strict definition, drugs with linear pharmacokinetics are dose proportional. Dose proportionality is clinically important because it means that dose adjustments will generate predictable changes in systemic drug exposure. For drugs that lack dose proportionality, V_d and CL will demonstrate concentration or time dependence, or both, making it difficult to predict the effect of dose adjustments on drug concentration (Fig. 15.2). Factors that can contribute to a lack of dose proportional pharmacokinetics include saturable oral absorption,[15] capacity-limited distribution or protein binding,[16] and/or saturable metabolism.[17] Dose proportionality of anticancer agents is typically assessed in Phase 1 dose-escalation trials in which small groups of patients are treated at a single dose level using a parallel study design, although the statistical power of such studies to detect deviations from dose proportionality is poor. An alternative, more robust study design is a crossover study in which each patient receives a low dose, an intermediate dose, and a high dose over consecutive cycles of treatment.[18] However, such studies are relatively rare in oncology because of the required use of low, potentially ineffective doses, which may raise ethical concerns for patients.

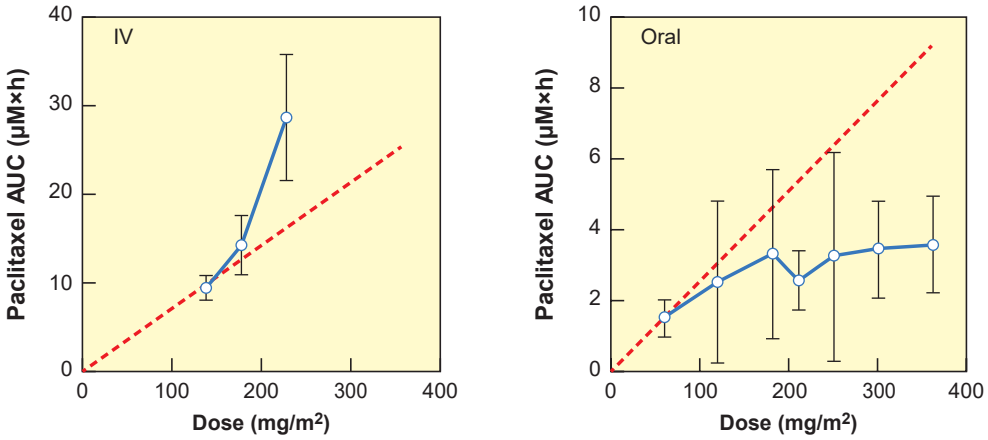


Figure 15.2 Effect of drug dose on systemic exposure to paclitaxel following intravenous (IV) or oral administration in patients with cancer. Data are expressed as mean values (symbols) and standard deviation (error bars). The *dashed line* indicates the hypothetical dose-proportional increase in the area under the plasma concentration time curve (AUC). (Data derived from van Zuylen L, Karlsson MO, Verweij J, et al. Pharmacokinetic modeling of paclitaxel encapsulation in Cremophor EL micelles. *Cancer Chemother Pharmacol* 2001;47:309–318, and Malingre MM, Terwogt JM, Beijnen JH, et al. Phase I and pharmacokinetic study of oral paclitaxel. *J Clin Oncol* 2000;18:2468–2475, respectively.)

TABLE 15.1

Examples of Systemic Exposure as a Pharmacodynamic Marker of Anticancer Drug Effects

Drug	Side Effect	Response/Survival
Carboplatin	Thrombocytopenia	Ovarian cancer
Cisplatin	Nephrotoxicity	Head and neck cancer
Cyclophosphamide	Cardiotoxicity	
Docetaxel	Neutropenia	Non–small-cell lung cancer
Doxorubicin	Neutropenia	
Epirubicin	Neutropenia	
Erlotinib	Skin rash	Non–small-cell lung and head and neck cancer
Etoposide		Non–small-cell lung cancer
5-Fluorouracil	Diarrhea, mucositis	Head and neck cancer
Imatinib		Chronic myeloid leukemia
Irinotecan	Diarrhea, neutropenia	
6-Mercaptopurine		Acute lymphoblastic leukemia
Methotrexate	Mucositis	Acute lymphoblastic leukemia
Nilotinib	Anemia, QT-interval prolongation	
Paclitaxel	Neutropenia	
Sorafenib	Hypertension, hand-foot skin reaction	Renal cell cancer
Sunitinib	Neutropenia	Renal cell cancer
Teniposide		Lymphoma

PHARMACODYNAMIC CONCEPTS

Pharmacodynamic models relate clinical drug effects with drug dose, concentration, or other pharmacokinetic parameters indicative of drug exposures (Table 15.1). In oncology, pharmacodynamic variability may account for substantial differences in clinical outcomes, even when systemic exposures are uniform. Variability in pharmacodynamic response may be heavily influenced by clinical covariates such as age, gender, prior chemotherapy, prior radiotherapy, concomitant medications, or other variables.[19] The pharmacokinetic parameters that are most often correlated with drug effects are markers of drug exposure, such as AUC. In general, the specific parameter used as the independent variable in a pharmacodynamic analysis depends on the particular characteristics of the study drug.

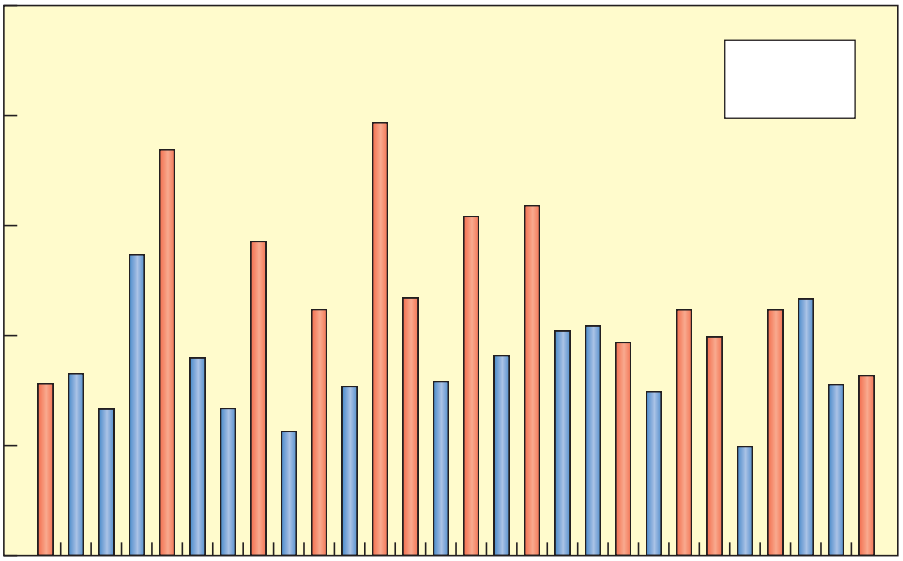
In oncology, pharmacodynamic studies of drug effects have most often focused on toxicity endpoints.[20] Continuous response variables, such as the percentage fall in the absolute blood count from baseline, are easily analyzed using nonlinear regression methods. Dose-limiting neutropenia has been frequently analyzed using a sigmoid maximum effect model described by the modified Hill equation. The pharmacodynamic analysis of subjectively graded clinical endpoints, such as common toxicity criteria scores on a 4-point scale, may require more sophisticated statistical methods.[21,22] Logistical regression methods have been used to model these types of categorical (ordinal) response or outcome variables.

Physiologic pharmacodynamic models describing the severity and time course of drug-related myelosuppression have been derived using population mixed-effect methods for several agents, including paclitaxel[23,24] and pemetrexed.[25] The ability of these models to predict both the severity and duration of drug-induced neutropenia substantially enhances their clinical usefulness.[26] In contrast to small-molecule therapeutics, large-molecule therapeutics such as monoclonal antibodies may not demonstrate toxicities directly related to dose levels. For these agents, a thorough understanding of the pharmacokinetic/pharmacodynamic relationships using modeling approaches may be critical for optimal dose selection.[27]

The antitumor activity of certain chemotherapeutic agents is highly schedule dependent. For such drugs, the dose fractionated over several days can produce a different antitumor response or toxicity profile compared with the same dose given over a shorter period. For example, the efficacy of etoposide in the treatment of small-cell lung cancer is markedly increased when an identical total dose of etoposide is administered by a 5-day divided-dose schedule rather than a 24-hour infusion.[28] Pharmacokinetic analysis in that study showed that both schedules produced very similar overall drug exposure (as measured by AUC), but that the divided-dose schedule produced twice the duration of exposure to an etoposide plasma concentration of >1 μg/mL. This finding has led to the use of prolonged oral administration of etoposide to treat patients with cancer.[29] Similar schedule dependence has been demonstrated for a number of other anticancer agents, notably paclitaxel[30,31] and topotecan.[32] For these agents, the variability in clinically tested treatment schedules is enormous, ranging from short intravenous infusions of less than 30 minutes to 21-day or even 7-week continuous infusion administrations, with large differences in experienced toxicity profiles.

VARIABILITY IN PHARMACOKINETICS/PHARMACODYNAMICS

There is often a marked variation in drug handling between individual patients, resulting in variability in pharmacokinetic parameters (Fig. 15.3), which will often lead to variability in the pharmacodynamic effects of a given dose of a drug.[33] That is, an identical dose of drug may result in acceptable toxicity in one patient, and unacceptable and possibly life-threatening toxicity in another, or a clinical response in one individual and cancer progression in another. The principal underlying sources of this interindividual pharmacokinetic/pharmacodynamic variability are discussed in the following paragraphs.



Body Size and Body Composition

The traditional method of individualizing anticancer drug dosage is by using body surface area (BSA).[34] However, the usefulness of normalizing an anticancer drug dose to BSA in adults has been questioned, because, for many drugs, there is no relationship between BSA and CL.[35] Likewise, attempts to replace BSA as a size metric in dose calculation with alternate descriptors such as lean body weight, either in an average population or in individuals at the outer extremes of weight (i.e., frail, severely obese patients) have failed for many anticancer agents.[36,37] It should be pointed out that BSA is a much more important consideration in drug dose calculation for pediatric patients as compared to adults, because of the larger size range in the former population.[38] Based in part on the failure to reduce interindividual pharmacokinetic variability with the use of BSA normalization to obtain a starting dose, many of the more recently developed molecularly targeted agents are currently administered using a flat-fixed dose irrespective of an individual's BSA.[37]

Age

Changes in body composition and organ function at the extremes of age can affect both drug disposition and drug effect.[39] For example, maturational processes in infancy may alter the absorption and distribution of drugs as well as change the capacity for drug metabolism and excretion.[4] The importance of understanding the influence of age on the pharmacokinetics and pharmacodynamics of individual anticancer agents has increased steadily as treatment for the malignancies of infants,[40] adolescents,[41] and the elderly[42] has advanced. Although pediatric cancers remain rare compared with cancers in adults and the elderly population, in particular, optimizing treatment in a patient group with a high cure rate and a long expected survival becomes critical to minimize the incidence of preventable late complications while maintaining efficacy.

Pathophysiologic Changes

Effects of Disease

Pathophysiologic changes associated with particular malignancies may cause dramatic alterations in drug disposition. For example, increases in the clearance of both antipyrine and lorazepam were noted after remission induction compared with the time of diagnosis in children with acute lymphoblastic leukemia (ALL).[43] The clearance of unbound teniposide is lower in children with ALL in relapse than during first remission.[44] Because leukemic infiltration of the liver at the time of diagnosis is common, drugs metabolized by the liver may have a reduced clearance, as has been documented in preclinical models.[45]

Furthermore, in mouse models, certain tumors elicited an acute phase response that coincided with downregulation of human CYP3A4 in the liver as well as the mouse ortholog Cyp3a11.[46] The reduction of murine hepatic Cyp3a gene expression in tumor-bearing mice resulted in decreased Cyp3a protein expression and, consequently, a significant reduction in Cyp3a-mediated metabolism of midazolam. These findings support the possibility that tumor-derived inflammation may alter the pharmacokinetic and pharmacodynamic properties of CYP3A4 substrates, leading to reduced metabolism of drugs in humans.[47] This supports a possible need for disease-specific design of early clinical trials with anticancer drugs,[48] as has been recommended for docetaxel.[49]

Effects of Renal Impairment

The potential impact of pathophysiologic status on interindividual pharmacokinetic variability can be due to either the disease itself or to a dysfunction of specific organs involved in drug elimination. For example, if urinary excretion is an important elimination route for a given drug, any decrement in renal function could lead to decreased drug clearance, which may result in drug accumulation

and toxicity.[50] Therefore, it would be logical to decrease the drug dose relative to the degree of impaired renal function in order to maintain plasma concentrations within a target therapeutic window. The best known example of this a priori dose adjustment of an anticancer agent remains carboplatin, which is excreted renally almost entirely by glomerular filtration. Various strategies have been developed to estimate carboplatin doses based on renal function among patients, either using creatinine clearance[51] or glomerular filtration rates as measured by a radioisotope method.[52] The application of these procedures has led to a substantial reduction in pharmacokinetic variability, such that carboplatin is currently one of the few drugs routinely administered to achieve a target exposure rather than on a milligram per square meter or milligram per kilogram basis.

The U.S. Food and Drug Administration (FDA) has developed a guidance on the impact of renal impairment on the pharmacokinetics, dosing, and labeling of drugs.[53] The impact of this guidance has been assessed following a survey of 94 new drug applications for small-molecule new molecular entities approved over the years 2003 to 2007. The survey results indicated that 41% of the applications that included renal impairment study data resulted in a recommendation of dose adjustment in renal impairment.[54] Interestingly, the survey results provided evidence that renal impairment can affect the pharmacokinetics of drugs that are predominantly eliminated by nonrenal processes such as metabolism and/or active transport. The latter finding supports the FDA recommendation to evaluate pharmacokinetic/pharmacodynamic alterations in renal impairment for those drugs that are predominantly eliminated by nonrenal processes, in addition to those that are mainly excreted unchanged by the kidneys. A striking example of a drug in the former category is imatinib, an agent that is predominantly eliminated by hepatic pathways but where predialysis renal impairment is associated with dramatically reduced drug clearance,[55] presumably due to a transporter-mediated process.[56]

Effects of Hepatic Impairment

In contrast to the predictable decline in renal clearance of drugs when glomerular filtration is impaired, it is difficult to make general predictions on the effect of impaired liver function on drug clearance. The major problem is that commonly applied criteria to establish hepatic impairment are typically not good indicators of drug-metabolizing enzyme activity and that several alternative hepatic function tests, such as indocyanine green and antipyrine, have relatively limited value in predicting anticancer drug pharmacokinetics. An alternative dynamic measure of liver function has been proposed, which is based on totaled values (scored to the World Health Organization [WHO] grading system) of serum bilirubin, alkaline phosphatase, and either alanine aminotransferase or aspartate aminotransferase to give a hepatic dysfunction score.[57] Based on pharmacokinetic studies in patients with normal and impaired hepatic function, guidelines have been proposed for dose adjustments of several agents when administered to patients with severe liver dysfunction.[58] It should be emphasized that no uniform criteria have been used in the conduct of these studies and that, ultimately, substantial advances could be made through an a priori determination of the hepatic activity of enzymes of pertinent relevance to the chemotherapeutic drug(s) of interest, as has been done for docetaxel.[59]

Effects of Serum Proteins

The binding of drugs to serum proteins, particularly those that are highly bound, may also have significant clinical implications for a therapeutic outcome.[60] Although protein binding is a major determinant of drug action, it is clearly only one of a myriad of factors that influence the disposition of anticancer drugs.[16] The extent of protein binding is a function of drug and protein concentrations, the affinity constants for the drug–protein interaction, and the number of protein-binding sites per class of binding site. Because only the unbound (or free) drug in plasma water is available for distribution, the therapeutic response will correlate with free drug concentration rather than total drug concentration. Several clinical situations, including liver and renal disease, can significantly decrease the extent of serum binding and may lead to higher free drug concentrations and a possible risk of unexpected toxicity, although the total (free plus bound forms) plasma drug concentrations are unaltered.[61] It is important to realize, however, that after therapeutic doses of most anticancer drugs, binding to serum proteins is independent of drug concentration, suggesting that the total plasma concentration is reflective of the unbound concentration. For some anticancer agents, including etoposide[62] and paclitaxel,[63] however, protein binding is highly dependent on dose and schedule.

Sex Dependence

A number of pharmacokinetic analyses have suggested that male gender is positively correlated with the maximum elimination capacity of various anticancer drugs (e.g., paclitaxel)[8] or with increased clearance (e.g., imatinib)[64] compared with female gender. These observations have added to a growing body of evidence that the pharmacokinetic profile of various anticancer drugs exhibits significant sexual dimorphism, which is rarely considered in the design of clinical trials during oncology drug development.

Drug Interactions

Coadministration of Other Chemotherapeutic Drugs

Favorable and unfavorable interactions between drugs must be considered in developing combination regimens. These interactions may influence the effectiveness of each of the components of the combination, and typically occur when the pharmacokinetic profile of one drug is altered by the other. Such interactions are important in the design of trials evaluating drug combinations because, occasionally, the outcome of concurrent drug administration is diminished therapeutic efficacy or increased toxicity of one or more of the administered agents. Although a recent survey indicated that clinically significant pharmacokinetic interactions are relatively rare in Phase I trials of oncology drug combinations,[65] interactions appear to be more common for combinations of tyrosine kinase inhibitors with cytotoxic chemotherapeutics.[66]

Coadministration of Nonchemotherapeutic Drugs

Many prescription and over-the-counter medications have the potential to cause interactions with anticancer agents by altering their pharmacokinetic characteristics and leading to clinically significant phenotypes. Most clinically relevant drug interactions in this category are due to changes in metabolic routes related to an altered expression or function of cytochrome P450 (CYP) isozymes. This class of enzymes, particularly the CYP3A4 isoform, is responsible for the oxidation of a large proportion of currently approved anticancer drugs. Elevated CYP activity (induction), translated into a more rapid metabolic rate, may result in a decrease in plasma concentrations and to a loss of therapeutic effect. For example, anticonvulsant drugs such as phenytoin, phenobarbital, and carbamazepine can induce drug-metabolizing enzymes and thereby increase the clearance of various anticancer agents.[33]

Conversely, the suppression (inhibition) of CYP activity, for example with ketoconazole,[13,67] may trigger a rise in plasma concentrations and can lead to exaggerated toxicity commensurate with overdose. It should be borne in mind that several pharmacokinetic parameters could be altered simultaneously. Especially in the development of anticancer agents given by the oral route,

TABLE 15.2

Effect of Food on Exposure to Select Oral Anticancer Agents

Drug	Food	Effect on Drug Exposure	Manufacturer's Recommendations
Abiraterone	High-fat meal	↑ AUC 1,000%	Without food
Dasatinib	High-fat meal	↑ AUC 14%	With or without food
Erlotinib	High-fat, high-calorie breakfast	Single dose, ↑ AUC 200% Multiple dose, ↑ AUC 37%–66%	Without food[a]
Gefitinib	High-fat breakfast	↓ AUC 14%, ↓ Cmax 35%	With or without food
	High-fat breakfast	↑ AUC 32%, ↑ Cmax 35%	
Imatinib	High-fat meal	No change	With food and a large glass of water[b]
		Variability (% CV) ↓ 37%	
Lapatinib	Low-fat meal (5% fat, 500 calories)	↑ AUC 167%, ↑ Cmax 142%	Without food[c]
	High-fat meal (50% fat, 1,000 calories)	↑ AUC 325%, ↑ Cmax 203%	
Nilotinib	High-fat meal	↑ AUC 82%	Without food
Sorafenib	Moderate-fat meal (30% fat, 700 calories)	No change in bioavailability	Without food
	High-fat meal (50% fat, 900 calories)	↓ Bioavailability 29%	
Sunitinib	High-fat, high-calorie meal	↑ AUC 18%	With or without food
Everolimus	High-fat meal	↓ AUC 16%, ↓ Cmax 60%	With or without food
Vismodegib	High-fat meal	↑ AUC 74% for single dose; no effect at steady state	With or without food
Vorinostat	High-fat meal	↑ AUC 37%	With food[d]

[a] Recommended without food because the approved dose is the maximum tolerated dose.
[b] Recommended with food to reduce nausea.
[c] Recommended without food to achieve consistent drug exposure; was taken without food in clinical trials.
[d] Was taken with food in clinical trials.
AUC, area under the plasma concentration time curve; Cmax, maximum plasma concentration; CV, coefficient of variation.

oral bioavailability plays a crucial role[9]; this parameter is contingent on adequate absorption and the circumvention of intestinal and, subsequently, hepatic metabolism of the drug. It has been suggested that the prevalence of drug–drug interactions is particularly high in cancer patients receiving oral chemotherapy,[68] especially for agents that are weak bases that exhibit pH-dependent solubility.[69]

An additional consideration is related to a possible influence of food intake on the extent of drug absorption after oral administration, which can increase, decrease, or remain unchanged depending on specific physicochemical properties of the drug in question (Table 15.2). The relatively narrow therapeutic index of most of these agents means that significant inter- and intrapatient variability would predispose some individuals to excessive toxicity or, conversely, inadequate efficacy.[12]

Coadministration of Complementary and Alternative Medicine

Surveys within the past decade estimate the prevalence of complementary and alternative medicine (CAM) use in oncology patients to be as high as 87%, and in many cases the treating physician is not aware of the patients' CAM use.[70] With a larger number of participants to phase I clinical trials[71] using herbal treatments combined with allopathic therapies, the risk for herb–drug interactions is a growing concern, and there is an increasing need to understand possible adverse drug interactions in oncology at the early stages of drug development.

A number of clinically important pharmacokinetic interactions involving CAM and cancer drugs have now been recognized, although causal relationships have not always been established.[72] Most of the observed interactions point to the herbs affecting several isoforms of the CYP family, either through inhibition or induction. In the context of chemotherapeutic drugs, St. John's wort,[73] garlic,[74] milk thistle,[75] and Echinacea[11] have been formally evaluated for their pharmacokinetic drug–interaction potential in cancer patients. However, various other herbs have the potential to significantly modulate the expression and/or activity of drug-metabolizing enzymes and drug transporters (Table 15.3), including ginkgo, ginseng, and kava.[70] Because of the high prevalence of herbal medicine use, physicians should include herb usage in their routine drug histories in order to have an opportunity to outline to individual patients which potential hazards should be taken into consideration prior to participation in a clinical trial.

Inherited Genetic Factors

The discipline of pharmacogenetics describes differences in the pharmacokinetics and pharmacodynamics of drugs as a result of inherited variation in drug metabolizing enzymes, drug transporters, and drug targets between patients.[76] These inherited variations are occasionally responsible for extensive interpatient variability in drug exposure or effects. Severe toxicity might occur in the absence of a typical metabolism of active compounds, while the therapeutic effect of a drug could be diminished in the case of an absence of activation of a prodrug, such as irinotecan.[77] The importance and detectability of polymorphisms for a given enzyme or transporter depends on the contribution of the variant gene product to pharmacologic response, the availability of alternative pathways of elimination, and the frequency of occurrence of the variant allele. Although many substrates have been identified for the known polymorphic drug metabolizing enzymes

TABLE 15.3

Effects of Common Herbal Products on Exposure to Anticancer Agents

Botanical	Concurrent Chemotherapy/Condition (Suspected Effect)
Ephedra	Avoid with all cardiovascular chemotherapy (synergistic increase in blood pressure)
Ginkgo	Caution with camptothecins, cyclophosphamide, TK inhibitors, epipodophyllotoxins, taxanes, and vinca alkaloids (CYP3A4 and CYP2C19 inhibition); discourage with alkylating agents, antitumor antibiotics, and platinum analogs (free-radical scavenging)
Ginseng	Discourage in patients with estrogen-receptor–positive breast cancer and endometrial cancer (stimulation of tumor growth)
Green tea	Discourage with erlotinib and pazopanib (CYP1A2 induction)
Japanese arrowroot	Avoid with methotrexate (ABC and OAT transporter inhibition)
St. John's wort	Avoid with all concurrent chemotherapy (CYP2B6, CYP2C9, CYP2C19, CYP2E1, CYP3A4, and ABCB1 induction)
Valerian	Caution with tamoxifen (CYP2C9 inhibition), cyclophosphamide, and teniposide (CYP2C19 inhibition)
Kava-kava	Avoid in all patients with preexisting liver disease, with evidence of hepatic injury (herb-induced hepatotoxicity), and/or in combination with hepatotoxic chemotherapy; caution with camptothecins, cyclophosphamide, TK inhibitors, epipodophyllotoxins, taxanes, and Vinca alkaloids (CYP3A4 induction)

TK, tyrosine kinase; CYP, cytochrome P450; ABC, ATP-binding cassette; OAT, organic anion transporter.

and transporters, the contribution of a genetically determined source of interindividual pharmacokinetic variability has been established for only a few cancer chemotherapeutic agents. Most of these cases involve agents for which elimination is critically dependent on a rate-limiting breakdown by a polymorphic enzyme (e.g., 6-mercaptopurine by thiopurine-S-methyltransferase; 5-fluorouracil by dihydropyrimidine dehydrogenase) or when a polymorphic enzyme is involved in the formation of a toxic metabolite (e.g., tamoxifen by CYP2D6).[78]

In addition to drug metabolism, pharmacokinetic processes are highly dependent on the interplay with drug transport in organs such as the intestines, kidneys, and liver. Genetically determined variation in drug transporter function or expression is now increasingly recognized to have a significant role as a determinant of intersubject variability in response to various commonly prescribed drugs.[79] The most extensively studied class of drug transporters are those encoded by the family of ATP-binding cassette (ABC) genes, some of which also play a role in the resistance of malignant cells to anticancer agents. Among the 48 known ABC gene products, ABCB1 (P-glycoprotein), ABCC1 (multidrug-resistance associated protein-1 [MRP1]) and its homologue ABCC2 (MRP2; cMOAT), and ABCG2 (breast cancer resistance protein [BCRP]) are known to influence the oral absorption and disposition of a wide variety of drugs.[80] As a result, the expression levels of these proteins in humans have important consequences for an individual's susceptibility to certain anticancer drug–induced side effects, interactions, and treatment efficacy, for example, in the case of genetic variation in ABCG2 in relation to gefitinib-induced diarrhea.[81]

Similar to the discoveries of functional genetic variations in drug efflux transporters of the ABC family, there have been considerable advances in the identification of inherited variants in transporters that facilitate cellular drug uptake in tissues that play an important role in drug elimination, such as the liver (Fig. 15.4). Among these, members of the organic anion-transporting polypeptides (OATP), organic anion transporters (OAT), and organic cation transporters (OCT) can mediate the cellular uptake of a large number of structurally divergent compounds.[82,83] Accordingly, functionally relevant polymorphisms in these influx transporters may contribute to interindividual and interethnic variability in drug disposition and response,[84] for example, in the case of the impact of polymorphic variants in the OCT1 gene *SLC22A1* on the survival of patients with chronic myeloid leukemia receiving treatment with imatinib.[85]

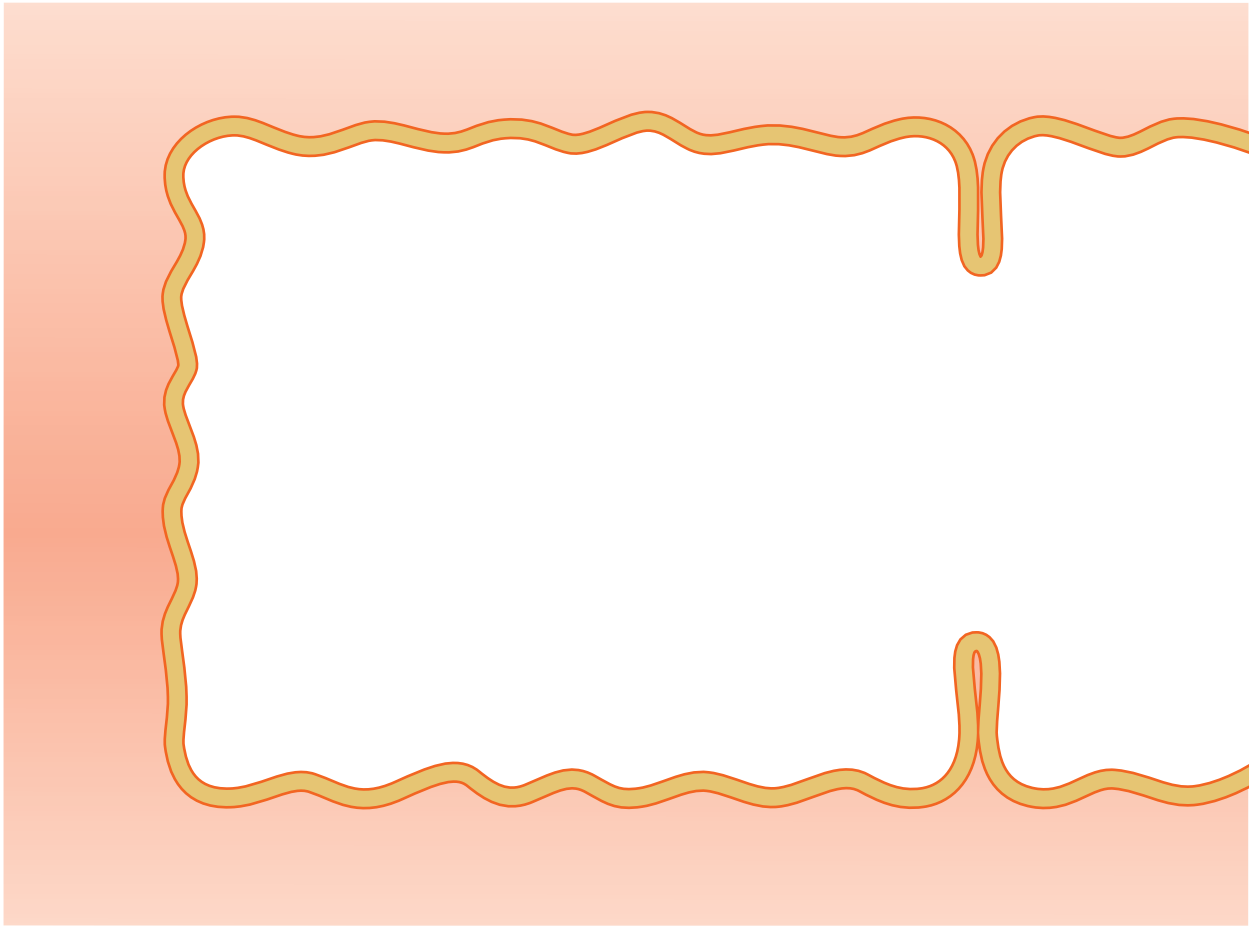
DOSE-ADAPTATION USING PHARMACOKINETIC/PHARMACODYNAMIC PRINCIPLES

Therapeutic Drug Monitoring

Prolonged infusion schedules of anticancer drugs offer a very convenient setting for dose adaptation in individual patients. At the time required to achieve steady-state concentration, it is possible to modify the infusion rate for the remainder of the treatment course if a relationship is known between this steady-state concentration and a desired pharmacodynamic endpoint. This method has been successfully used to adapt the dose during continuous infusions of 5-fluorouracil and etoposide, and for repeated oral administration of etoposide or repeated intravenous administration of cisplatin.[86] Methotrexate plasma concentrations are routinely monitored to identify patients at high risk of toxicity and to adjust leucovorin rescue in patients with delayed drug excretion. This monitoring has significantly reduced the incidence of serious toxicity, including toxic death, and in fact, has improved outcome by eliminating unacceptably low systemic exposure levels.[87] Therapeutic drug monitoring has also been applied to or is currently under investigation for several more recently developed anticancer drugs, including imatinib[88–90] and sorafenib.[91]

Feedback-Controlled Dosing

It remains to be determined how information on interindividual pharmacokinetic variability can eventually be used to devise an optimal dosage regimen of a drug for the treatment of a given disease in an individual patient. Obviously, the desired objective would be most efficiently achieved if the individual's dosage requirements could be calculated prior to administering the drug. While this ideal cannot be met completely in clinical practice, with the notable exception of carboplatin, some success may be achieved by adopting feedback-controlled dosing. In the adaptive dosage with feedback control, population-based predictive models are used initially, but allow the possibility of dosage alteration based on feedback revision. In this approach, patients are first treated with standard dose and, during treatment, pharmacokinetic information is estimated by a limited-sampling strategy and compared with that predicted from the population model with which treatment was initiated. On the basis of the comparison,



more patient-specific pharmacokinetic parameters are calculated, and dosage is adjusted accordingly to maintain the target exposure measure producing the desired pharmacodynamic effect. Despite its mathematical complexity, this approach may be the only way to deliver the desired and precise exposure of an anticancer agent.

The study of population pharmacokinetics seeks to identify the measurable factors that cause changes in the dose-concentration relationship and the extent of these alterations so that, if these are associated with clinically significant shifts in the therapeutic index, dosage can be appropriately modified in the individual patient. It is obvious that a careful collection of data during the development of drugs and subsequent analyses could be helpful to collect some essential information on the drug. Unfortunately, important information is often lost by failing to analyze this data or due to the fact that the relevant samples or data were never collected. Historically, this has resulted in the notion that tools for the identification of patient population subgroups are inadequate for most of the currently approved anticancer drugs.

However, the use of population pharmacokinetic models is increasingly studied in an attempt to accommodate as much of the pharmacokinetic variability as possible in terms of measurable characteristics. This type of analysis has been conducted for a number of clinically important anticancer drugs, including carboplatin,[92] docetaxel,[93] topotecan,[94] gefitinib,[95] and erlotinib,[96] and provided mathematical equations based on morphometric, demographic, phenotypic enzyme activity, and/or physiologic characteristics of patients, in order to predict drug clearance with an acceptable degree of precision and bias.[97]

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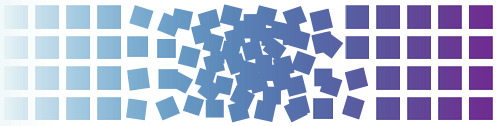
16 Pharmacogenomics



Christine M. Walko and Howard L. McLeod

INTRODUCTION

The evolution of understanding cancer biology has yielded many advances that have been translated into cancer treatment. Application of this knowledge has allowed for a shift in chemotherapeutics from traditional cytotoxic agents that worked by killing both healthy and malignant fast growing cells to chemical and biologic therapies aimed at targeting a specific gene or pathway critical to the particular cancer being treated.[1] This age of pathway-directed therapy has been made possible by the increased availability and feasibility of high throughput technology able to provide comprehensive and clinically useful molecular characterization of tumors. Translation of these efforts have resulted in improved degree to disease control for many common cancers including breast, colorectal, lung, and melanoma as well as long-term survival benefits for chronic myelogenous leukemia (CML), gastrointestinal stromal tumors (GIST), and childhood acute lymphoblastic leukemia (ALL).[2]

Pharmacogenomic-guided therapy aims the use information on DNA and RNA integrity to optimize not only the treatment choice for an individual patient, but also the dose and schedule of that treatment. The assessment of both somatic and germ-line mutations contribute to the overall individualization of cancer treatment. Somatic mutations are genetic variations found within the tumor DNA, but not DNA from the normal (germ-line) tissues, which also have functional consequences that influence disease outcomes and/or response to certain therapies. These types of mutations or biomarkers can be classified as either prognostic or predictive. Prognostic biomarkers identify subpopulations of patients with different disease courses or outcomes, independent of treatment. Predictive biomarkers identify subpopulations of patients most likely to have a response to a given therapy.[3] Germ-line mutations are heritable variations found within the individual and, in practical terms, are focused on DNA markers predictive for toxicity or therapeutic outcomes of a particular therapy as well as inheritable risk of certain cancers.[4] Pharmacogenomic mutations in the germ line provide some explanation for the interindividual and interracial variability in drug response and toxicity. For cancer chemotherapy, where cytotoxic agents are administered at doses close to their maximal tolerable dose, and therapeutic windows are relatively narrow, minor differences in individual drug handling may lead to severe toxicities. Therefore, an understanding of the sources of this variability would lead to the possibility of individualizing dosages or influencing clinical decisions that can improve patient care. Pharmacogenomics has putative utility in therapy selection, clinical study design, and as a tool to improve understanding of the pharmacology of a medication.

The term *pharmacogenetics* was initially used to define inherited differences in drug effects and typically focused on individual candidate genes. The field of pharmacogenomics now includes genomewide association studies and is used to describe genetic variations in all aspects of drug absorption, distribution, metabolism, and excretion in addition to drug targets and their downstream pathways.[5] Table 16.1 illustrates some current clinical examples of genotype-guided cancer chemotherapy. Variations in the DNA sequences encoding these proteins may take the form of deletions, insertions, repeats, frameshift mutations, nonsense mutations, and missense mutations, resulting in an inactive, truncated, unstable, or otherwise dysfunctional protein. The most common change involves single nucleotide substitutions, called single-nucleotide polymorphisms (SNP), which occur at approximately 1 per 1,000 base pairs on the human genome. Variability in toxicity or activity can also be mediated by postgenomic events, at the level of RNA, protein, or functional activity.

PHARMACOGENOMICS OF TUMOR RESPONSE

Tumor response to chemotherapy is regulated by a complex, multigenic network of genes that encompasses inherent characteristics of the tumor, differentially activated pathways of cell signaling, proliferation and DNA repair, factors that control drug delivery to the tumor cells (e.g., metabolism, transport), and cell death. These may in turn be modulated by previously administered treatment or drug exposure, which may upregulate target proteins or activate alternative pathways of drug resistance. The polygenic nature of drug response implies that a better understanding of genotype–phenotype associations would require more than the usual single-gene pharmacogenetic strategies employed to date. However, there are instances where the genomic context of a single gene within a cancer will be of high impact for specific therapeutic agents (see Table 16.1).

Pathway Directed Anticancer Therapy

One of the earliest success stories illustrating pathway-driven therapeutics is with CML. The hallmark chromosomal abnormality of this disease is the translocation of chromosomes 9 and 22 that ultimately produces the fusion gene *BCR-ABL*. This discovery in 1960 eventually led to the development of the targeted tyrosine-kinase inhibitor (TKI) imatinib and its subsequent Food and Drug Administration (FDA) approval for treatment of CML in 2001.[6] The International Randomized Study of Interferon and STI571 (IRIS) trial began enrollment in 2000 and compared imatinib with interferon and low-dose cytarabine, which was the previous standard of care for newly diagnosed patients with chronic-phase CML. All efficacy endpoints favored imatinib, including complete cytogenetic response of 76.2% with imatinib compared with 14.5% with interferon ($p < 0.001$).[7] Overall survival (OS) after 60 months of follow-up was 89% with imatinib.[8] This example is just one of many where a once fatal disease can now be considered more akin to a chronic disease, requiring a daily medication and regular physician follow-up, similar to hypertension or diabetes. Drug development has also kept pace with these advances and now several other agents, including dasatinib, nilotinib, bosutinib, and ponatinib, have joined imatinib as treatment options for CML.

The idea of changing treatment focus from a disease-based model to a pathway-driven model is also evolving. Human epidermal

TABLE 16.1

Clinical Examples of Genotype-Guided Cancer Chemotherapy

Somatic Mutation Examples		
Drug Target	**Drug(s)**	**Malignancy**
EML4-ALK	Crizotinib	Non–small-cell lung cancer
BCR-ABL	Dasatinib, imatinib, nilotinib, bosutinib, ponatinib	Chronic myelogenous leukemia
BRAF	Vemurafenib, dabrafenib	Melanoma
Epidermal growth factor receptor (EGFR)	Erlotinib, afatinib	Non–small-cell lung cancer
HER2	Trastuzumab, lapatinib, pertuzumab, Ado-trastuzumab emtansine	Breast cancer, gastric cancer
Janus kinase 2 (JAK2)	Ruxolitinib	Myelofibrosis
Kirsten rat sarcoma viral oncogene (KRAS)	Cetuximab, panitumumab	Colorectal cancer
Rearranged during transfection (RET)	Vandetanib	Medullary thyroid cancer
Germ-Line Mutation Examples		
Gene Mutation	**Drug**	**Effect**
Cytochrome P450 (CYP) 2C19	Voriconazole	Decreased serum levels of active drug and potential decreased efficacy in patients with high enzyme levels (ultrarapid metabolizers)
CYP2D6	Tamoxifen, codeine, ondansetron	Decreased production of active metabolite and potential decreased efficacy in patients with low enzyme levels
Dihydropyrimidine dehydrogenase (DPYD)	5-Fluorouracil	Decreased elimination and increased risk of myelosuppression, diarrhea, and mucositis in patients with low enzyme levels
Glucose-6-phosphate dehydrogenase (G6PD)	Rasburicase	Risk of severe hemolysis in patients with G6PD deficiency
Thiopurine methyltransferase (TPMT)	Mercaptopurine, thioguanine, azathioprine	Decreased methylation of the active metabolite resulting decreased elimination and increased risk of neutropenia in patients with low enzyme levels
UDP-glucuronosyltransferase (UGT) 1A1	Irinotecan	Decreased glucuronidation of the active metabolite resulting decreased elimination and increased risk of neutropenia and diarrhea in patients with low enzyme levels

growth factor receptor 2 (HER2) is a transmembrane receptor tyrosine kinase that is overexpressed or amplified in up to 25% of breast cancers. Trastuzumab is a humanized monoclonal antibody directed against HER2 and demonstrated improved response rates (RR) and time to disease progression in patients with metastatic HER2 positive breast cancer and improved disease-free survival (DFS) and OS in HER2-positive breast cancer patients treated with adjuvant trastuzumab.[9] Several additional agents are now available to target the HER2 pathway and vary in their pharmacology and mechanism of action. Lapatinib is an oral TKI directed against HER2 and the epidermal growth factor receptor (EGFR), pertuzumab is a humanized monoclonal antibody that binds at a different location than trastuzumab and inhibits the dimerization and subsequent activation of HER2 signaling, and ado-trastuzumab emtansine is an antibody-drug conjugate that targets HER2-positive cells and then releases the cytotoxic antimitotic agent emtansine through liposomal degradation of the linking compound. All of these agents illustrate the progress and pharmacologic diversity of pathway-directed therapy and remain as standard of care options for HER2-positive breast cancer in either the adjuvant and/or metastatic settings.[10] HER2 expression is not limited to breast cancer, however. Though less common, HER2 expression is seen in numerous solid tumors including bladder, gastric, prostate and non–small-cell lung cancer with varying degrees of incidence depending on the method of detection. Based on results from a large, open-label phase III randomized, international trial of 594 patients with gastric or gastroesophageal junction cancer expressing HER2 by either immunohistochemistry or gene amplification by fluorescence in situ hybridization, trastuzumab is also approved for treatment of metastatic gastric or gastroesophageal junction adenocarcinoma that expresses HER2. Patients randomized to chemotherapy in combination with trastuzumab had a median OS of 13.8 months compared with 11.1 months in the patients receiving chemotherapy alone (hazard ratio [HR], 0.74; 0.60 to 0.91, $p = 0.0046$).[11] Numerous examples also support that pathway-directed therapy will cross the boundaries of disease sites and that tumor genetics will become one of the biggest determining factors for treatment.

Simple expression of the drug target does not always translate into desired clinical outcomes though. Cetuximab and panitumumab are monoclonal antibodies directed against EGFR; however, it was found that colorectal cancer (CRC) patients who did not have detectable EGFR still experienced responses to these agents similar in extent to EGFR-positive patients. Kirsten rat sarcoma viral oncogene (KRAS) is a downstream effector of the EGFR pathway. Ligand binding to EGFR on the cell surface activates pathway signaling through the KRAS-RAF-mitogen-activated

protein kinase (MAPK) pathway, which is thought to control cell growth, differentiation, and apoptosis.[12] Eventually it was found that CRC patients with a KRAS mutation did not derive benefit from cetuximab or panitumumab. The RR in CRC receiving either cetuximab or panitumumab who were KRAS wild type was 10% to 40% compared with near zero percent in those with KRAS mutations.[13] This finding was the result of a retrospective analysis of small group of patients and was confirmed in large, prospective trials. Additionally, it underscores the importance of tissue collection for biomarker assessment in trials with novel therapeutics. A recent clinical trial genomic analysis suggests that mutations in *NRAS* may also have value in predicting the utility of EGFR antibody therapy in colorectal cancer. Although the predictive value of KRAS mutation status in colorectal cancer has been well established in clinical trials, the role of KRAS in lung cancer and other malignancies is less well elucidated. Lung cancers harboring KRAS mutations have been shown to have less clinical benefit from the EGFR-targeted erlotinib in some trials, although this has not consistently been the case across all trials. Additionally, lung cancer KRAS mutation status does not appear to reproducibly predict clinical benefit from the EGFR-targeted monoclonal antibodies, as is the case in colorectal cancer.[14] Unlike the HER2 example discussed previously, the clinical application of some genetic mutations will differ between tissue of origin.

Deeper investigations and understandings of mutations driving oncogenic pathways can also elucidate mechanisms of resistance and practical therapeutic strategies for treatment and prevention. Approximately half of all cutaneous melanomas carry mutations in *BRAF*, with the most common being the V600E mutation. Vemurafenib is a TKI directed against mutated BRAF that demonstrated improvements in both progression-free survival (PFS) and OS when compared with the cytotoxic agent dacarbazine in previously untreated patients with metastatic melanoma carrying the BRAF V600E mutation. Vemurafenib demonstrated a 63% relative reduction in the risk of death compared with dacarbazine ($p < 0.001$) along with a higher response rate (48% compared with 5% for dacarbazine).[15] Based on these results, vemurafenib was the first BRAF targeted TKI approved by the FDA and was soon joined by dabrafenib. Although dramatic responses to these agents have been observed, relapse almost universally occurs after a median of 6 to 8 months. Activating BRAF mutations, like V600E, result in uncontrolled activity of the MAPK pathway through activation of the downstream kinase MEK, which when phosphorylated, subsequently activates extracellular signal-regulated kinase (ERK), which ultimately translocates to the cell nucleus, resulting in cell proliferation and survival (Fig. 16.1).[16] An assessment of serial biopsies from patients treated with vemurafenib suggested numerous mechanisms for acquired resistance, including the appearance of secondary mutations in MEK.[17] This finding supports the clinical rationale for using combination therapy with a BRAF and a MEK inhibitor. The combination of dabrafenib (BRAF inhibitor) and trametinib (MEK inhibitor) was assessed in 247 metastatic melanoma patients with BRAF V600 mutations compared with dabrafenib alone. Median PFS was 9.4 months in the combination group compared with 5.8 months in the patients who received single agent therapy (HR, 0.39; 0.25 to 0.62, $p < 0.001$). A complete or partial response was also higher in the combination therapy group (76% compared with 54%, $p = 0.03$). The occurrence of cutaneous squamous cell carcinoma, a known side effect of single-agent BRAF inhibitor therapy due to paradoxical activation of RAF in nonmutated cells, was also decreased in the combination therapy group (7% compared with 19%, $p = 0.09$), further supporting the evidence of downstream inhibition.[18] Although combination therapy does prolong the time to disease progression, resistance still occurs in patients through a variety of mechanisms. Utilization of sequential biopsies and a genetic assessment will help to inform rationale combination and sequential pathway-driven therapy trials that will ultimately aid in better understanding and mitigation of common mechanism of resistance.

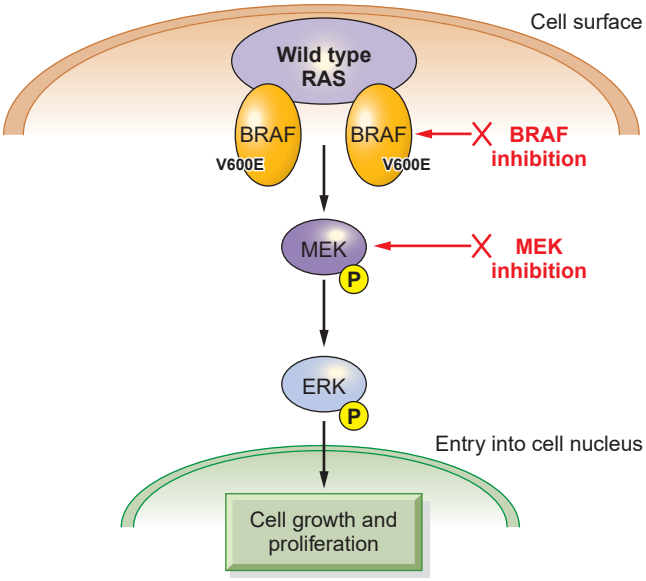


Figure 16.1 MAPK pathway in BRAF mutated melanoma. The BRAF V600E mutation results in activation of the MAPK pathway independent of growth factor binding, initially by phosphorylation (P) of MEK. MEK subsequently phosphorylates ERK. ERK then translocates to the cell nucleus and causes transcription of cellular factors, resulting in cell proliferation and survival. Because one mechanism of resistance to BRAF inhibition is through mutations in MEK, inhibition at both the upstream target of BRAF and the downstream site of MEK can prolong the clinical benefit of the BRAF inhibitor.

Although advances in basic science and drug development have translated many oncogenic driver mutations across tumor types into pathway-directed therapy, this is not the case for the majority. There are numerous examples of functionally relevant recurrent driver mutations that affect protein targets that are not currently druggable. Regardless of malignancy, one of the most commonly mutated tumor suppressors is the protein p53. Mutations can result in p53 acquiring oncogenic functions that enable proliferation, invasion, metastasis, and cell survival as well as coordinating with different proteins, such as EGFR, to enhance or inhibit its effects. However, a clinical application of p53 mutation data or directly targeting p53 has been limited, to date.[19] *PIK3CA* encodes a catalytic subunit of phophoinositol-3 kinase (PI3K), which includes four distinct subfamily kinases involved in regulating cell growth, motility, proliferation, and survival. Direct inhibitors of the kinase, as well as downstream targets, including AKT (protein kinase B [PKB]) and mammalian target of rapamycin (mTOR), are being assessed to target these mutations. Therapeutic challenges include understanding the complex signaling network germane to each cancer and the role of kinases in each subfamily.[20] Both the examples of p53 and PI3K illustrate the challenge of translating the multitude of somatic mutations into applications of available therapeutic agents.

Application of Genomewide Gene Expression Profiling to Guide Therapy

Single gene approaches may not reflect the overall complexity of genetic regulation of chemotherapy responses. Genomic strategies using global gene expression data are able to provide a more complete picture of the tumor through disease classification.[21] These strategies may identify subgroups of patients with early disease that need adjuvant chemotherapy, those who will not benefit from standard therapy, or help with the selection of chemotherapy from a menu of potentially active agents. Oncotype Dx

is a 21-gene assay with 16 tumor-associated genes and 5 reference genes used to predict the risk of distant local recurrence in estrogen receptor (ER)-positive, HER2-negative patients with node-negative or select node-positive breast cancer. Additionally, the test also provides predictive information on which patients may benefit from the addition of chemotherapy to hormonal therapy alone. The test ultimately reports a recurrence score (RS) on a continuous scale from zero to 100. Patients with an RS <18 are considered low risk, with a 10-year distant recurrence rate (DRR) of 6.8% (95% confidence interval [CI], 4 to 9.6); RS scores of 18 to 30 are at intermediate risk, with a 10-year DRR of 14.3% (CI, 8.3 to 20.3); and RS scores ≥31 are at high risk, with a 10-year DRR of 30.5% (CI, 23.6 to 37.4).[22] Additionally, high-risk patients have the largest benefit from the addition of chemotherapy to hormonal therapy (HR, 0.26; 0.13 to 0.53), whereas low-risk patients have little benefit from the addition of chemotherapy and could consider hormonal treatment alone (HR, 1.31; 0.46 to 3.78). Intermediate risk patients are harder to classify, and clinical trials are underway to further address treatment recommendations for this group of patients.[23] These type of assays are also in development and in clinical trials for a variety of other solid tumor and hematologic malignancies.

Genetic-Guided Therapy Practical Issues in Somatic Analysis

Currently, targeted DNA capture is the most common type of somatic genetic screening and involves focusing on a few relevant candidate genes followed by deeper sequencing. These types of techniques can reveal common genes associated with a particular malignancy but also may uncover a signaling pathway that would not be obviously associated with a particular histology or tumor site. Application of a next-generation sequencing assay in 40 CRC and 24 non–small-cell lung cancer (NSCLC) tissue samples that assessed 145 cancer-relevant genes demonstrated that somatic mutations were seen in 98% of the CRC tumors and 83% of the NSCLCs (Fig. 16.2).[24] The evolution of sequencing strategies and decreasing costs has made whole genome sequencing more available in the clinical setting, and several companies offer commercially available tumor profiling services. Several limitations exist that currently restrict the broad clinical implementation of these assays, however. Although germ-line genetic assessments can be done on a peripheral blood sample or buccal swab, somatic assessments typically require biopsy tissue, which is often in limited supply and of varying quality or may not be feasible depending on the site of the cancer. Ongoing studies are assessing the value of liquid biopsies of circulating tumor DNA.[25] Optimizing and creating uniformity in quality control of gene panel or whole-genome assessment is also needed to decrease the reporting of uncertain or erroneous identification of mutations. Once sequencing is completed, a predictive analysis is needed for the 25% to 80% of instances where variants of unknown significance are identified in genes of interest. Translation of genomic sequencing into clinical practice will require a diverse team, including pathologists, medical oncologists, surgical oncologists, information technologists, geneticists, and pharmacologists.

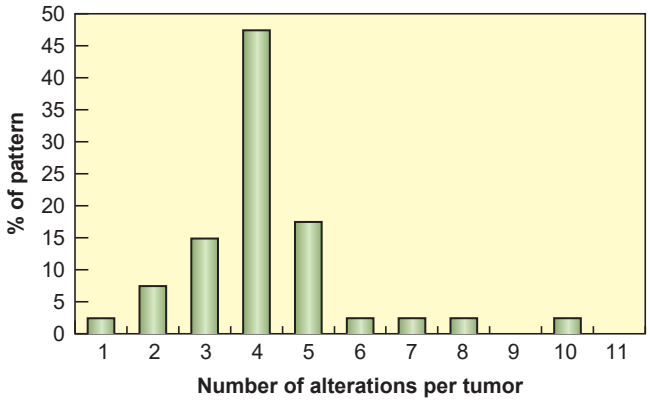


Figure 16.2 Number of alterations per tumor. Deep sequencing of 145 genes in 40 colorectal cancers found a spectrum of incidence of somatic mutations, with more than half occurring in genes that are *druggable* with medication that is either FDA approved or in late stage clinical development.

PHARMACOGENOMICS OF CHEMOTHERAPY DRUG TOXICITY

A drug's disposition and pharmacodynamic effects can be influenced by a number of variables, including patient age, diet, concomitant medications, and underlying disease processes. However, an individual's genetic constitution is an important regulator of variability in drug effect. Differences in drug effects are more pronounced between individuals compared to within an individual. Indeed, studies in monozygotic and dizygotic twins identified that 20% to 80% of the variation in drug disposition is mediated by inheritance.[26] Drug-metabolizing enzymes, cellular transporters, and tissue receptors are governed by genetic variation.

Advances in the treatment of most common malignancies have resulted in the availability of multiple distinct combination chemotherapy regimens with similar or equal anticancer efficacy. Therefore, differences in systemic toxicity have become a major determinant in the selection of therapy. The majority of pharmacogenomic examples affecting adverse events or efficacy from cytotoxic drugs involve hepatic metabolizing enzymes that detoxify or biotransform xenobiotics.[27,28]

Thiopurine Methyltransferase

One of the best-studied pharmacogenetic syndrome involves the metabolism of the thiopurine drugs—6-mercaptopurine (6MP), 6-thioguanine, and azathioprine—which have wide applications, including maintenance therapy for childhood ALL and adult leukemias. These prodrugs must be activated to thioguanine nucleotides in order to have antiproliferative effects. However, most of the variability in the formation of active metabolites is mediated by methylation via thiopurine methyltransferase (TPMT).[29] TPMT is a cytosolic enzyme that catalyzes S-methylation of thiopurine agents, resulting in an inactive metabolite. Erythrocyte TPMT activity has a trimodal distribution, with 90% of patients having high activity, 10% intermediate activity, and 0.3% with very low or no detectable activity. TPMT deficiency results in higher intracellular activation of 6MP to form thioguanine nucleotides, resulting in severe or fatal hematologic toxicity from standard doses of therapy.[30] The variable activity results from polymorphism in the TPMT gene, located on chromosome locus 6p22.3. Genetic variants at codon 238 (TPMT*2), codon 719 (TPMT*3C), or both codons 460 and 719 (TPMT*3A) are the most clinically significant, accounting for 95% of the patients with reduced TPMT activity.[31] Heterozygotes (one wild type and one variant allele) are common (10% of patients), and have elevated levels of active metabolites (twofold more than homozygous wild type), and required more cumulative dose reductions of 6MP for maintenance ALL chemotherapy compared to homozygous wild-type patients (Fig. 16.3).[32] Patients with a homozygous variant TPMT genotype are at a fourfold risk of severe toxicity, compared with wild-type patients.[31] TPMT genotype tests are now available commercially in a Clinical Laboratory Improvement Amendments (CLIA)-certified environment. To date, patients homozygous for TPMT variant alleles appear to tolerate 10%, and heterozygotes appear to tolerate 65% of the recommended doses of 6MP, with no apparent

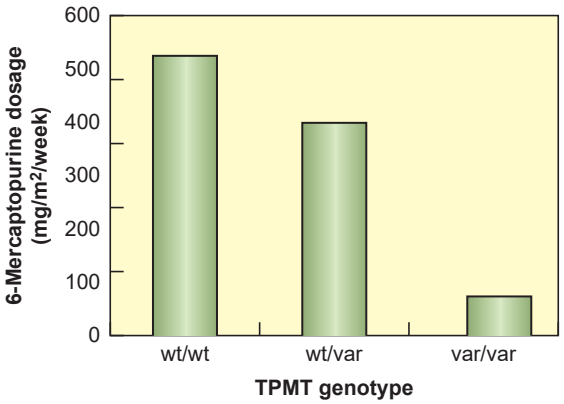


Figure 16.3 Relationship between TPMT genotype and required 6MP dose. Compared with homozygous wild-type patients, those heterozygous for a thiopurine methyltransferase (TPMT) variant allele generally require at least a 30% dose reduction in 6MP, whereas homozygous variant patients require substantial dose reductions of approximately 90% that of wild-type patients.

decrease in clinical efficacy (Fig. 16.3).[32] This has formed the basis for prospective, TPMT genotype-guided dosing of 6MP to avoid severe toxicity. Clinical Pharmacogenomics Implementation Consortium (CPIC) Guidelines recommend that homozygous wild-type patients be started at the full standard dose. Heterozygous patients should start with reduced doses at 30% to 70% of the full dose with adjustments made after 2 to 4 weeks based on myelosuppression and disease-specific guidelines. Homozygous variant patients should start with 10% of the full dose due to the extremely high levels of the active metabolite and potential for fatal toxicity at standard doses. Adjustments should be made after 4 to 6 weeks based on myelosuppression and disease-specific guidelines.[33]

Dihydropyrimidine Dehydrogenase (DPD)

Although 5-fluorouracil (5FU) has been available for over 40 years, it remains the cornerstone of colorectal cancer chemotherapy, both in the adjuvant and metastatic settings. Additionally, the oral prodrug capecitabine ultimately undergoes activation to 5FU and is commonly used in gastrointestinal and breast malignancies. 5FU is a prodrug that is activated intracellularly to 5-fluoro-2′-deoxyuridine monophosphate (5FdUMP), which inhibits thymidylate synthase (TS), among other mechanisms of action. TS inhibition results in impaired de novo pyrimidine synthesis and suppression of DNA synthesis. Approximately 85% of a 5FU dose is catabolized by dihydropyrimidine dehydrogenase (DPD) to inactive metabolites. Therefore, DPD is a primary regulator of 5FU activity. DPD deficiency has been described, resulting in higher 5FU blood levels, greater formation of active metabolites, and severe or fatal clinical toxicity, predominately myelosuppression, mucositis, and cerebellar toxicity.[34] In theory, this toxicity could be reduced or avoided by screening for DPD activity in surrogate tissues, such as peripheral mononuclear cells. However, the technical requirements for preparation of these samples make it impractical for many practice sites. Understanding the molecular basis for DPD deficiency will provide an approach for prospective identification of patients at high risk for severe 5FU toxicity. The gene encoding DPD is composed of 23 exons, and at least 23 SNPs have been found.[35] Studies in DPD-deficient patients have identified several distinct molecular variants associated with low enzyme activity. Many of these are rare, and base substitutions, splicing defects, and frame shift mutations, have been described. The prevalent variation is the splice recognition site in intron 14 (DPYD*2A), where a G to A substitution results in the skipping of exon 14, resulting in an inactive enzyme.[36–38] This polymorphism has been associated with severe DPD deficiency in heterozygous patients, with a homozygous genotype associated with a mental retardation syndrome. Patients with severe 5FU toxicity may harbor one or more variant alleles of DPD, and a recent study showed that 61% of cancer patients experiencing severe 5FU toxicities had decreased DPD activity in peripheral mononuclear cells, and DPYD*2A was commonly found.[39] In the patients with grade 4 neutropenia, 50% harbored at least one DPYD*2A. It is estimated that in the Caucasian population, homozygotes for the variant alleles have an incidence of 0.1% and heterozygotes occur at an incidence of 0.5% to 2%. There are additional DPD mutations that have been associated with impaired enzyme activity, including DPYD *3 and DPYD*13. CPIC guidelines recommend standard dosing for homozygous wild-type patients. Reducing the dose by at least 50% in heterozygous patients (*1/*2A) is recommended, followed by dose adjustment based on toxicity and/or pharmacokinetic testing. The use of an alternative agent is recommended in homozygous-variant patients (*2A/*2A).[34] There are many patients with severe 5FU toxicity that have normal DPD activity. This highlights that many factors, including multiple genes, are potential causes of 5FU toxicity, and there will not be one simple test to avoid this important clinical problem.

Cytochrome P450 2D6

Tamoxifen is a selective estrogen-receptor modulator used in ER-positive breast cancer in both the localized and metastatic settings. It is the drug of choice for premenopausal women and is a treatment option, along with aromatase inhibitors, for postmenopausal women. The low cost of tamoxifen also makes it a preferred therapy regardless of menopausal status in numerous countries. Tamoxifen metabolism is complex, with extensive metabolism through numerous phase I and II enzymes that produce several primary and secondary metabolites and their corresponding isomers, each possessing different antiestrogen effects.[40] The primary active metabolite is believed to be endoxifen, which is produced by the CYP3A4/5 mediated-conversion of tamoxifen to N-desmethyltamoxifen, which is then further converted to endoxifen (4-hydroxy-N-desmethyltamoxifen) via cytochrome P450 2D6 (CYP2D6). A direct relationship between endoxifen concentration and its antiestrogen effects has been demonstrated, potentially suggesting that a threshold concentration may be needed for optimal clinical effect.[41] CYP2D6 is highly polymorphic, with more than 80 allelic CYP2D6 variants described. These alleles vary in enzyme activity and prevalence with respect to race and ethnicity.[42] Based on genotype, patients can be classified by phenotype into ultrarapid metabolizers (UM; approximately 1% to 2% of patients [common alleles include *1xN, *2xN]) who carry more than two functional allele copies, extensive metabolizers (EM; 77% to 92% [e.g., *1, *2]), intermediate metabolizers (IM; 2% to 11% [e.g., *10, *17, *41]), or poor metabolizers (PM; 5% to 10% [*3, *4, *5]).[43] UM patients have the highest concentrations of endoxifen, followed by EM patients, then IM patients, and finally, PM patients have the lowest concentration. Up to a sixfold variation in endoxifen levels may be seen between homozygous PM and homozygous EM patients.[40]

The relationship between CYP2D6 genotype, endoxifen concentrations, and disease outcomes has been investigated in numerous clinical trials. One of the largest retrospective trials assessed this relationship in 1,325 women treated with adjuvant tamoxifen 20 mg daily. Approximately 46% of the patients were classified as EM, 48% were IM, and 5.9% were PM. A statistically significant increased risk of disease recurrence was seen in the IM and PM patients compared with the EM patients (HR, 1.40; 95% CI, 1.04 to 1.90 for IM; and HR 1.90, 95% CI, 1.10 to 3.28 for PM).[44] A large meta-analysis of 4,973 tamoxifen-treated patients across 12 international studies conducted by the International Tamoxifen Pharmacogenomics Consortium also supported this relationship.

CYP2D6 PM phenotypes were associated with decreased DFS (HR 1.25, 95% CI, 1.06 to 1.47, p = 0.009) when only considering the data from trials with postmenopausal women with ER-positive breast cancer who received tamoxifen 20 mg daily for 5 years.[45]

Not all trial results have been consistent, however, and dosing guidelines for genotype-guided therapy do not yet exist. Clinical trials do support the potential for genotype-guided therapy. IM patients who received an increased dose of 40 mg daily instead of the standard 20 mg were shown to have endoxifen concentrations similar to that of EM patients (p = 0.25).[46] This suggests that genotype-guided therapy with increased dose recommendations may be feasible, but additional prospective trials are needed to determine the clinical efficacy of this intervention.

CONCLUSIONS AND FUTURE DIRECTIONS

Genomic-driven cancer medicine is being translated into clinical practice through increased understanding of somatic mutations in a specific tumor that can be translated to pathway-directed therapeutics as well as germ-line mutations that affect the pharmacokinetics and pharmacodynamics of individual medications. For the practicing oncologist, knowledge of pharmacogenomics is necessary because therapeutic decisions of drug selection and dosage are being based on more molecularly and genetically defined variables than the current phenotypic information of tumor type, immunohistochemistry, and body surface area. Health-care policy changes preferring the bundling of care and reimbursement based on diagnosis coding may further drive individualized therapy where the goal is to optimize both treatment responses while minimizing toxicity. However, with advances always come challenges. Reimbursement for multiplex genomic testing is not universal, so deciding who and when to initiate testing is a consideration. Optimizing turnaround time, especially for referral patients who have had biopsies performed elsewhere, will require requesting this archived tissue prior to or during the initial patient visit to facilitate minimizing treatment delays. Although some variants have strong evidence supporting treatment recommendations, many currently do not yet. Multidisciplinary committees charged with reviewing the level of evidence for each genetic result and providing clinically actionable recommendations will be essential for translating these multigene tumor assay results into routine clinical practice. Decision tools and development of treatment guidelines will further assist with routine integration of this technology, especially for oncologists at smaller practice sites. Oncology fellowship training programs will also need to be expanded to ensure competence of new practitioners in the area of genomic-guided therapies.

Regardless of these challenges, the treatment paradigm of genomic-driven medicine and individualizing therapy has permitted the field of oncology to move beyond the limitations of nonselective cytotoxic therapy and toward the more optimal selection and dosing of oncology agents.

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17 Alkylating Agents

Kenneth D. Tew

PERSPECTIVES

Alkylating agents were the first anticancer molecules developed, and they are still used today. After more than 50 years of use, the basic chemistry and pharmacology of this drug family is well understood and has not changed substantially. The family contains six major classes: nitrogen mustards, aziridines, alkyl sulfonates, epoxides, nitrosoureas, and triazene compounds, although a few nonstandard agents have recently been developed. Most epoxides tend to be quite nonspecific with respect to their reactivity and, as such, few have useful clinical characteristics. This chapter provides perspective on how the limited varieties of alkylating agents continue to be useful in the therapeutic management of cancer patients.

The alkylating agents are a diverse group of anticancer agents with the commonality that they react in a manner such that an electrophilic alkyl group or a substituted alkyl group can covalently bind to cellular nucleophilic sites. Electrophilicity is achieved through the formation of carbonium ion intermediates and can result in transition complexes with target molecules. Ultimately, reactions result in the formation of covalent linkages by alkylation with a broad range of nucleophilic groups, including bases in DNA, and these are believed responsible for ultimate cytotoxicity and therapeutic effect. Although the alkylating agents react with cells in all phases of the cell cycle, their efficacy and toxicity result from interference with rapidly proliferating tissues. From a historical perspective, the vesicant properties of mustard gas used during World War I were shown to be accompanied by the suppression of lymphoid and hematologic functions in experimental animals[1] and led to the development of mechlorethamine as the first alkylating agent used in the management of human cancer.[2] Subsequently, a number of related drugs have been developed, and these have roles in the treatment of a range of leukemias, lymphomas, and solid tumors. Most of the alkylating agents cause dose-limiting toxicities to the bone marrow and, to a lesser degree, the intestinal mucosa, with other organ systems also affected contingent on the individual drug, dosage, and duration of therapy. Despite the present trend toward targeted therapies, this class of "nonspecific" drugs maintains an essential role in cancer chemotherapy.

Because of the classic nature of the drug family, there have been relatively few advances in either their use or utility since publication of the previous edition of this book.

CHEMISTRY

Alkylating reactions are generally classified through their kinetic properties as S_N1 (nucleophilic substitution, first order) or S_N2 (nucleophilic substitution, second order) (Fig. 17.1). The first-order kinetics of the S_N1 reactions depend on the concentration of the original alkylating agent. The rate-limiting step is the initial formation of the reactive intermediate, and the rate is essentially independent of the concentration of the substrate. The S_N2 alkylation reaction is a bimolecular nucleophilic displacement with second-order kinetics, where the rate depends on the concentration of both alkylating agent and target nucleophile. Reactivity of electrophiles[3] suggests that the rates of alkylation of cellular nucleophiles (including thiols, phosphates, amino and imidazole groups of amino acids, and various reactive sites in nucleic acid bases) are most dependent on their potential energy states, which can be defined as "hard" or "soft," based on the polarizability of their reactive centers.[4] Although the metabolism and metabolites of nitrogen mustards and nitrosoureas differ, the active alkylating species of each is the alkyl carbonium ion (see Fig. 17.1), a highly polarized hard electrophile as a consequence of its highly positive charge density at the electrophilic center. Alkyl carbonium ions will react most readily with hard nucleophiles (possessing a highly polarized negative charge density), where the high-energy transition state (a potential energy barrier to the reaction) is most favorable. In specific terms, an active alkylating species from a nitrogen mustard will demonstrate selectivity for cellular nucleophiles in the following order: (1) oxygen in phosphate groups of RNA and DNA, (2) oxygens of purines and pyrimidines, (3) amino groups of purine bases, (4) primary and secondary amino groups of proteins, (5) sulfur atoms of methionine, and (6) thiol groups of cysteinyl residues of protein and glutathione.[3] The least favored reactions will still occur, but at much slower rates unless they are catalyzed.

Alkylation through highly reactive intermediates (e.g., mechlorethamine) would be expected to be less selective in their targets than the less reactive S_N2 reagents (e.g., busulfan). However, the therapeutic and toxic effects of alkylating agents do not correlate directly with their chemical reactivity. Clinically useful agents include drugs with S_N1 or S_N2 characteristics, and some with both.[5] These differ in their toxicity profiles and antitumor activity, but more as a consequence of differences in pharmacokinetics, lipid solubility, penetration of the central nervous system (CNS), membrane transport, metabolism and detoxification, and specific enzymatic reactions capable of repairing alkylation sites on DNA.

CLASSIFICATION

The major classes of clinically useful alkylating agents are illustrated in Table 17.1 and summarized in the following sections. Doses and schedules of the various agents are shown in Table 17.2.

Alkyl Sulfonates

Busulfan is used for the treatment of chronic myelogenous leukemia. It exhibits S_N2 alkylation kinetics and shows nucleophilic selectivity for thiol groups, suggesting that it may exert cytotoxicity through protein alkylation rather than through DNA. In contrast to the nitrogen mustards and nitrosoureas, busulfan has a greater effect on myeloid cells than lymphoid cells, thus the reason for its use against chronic myelogenous leukemia.[6]

Aziridines

Aziridines are analogs of ring-closed intermediates of nitrogen mustards and are less chemically reactive, but they have

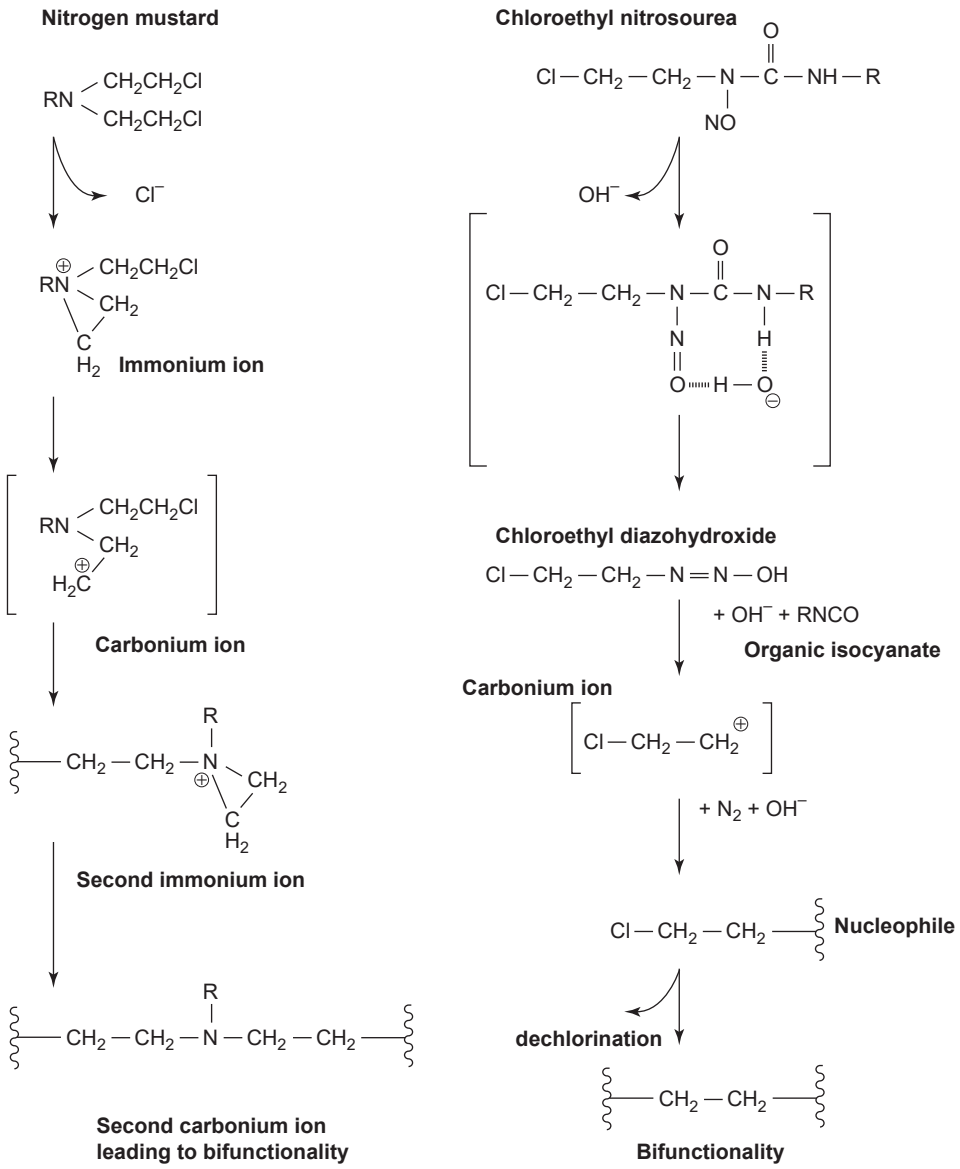

Figure 17.1 Comparative decomposition and metabolism of a typical nitrogen mustard compared to a nitrosourea. Although intermediate metabolites are distinct, the active alkylating species is a carbonium ion in each case. This electrophilic moiety reacts with target cellular nucleophiles.

equivalent therapeutic properties. Thiotepa has been used in the treatment of carcinoma of the breast, ovary, for a variety of CNS diseases, and with increasing frequency as a component of high-dose chemotherapy regimens.[7] Thiotepa and its primary desulfurated metabolite triethylenethiophosphoramide (TEPA) alkylate through aziridine ring openings, a mechanism similar to the nitrogen mustards.

Triazines

Perhaps the newest clinical development in the alkylating agent field is the emergence of temozolomide (TMZ). This agent acts as a prodrug and is an imidazotetrazine analog that undergoes spontaneous activation in solution to produce 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide (MTIC), a triazine derivative. It crosses the blood–brain barrier with concentrations in the CNS approximating 30% of plasma concentrations.[8] Resistance to the methylating agent occurs quite frequently and has adversely affected the rate and durability of the clinical responses of patients. However, because of its favorable toxicity and pharmacokinetics, TMZ is being combined with numerous other classes of anticancer drugs in an effort to improve response rates in diseases such as malignant melanomas, gliomas, brain metastasis from solid tumors, and refractory leukemias. Many of these trials are currently underway.[9]

Nitrogen Mustards

Bischloroethylamines or nitrogen mustards are extensively administered in the clinic. As an initial step in alkylation, chlorine acts as a leaving group and the β-carbon reacts with the nucleophilic nitrogen atom to form the cyclic, positively charged, reactive aziridinium moiety. Reaction of the aziridinium ring with an electron-rich nucleophile creates an initial alkylation product. The remaining chloroethyl group achieves bifunctionality through the formation of a second aziridinium. Melphalan (L-phenylalanine mustard), chlorambucil, cyclophosphamide, and ifosfamide (see Table 17.1) replaced mechlorethamine as primary therapeutic agents. These derivatives have electron-withdrawing groups substituted on the nitrogen atom, reducing the nucleophilicity of the nitrogen and rendering them less reactive, but enhancing their antitumor efficacy.

TABLE 17.1

Major Classes of Clinically Useful Alkylating Agents

Drug	Main Therapeutic Uses	Clinical Pharmacology	Major Toxicities	Notes
ALKYL SULFONATES				
Busulfan	Bone marrow transplantation, especially in chronic myelogenous leukemia	Bioavailability, 80%; protein bound, 33%; $t_{1/2}$, 2.5 h	Pulmonary fibrosis, hyperpigmentation thrombocytopenia, lowered blood platelet count and activity	Oral or parenteral; high dose causes hepatic veno-occlusive disease
ETHYLENEIMINES/METHYLMELAMINES				
Altretamine		Protein bound, 94%; $t_{1/2}$, 5–10 h	Nausea, vomiting, diarrhea, and neurotoxicity	Not widely used
Thio TEPA	Breast, ovarian, and bladder cancer; also bone marrow transplant	$t_{1/2}$, 2.5 h; urinary excretion at 24 h, 25%; substrate for CYP2B6 and CYP2C11	Myelosuppression	Nadirs of leukopenia, occur 2 wk; thrombocytopenia, 3 wk (correlates with AUC of parent drug)
NITROGEN MUSTARDS				
Mechlorethamine	Hodgkin lymphoma		Nausea, vomiting, myelosuppression	Precursor for other clinical mustards
Melphalan (L-phenylalanine mustard)	Multiple myeloma and ovarian cancer, and occasionally malignant melanoma	Bioavailability 25%–90%; $t_{1/2}$, 1.5 h; urinary excretion at 24 h, 13%; clearance, 9 mL/min/kg	Nausea, vomiting, myelosuppression	Causes less mucosal damage than others in class
Chlorambucil	Chronic lymphocytic leukemia	$t_{1/2}$, 1.5 h; urinary excretion at 24 h, 50%	Myelosuppression, gastrointestinal distress, CNS, skin reactions, hepatotoxicity	Oral
Cyclophosphamide	Variety of lymphomas, leukemias, and solid tumors	Bioavailability, >75%; protein bound, >60%; $t_{1/2}$, 3–12 h; urinary excretion at 24 h, <15%	Nausea and vomiting, bone marrow suppression, diarrhea, darkening of the skin/nails, alopecia (hair loss), lethargy, hemorrhagic cystitis	IV; primary excretion route is urine
Ifosfamide	Testicular, breast cancer; lymphoma (non-Hodgkin); soft tissue sarcoma; osteogenic sarcoma; lung, cervical, ovarian, bone cancer	$t_{1/2}$, 15 h; urinary excretion at 24 h, 15%	As for cyclophosphamide	Ifosfamide is often used in conjunction with mesna to avoid cystinuria
NITROSOUREAS				
Carmustine	Glioma, glioblastoma multiforme, medulloblastoma and astrocytoma, multiple myeloma and lymphoma (Hodgkin and non-Hodgkin)	Bioavailability, 25%; protein bound, 80%; $t_{1/2}$, 30 min	Bone marrow and pulmonary toxicities are a function of lifetime cumulative dose	Clinically, nitrosoureas do not share cross-resistance with nitrogen mustards in lymphoma treatment
Streptozotocin	Cancers of the islets of Langerhans	$t_{1/2}$, 35 min; excreted in the urine (15%), feces (<1%), and in the expired air	Nausea and vomiting; nephrotoxicity can range from transient protein urea and azotemia to permanent tubular damage; can also cause aberrations of glucose metabolism	A natural product from *Streptomyces achromogenes*
TRIAZENES				
Dacarbazine	Malignant melanoma and Hodgkin lymphoma	$t_{1/2}$, 5 h; protein bound, 5% hepatic metabolism	Nausea, vomiting, myelosuppression	IV or IM
Temozolomide	Glioblastoma; astrocytoma; metastatic melanoma	Protein bound, 15%; $t_{1/2}$, 1.8 h; clearance, 5.5 l/h/m^2	Nausea, vomiting, myelosuppression	Oral; derivative of imidazotetrazine, prodrug of dacarbazine; rapidly absorbed

$t_{1/2}$, half-life; TEPA, triethylenethiophosphoramide; AUC, area under curve; CNS, central nervous system; IV, intravenous; IM, intramuscular.

TABLE 17.2

Dose and Schedules of Clinically Useful Alkylating Agents

Alkylating Agent	Disease Sites and Dose Ranges Used Clinically	Notes
BCNU (Carmustine)	General antineoplastic 150–200 mg/m^2 (IV, every 6 wks) Cutaneous T-cell lymphoma 200–600 mg (topical solution) Adjunct to surgical resection of brain tumor 61.6 mg (implant)	Infusion 1–2 h; in combination, dose usually reduced by 25%–50% Side effects include irritant dermatitis, telangiectasia, erythema, and bone marrow suppression Up to 8 wafers (7.7 mg of carmustine) implanted
Busulfan	Chronic myelogenous leukemia and myeloproliferative disorders 4–8 mg (daily PO) 1.8 mg/m^2 (daily PO) Bone marrow transplant 640 mg/m^2 (daily PO)	Dispensed over 3–4 d, with cyclophosphamide
Carboplatin	Advanced ovarian cancer—monotherapy 360 mg/m^2 (IV, every 4 wks) Ovarian cancer—combination 300 mg/m^2 (IV, every 4 wks for 6 cycles) Ovarian cancer—IP 200–500 mg/m^2 (IP, 2 L dialysis fluid) Ovarian and other sites phase 1/2 setting—high-dose therapy 800–1,600 mg/m^2 (IV)	With cyclophosphamide Patients usually receive marrow transplantation or peripheral stem cell support
Cisplatin	Metastatic testicular cancer: 20 mg/m^2/d for 5 d of each cycle (IV) Metastatic ovarian cancer: 75–100 mg/m^2 (IV, once every 4 wks) Head and neck cancer: 100 mg/m^2 (IV) Bladder cancer: (combination prior to cystectomy) 50–70; initiate dosing at 50 mg/m^2 (IV, once every 3–4 wks) Metastatic breast cancer: 20 mg/m^2 (IV, days 1–5 every 3 wks) Cervical cancer: 70 mg/m^2 (IV, dosing cycled every 4 wks) Non–small-cell lung cancer: 75 mg/m^2 (IV, every 3 wks) Esophageal cancer: 75 mg/m^2 on day 1 of wks 1, 5, 8, and 11 (IV)	With other antineoplastic agents With cyclophosphamide (600 mg/m^2 once every 4 wks) With vincristine, bleomycin, and fluorouracil With methotrexate and fluorouracil MVAC regimen (methotrexate, vinblastine, doxorubicin, and cisplatin) used for cervical cancer Administration preceded by paclitaxel 135 mg/m^2 every 3 wks With radiation therapy
Cyclophosphamide	General antineoplastic 1–5 mg/kg (daily PO) 40–50 mg/kg (IV, in divided doses over 2–5 d) 40–50 mg/kg (IV, in divided doses over 2–5 d) 10–15 mg/kg (IV, every 7–10 d) 10–15 mg/kg (IV, every 7–10 d) 3–5 mg/kg (IV twice per wk) High-dose regimen in bone marrow transplantation and for other autoimmune disorders 200 mg/kg (IV) 1–2.5 mg/kg (daily PO 7–14 d/mo)	Dose used as monotherapy for patients with no hematologic toxicity
Dacarbazine	General antineoplastic 2–4.5 mg/kg/d (IV) 150 mg/m^2/d (IV)	Administered for 10 d, may be repeated at 4-week intervals With other anticancer agents; treatment lasts 5 d, may be repeated every 4 wks
Etoposide	Testicular cancer 50–100 mg/m^2/day (IV, slow infusion over 30–60+ min for 5 d) Small cell lung cancer 35–50 mg/m^2/day (IV, slow infusion over 30–60+ min for 4–5 d)	Alternatively, 100 mg/m^2/d on days 1, 3, and 5 may be used; doses for combination therapy and are repeated at 3- to 4-wk intervals after recovery from hematologic toxicity Doses are for combination therapy and repeated at 3- to 4-wk intervals after recovery from hematologic toxicity; oral dose is twice the IV, rounded to the nearest 50 mg

(continued)

TABLE 17.2

Dose and Schedules of Clinically Useful Alkylating Agents *(continued)*

Ifosfamide	General antineoplastic 1.2 g/m^2/d (IV, for 5 consecutive days)	Repeat every 3 wks
Melphalan	Multiple myeloma: 16 mg/m^2 (IV, infusion over 15–20 min) 6 mg (daily PO) Epithelial ovarian cancer: 0.2 mg/kg (daily PO)	2-week intervals for 4 doses, 4-wk intervals thereafter After 2–3 wks treatment, should be discontinued for up to 4 wks, then reinstituted at 2–4 mg/d Daily dose for a 5-d course, repeated every 4–5 wks
Streptozotocin	Pancreatic tumors 500 mg/m^2/d; 1,000 mg/m^2/d (IV; IV)	500 mg for 5 consecutive days every 6 wks, 1,000 mg is for 2 wks, followed by an increase in weekly dose not to exceed 1,500 mg/m^2/wk
Temozolomide	Brain tumors 150 mg/m^2 (daily PO)	Dose adjusted on the basis of blood counts
Thiotepa	General antineoplastic: 0.3–0.4 mg/kg (IV) Papillary carcinoma of the bladder: 60 mg/wk for 4 wks (bladder catheter) Control of serous effusions: 0.6–0.8 mg/kg (intracavitary)	Rapid administration given at 1- to 4-wk intervals 30 or 60 mL should be retained for 2 h, so the patient is usually dehydrated prior to administration of the drug

IV, intravenously; PO, by mouth; IP, intraperitoneal.

One distinguishing feature of melphalan is that an amino acid transporter responsible for uptake influences its efficacy across cell membranes.[10] Although a number of glutathione (GSH) conjugates of alkylating agents are effluxed through adenosine triphosphate–dependent membrane transporters,[11] specific uptake mechanisms are generally rare for cancer drugs. Cyclophosphamide and ifosfamide are prodrugs that require cytochrome P-450 metabolism to release active alkylating species. Cyclophosphamide continues to be the most widely used alkylating agent and has activity against a variety of tumors.[12] A cost saving with equivalent therapeutic activity was recently shown in a modified regimen of high-dose cyclophosphamide plus cyclosporine in patients with severe or very severe aplastic anemia.[13]

Nitrosoureas

The nitrosoureas form a diverse class of alkylating agents that have a distinct metabolism and pharmacology that separates them from others.[14] Under physiologic conditions, proton abstraction by a hydroxyl ion initiates spontaneous decomposition of the molecule to yield a diazonium hydroxide and an isocyanate (see Fig. 17.1). The chloroethyl carbonium ion generated is the active alkylating species. Through a subsequent dehalogenation step, a second electrophilic site imparts bifunctionality.[15] Thus, while cross-linking may occur similar to those lesions caused by nitrogen mustards, the chemistry leading to the endpoint is distinct. The isocyanate species generated are also electrophilic, showing nucleophilic selectivity toward sulfhydryl and amino groups that can inhibit a number of enzymes involved in nucleic acid synthesis and thiol balance.[16] Because carbamoylation is considered of minor importance to the therapeutic efficacy of clinically used nitrosoureas, chlorozotocin and streptozotocin were designed to undergo internal carbamoylation at the 1- or 3-OH group of the glucose ring, with the consequence that no carbamoylating species are produced.[17,18] Streptozotocin is also unusual in that most methylnitrosoureas have only modest therapeutic value. However, its lack of bone marrow toxicity and strong diabetogenic effect in animals led to its use in cancer of the pancreas (see Table 17.1).[19] The dose-limiting toxicities in humans are gastrointestinal and renal, but the drug has considerably less hematopoietic toxicity than the other nitrosoureas. Because of their lipophilicity and capacity to cross the blood–brain barrier, the chloroethylnitrosoureas were found to be effective against intracranially inoculated murine tumors. Indeed, early preclinical studies showed that many mouse tumors were quite responsive to nitrosoureas. The same extent of efficacy was not found in humans. Subsequent analyses demonstrated that an enzyme responsible for repair of O-6-alkyl guanine (O^6-methylguanine-DNA methyltransferase [MGMT], or the Mer/Mex phenotype)[20] was expressed at low levels in mice, but at high levels in humans, a contributory factor in the reduced clinical efficacy of nitrosoureas in humans. In the 1980s, in particular, a number of new nitrosoureas were tested in patients in Europe and Japan, but none established a regular role in standard cancer treatment regimens.

MGMT promoter methylation is crucial in MGMT gene silencing and can predict a favorable outcome in glioblastoma patients receiving alkylating agents.[21] This biomarker is on the verge of entering clinical decision making and is currently used to stratify or even select glioblastoma patients for clinical trials. In other subtypes of glioma, such as anaplastic gliomas, the relevance of MGMT promoter methylation might extend beyond the prediction of chemosensitivity, and could reflect a distinct molecular profile. At this time, the standardization of MGMT assays will be critical in establishing prospective prognostic or predictive effects. In addition, eventual clinical trials will need to determine, for each subtype of glioma, the extent to which methylation patterns are predictive or prognostic and whether such assays could be incorporated into an individualized approach to clinical practice.[21]

CLINICAL PHARMACOKINETICS/ PHARMACODYNAMICS

The pharmacokinetics of the alkylating agents are highly variable depending on the individual agent. Nevertheless, they are generally characterized by high reactivity and short half-lives. Although detailed studies on clinical pharmacology are available,[22] Table 17.1 summarizes some of the primary kinetic characteristics of the major clinically useful drugs. Mechlorethamine is unstable and is administered rapidly in a running intravenous infusion to avoid its rapid breakdown to inactive metabolites. In contrast, chlorambucil and cyclophosphamide are sufficiently stable to be given orally, and are rapidly and completely absorbed from the gastrointestinal tract, whereas others like melphalan have poor and variable

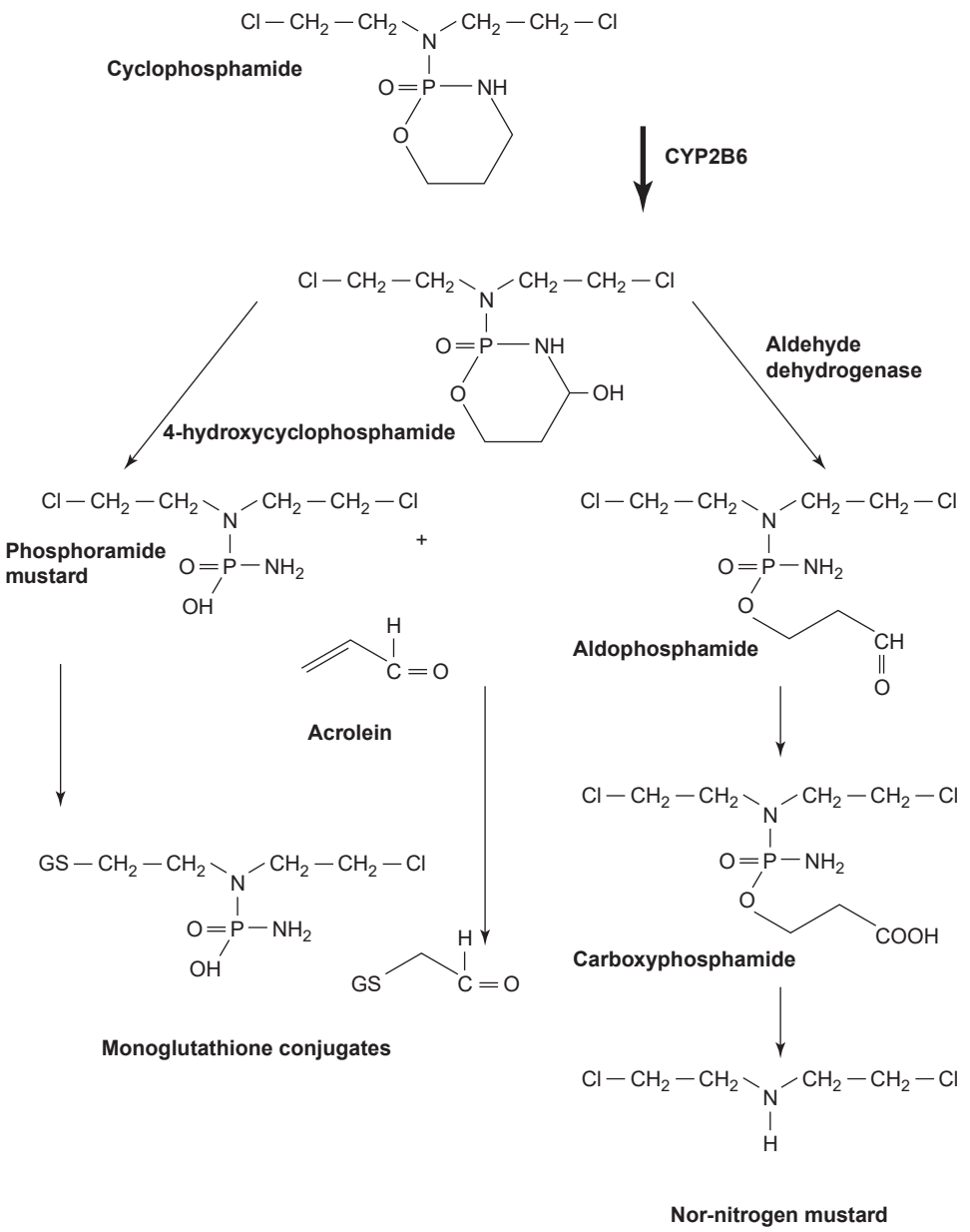

Figure 17.2 Activation and detoxification routes of metabolism for cyclophosphamide.

oral absorption. Cyclophosphamide,[23] ifosfamide, and dacarbazine are unusual in that they require activation by cytochrome P-450 in the liver before they can alkylate cellular constituents. The nitrosoureas also require activation, albeit nonenzymatic. The major route of metabolism of most alkylating agents is spontaneous hydrolysis, although many can also undergo some degree of enzymatic metabolism. This is particularly pertinent for phase II metabolic conversions where reactivity with nucleophilic thiols precedes conversion to mercapturates, with the result that most of the alkylating agents are excreted in the urine. One example of complex multistep metabolism is provided by cyclophosphamide (see Fig. 17.2). Activation by CYP2B6 is followed by the conversion of aldehyde dehydrogenase to reactive alkylating species or possible detoxification through GSH conjugation reactions. The latter is particularly important for acrolein because it is believed to contribute to the bladder toxicities associated with the drug.

The alkylating agents form covalent bonds with a number of nucleophilic groups present in proteins, RNA, and DNA (e.g., amino, carboxyl, sulfhydryl, imidazole, phosphate). Under physiologic conditions, the chloroethyl group of the nitrogen mustards undergoes cyclization, with the chloride acting as a leaving group forming an intermediate carbonium ion that attacks nucleophilic sites (see Fig. 17.1). Bifunctional alkylating agents (with two chloroethyl side chains) can undergo a subsequent cyclization to form a covalent bond with an adjacent nucleophilic group, resulting in DNA–DNA or DNA–protein cross-links. The N7 or O6 positions of guanine are particularly susceptible and may represent primary targets that determine both the cytotoxic and mutagenic consequences of therapy.[24] The nitrosoureas have a similar, but distinct, mechanism of action, spontaneously forming both alkylating and carbamoylating agents in aqueous media (see Fig. 17.1). The carbamoylating moieties are generally believed to be inconsequential to the therapeutic properties of the nitrosoureas.

THERAPEUTIC USES

The alkylating agents are frequently used in combination therapy to treat a variety of types of cancer. Perhaps the most versatile is cyclophosphamide, whereas the other alkylating agents are of

more restricted clinical use. Because of early successes, many disease states are managed with drug combinations that contain several alkylating agents. Cyclophosphamide is employed to treat a variety of immune-related diseases and to purge bone marrow in autologous marrow transplant situations.[25] A general summary of the clinical uses of the primary alkylating agents is shown in Table 17.1.

TOXICITIES

The alkylating agents show significant qualitative and quantitative variability in the sites and severities of their toxicities. The primary dose-limiting toxicity is suppression of bone marrow function, with secondary limiting effects on the proliferating cells of the intestinal mucosa.

Contraindications to the use of alkylating agents would identify patients with severely depressed bone marrow function and patients with hypersensitivity to these drugs. Other listed precautions to these drugs include carcinogenic and mutagenic effects and impairment of fertility. Precaution is also advised in patients with (1) leukopenia or thrombocytopenia, (2) previous exposure to chemotherapy or radiotherapy, (3) tumor cell infiltration of the bone marrow, and (4) impaired renal or hepatic function. These drugs can also increase toxicity in adrenalectomized patients and interfere with wound healing. A brief summary of dose-limiting toxicities is shown in Table 17.1, and a narrative of each follows here.

Nausea and Vomiting

Nausea and vomiting are frequent side effects of alkylating agent therapy and are not well controlled by conventional antiemetics.[24] They are a major source of patient discomfort and a significant cause of lack of drug compliance and even discontinuation of therapy. Frequency and extent are highly variable among patients. The overall frequency of nausea and vomiting is directly proportional to the dose of alkylating agent. The onset of nausea may occur within a few minutes of the administration of the drug or may be delayed for several hours.

Bone Marrow Toxicity

Bone marrow toxicity can involve all of the blood elements, leukocytes, platelets, and red cells.[26] The extent and time course of suppression show marked interindividual fluctuation. Relative platelet sparing is a characteristic of cyclophosphamide treatment. Even at the very high doses (<200 mg/kg) of cyclophosphamide (used in preparation for bone marrow transplantation), some recovery of hematopoietic elements occurs within 21 to 28 days. This stem cell–sparing property is further reflected by the fact that cumulative damage to the bone marrow is rarely seen when cyclophosphamide is given as a single agent, and repeated high doses can be given without progressive lowering of leukocyte and platelet counts. The biochemical basis for the stem cell–sparing effect of cyclophosphamide is related to the presence of high levels of aldehyde dehydrogenase in early bone marrow progenitor cells (see Fig. 17.2). Busulfan is particularly toxic to bone marrow stem cells,[26] and treatment can lead to prolonged hypoplasia. The hematopoietic depression produced by the nitrosoureas is characteristically delayed. The onset of leukocyte and platelet depression occurs 3 to 4 weeks after drug administration and may last an additional 2 to 3 weeks.[22,26] Thrombocytopenia appears earlier and usually is more severe than leukopenia. Even if the nitrosourea is given at 6-week intervals, hematopoietic recovery may not occur between courses, and the drug dose often must be decreased when repeated courses are used.

Renal and Bladder Toxicity

Hemorrhagic cystitis is unique to the oxazaphosphorines (cyclophosphamide and ifosfamide) and may range from a mild cystitis to severe bladder damage with massive hemorrhage.[27] This toxicity is caused by the excretion of toxic metabolites (particularly acrolein) (see Fig. 17.2) in the urine, with subsequent direct irritation of the bladder mucosa. The incidence and severity can be lessened by adequate hydration and continuous irrigation of the bladder with a solution containing 2-mercaptoethane sulfonate (MESNA) and frequent bladder emptying.[26] MESNA is given in divided doses every 4 hours in dosages of 60% of those of the alkylating agent.

At high cumulative doses, all commonly used nitrosoureas can produce a dose-related renal toxicity that can result in renal failure and death.[29] In patients developing clinical evidence of toxicity, increases in serum creatinine usually appear after the completion of therapy and may be first detected up to 2 years after treatment.

Interstitial Pneumonitis and Pulmonary Fibrosis

Long-term busulfan therapy can lead to the gradual onset of fever, a nonproductive cough, and dyspnea, followed by tachypnea and cyanosis, and progressing to severe pulmonary insufficiency and death.[30] If busulfan is stopped before the onset of clinical symptoms, pulmonary function may stabilize, but if clinical symptoms are manifest, the condition may be rapidly fatal. Cyclophosphamide, bischloroethylnitrosourea, and methyl-1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea in cumulative doses exceeding 1,000 mg/m^2 may also lead to similar side effects.[31] Other alkylating agents, including melphalan, chlorambucil, and mitomycin C, can lead to pulmonary fibrosis after therapy.[32] This effect is probably caused by a direct cytotoxicity of the alkylating agent to pulmonary epithelium, resulting in alveolitis and fibrosis.

Gonadal Toxicity, Teratogenesis, and Carcinogenesis

Alkylating agents can have profound toxic effects on reproductive tissue.[33] A depletion of testicular germ (but not Sertoli) cells is accompanied by aspermia. In patients with a total absence of germ cells, an increase in plasma levels of follicle-stimulating hormone occurs. However, patients in remission and off alkylating agents for 2 to 7 years show complete spermatogenesis, indicating that testicular damage is reversible.

In women, a high incidence of amenorrhea and ovarian atrophy is associated with cyclophosphamide or melphalan therapy.[34] This seems to be age related because it developed after lower doses in older compared with younger patients, and was less likely to be reversible in the older cohort. A pathologic analysis reveals the absence of mature or primordial follicles, and endocrinology studies demonstrate decreased estrogen and progesterone levels and elevated serum follicle-stimulating hormone and luteinizing hormone levels typical of menopause.

The DNA-damaging properties of alkylating agents ensure that they are all teratogenic and carcinogenic to some degree. The administration of alkylating agents during the first trimester of pregnancy presents a definitive risk of a malformed fetus, but the administration of such drugs during the second and third trimesters does not increase the risk of fetal malformation above normal.[35]

Development of second cancer as a consequence of alkylating agent therapy has been documented. For example, a fulminant acute myeloid leukemia characterized by a preceding phase of myelodysplasia is found in some patients treated with melphalan, cyclophosphamide (which is much less leukemogenic than melphalan), chlorambucil, and the nitrosoureas.[33] This circumstance probably reflects the fact that these have been the most

widely used of the alkylating agents. Also, the preponderance of patients with multiple myeloma, Hodgkin lymphoma, and carcinoma of the ovary in the reports of leukemogenesis is probably because patients with these diseases may have good responses and are often treated with alkylating agents for a number of years. The rate of occurrence of acute leukemia in patients with ovarian cancer who survive for 10 years after treatment with alkylating agents might be as high as 10%. Acute leukemia has been the most frequently described second malignancy, and it usually develops 1 to 4 years after drug exposure.[36] Other malignancies, including solid tumors, also have been reported to develop in patients treated with alkylating agents.[37]

The last four decades have yielded a significant improvement in the survival of children diagnosed with cancer (5-year survival is approximately 80%). As many as two-thirds of the survivors of childhood malignancies can experience delayed drug toxicities that may be severe or even life threatening. Such complications include impairment in growth and development, neurocognitive dysfunction, cardiopulmonary compromise, endocrine dysfunction, renal impairment, gastrointestinal dysfunction, musculoskeletal sequelae, and second cancers.[38]

Alopecia

The degree of alopecia after cyclophosphamide administration may be quite severe, especially when this drug is used in combination with vincristine sulfate or doxorubicin hydrochloride.[39] Regrowth of hair inevitably occurs after the cessation of therapy, but may be associated with a change in the color and greater curl. Use of a tourniquet or ice pack applied to the scalp during and for a short period after cyclophosphamide administration reduces the impact.

Allergic Reactions

Alkylating agents covalently bind to proteins, and these conjugates can act as haptens and produce allergic reactions.[40] An increasing number of reports of skin eruption, angioneurotic edema, urticaria, and anaphylactic reactions after the systemic administration of alkylating agents have appeared.

Immunosuppression

Alkylating agents suppress both humoral and cellular immunity in a variety of experimental systems.[41] The most immunosuppressive is cyclophosphamide, reported to cause (1) selective suppression of B-lymphocyte function, (2) depletion of B-lymphocytes, and (3) suppression of lymphocyte functions that are mediated by T cells, such as the graft-versus-host response and delayed hypersensitivity. Most intermittent antitumor regimens do not uniformly produce profound immunosuppression, and recovery is usually prompt. Sustained drug treatments can lead to severe lymphocyte depletion and profound immunosuppression and may be accompanied by an increase of viral, fungal, and protozoal infections.[41]

COMPLICATIONS WITH HIGH-DOSE ALKYLATING AGENT THERAPY

At standard doses, alkylating agents produce myelosuppression as their dose-limiting toxicity. Less severe effects on the gastrointestinal epithelium, lungs, bladder, and kidneys may become problems with long-term treatment, but rarely limit initial therapy. For this reason, and because of their steep dose response to tumor-killing curves, the alkylating agents have become a logical tool, either alone or in combination, for high-dose chemotherapy regimens in which bone marrow toxicity is expected, and is accommodated by bone marrow transplantation, stem cell reconstitution from peripheral blood monocytes, and growth factor rescue. In this high-dose setting, toxicities that affect the gut, lungs, liver, and CNS become dose limiting and life threatening.[42] The highly lipid-soluble alkylators, especially ifosfamide, busulfan, the nitrosoureas, and thiotepa, cause CNS dysfunction, including seizures, altered mental status, cerebellar dysfunction, cranial nerve palsies, and coma.[43] High-dose ifosfamide is most frequently the cause of neurotoxicity.[44] Clinical manifestations of grade 4 neurotoxicities were reported in approximately one-fourth of those patients receiving ifosfamide. The side-chain *N*-linked chloroethyl moiety of ifosfamide (see Table 17.1) is more likely than the bischloroethyl group of cyclophosphamide to undergo oxidation and subsequent *N*-deethylation and lead to the formation of chloroacetaldehyde. High-dose busulfan is also frequently used in a variety of conditioning regimens for hematopoietic cell transplantation. In this setting, busulfan causes neurotoxicity manifesting in seizures that generally are tonic–clonic in character. Phenytoin has been the preferred drug to treat busulfan-induced seizures, although some emerging clinical data support the use of benzodiazepines, most notably clonazepam and lorazepam, to prevent busulfan-induced seizures. Moreover, the second-generation antiepileptic drug levetiracetam possesses the characteristics of optimal prophylaxis for busulfan-induced seizures.[45] At least one recent study has suggested that a polymorphism in the glutathione S-transferase A2 family may be predictive of transplant-related mortality after allogeneic stem cell transplantation,[46] perhaps indicating that a pharmacogenetic approach might be possible in this disease setting. Moreover, in a preclinical setting, a proteomic analysis identified thioredoxin as a potentially important adjuvant therapy in enhancing donor cell graft enhancement in bone marrow transplantation.[47] The possibility that this approach may benefit patients following alkylating agent–based ablation remains to be tested in a clinical setting.

Cyclophosphamide at doses exceeding 100 mg/kg during a 48-hour period (preparatory to bone marrow transplantation) can cause cardiac toxicity.[48] No evidence exists for cumulative damage to the heart after repeated moderate or low doses of the drug. Cardiac toxicity occurs with greatest frequency in patients older than 50 years or in those previously treated with anthracyclines.[48]

ALKYLATING AGENT–STEROID CONJUGATES

Adapting the rationale that steroid receptors may function to localize and concentrate attached drug species intracellularly in hormone-responsive cancers, a number of synthetic conjugates of nitrogen mustards and steroids have been developed. Of these, two made the transition into clinical use.

Prednimustine is an ester-linked conjugate of chlorambucil and prednisolone designed to function as a prodrug for chlorambucil. Release of the alkylating agent occurs after cleavage by serum esterases,[49] which can release the ester link of prednimustine, producing the hormone and active alkylating drug. The elimination phase of chlorambucil in patient plasma is significantly longer after the administration of prednimustine than after chlorambucil. Estramustine is a carbamate ester–linked conjugate of nor-nitrogen mustard and estradiol. Unlike prednimustine, the pharmacology of estramustine is governed by the presence of the carbamate group in the steroid–mustard linkage. The relative resistance of the carbamate bond to enzymatic cleavage eliminates the alkylating activity of the molecule and conveys an entirely new pharmacology.[50] The crystal structural and mechanism of action studies showed that estramustine has antimitotic activity, an activity shared by some other steroids.[51] Estramustine has found a clinical niche used in combination with other antimitotic drugs in the management of hormone refractory prostate cancer.[52]

DRUG RESISTANCE AND MODULATION

As with all drugs, intrinsic or acquired resistance to alkylating agents occurs and limits the therapeutic utility of this class of anticancer drugs.[53] A plethora of preclinical studies have characterized mechanisms by which cells develop resistance and, to a lesser degree, these have been shown to occur clinically. Because alkylating agents have a narrow therapeutic index, the emergence of resistance can have a significant impact on clinical success. Some of the factors that can contribute to the expression of resistance to alkylating agents include (1) alterations in drug uptake or transport, (2) increased repair of drug-induced nucleic acid damage, (3) failure to activate alkylating agent prodrugs, (4) increased scavenging of drug species by nonessential cellular nucleophiles, (5) increased enzymatic detoxification of drug species, and (6) altered expression of genes coding for cellular commitment to apoptosis.

RECENT DEVELOPMENTS

In the era of directed targeted therapies, the lack of specificity of alkylating agents would seem to limit the likelihood that novel drugs will be forthcoming. High toxicities, narrow therapeutic indices, and chemical instabilities are all properties that consign this drug class to the lower echelons of popularity in drug-discovery platforms. Although covalent bonding to specific target sites is one approach to direct targeting, the random electrophilic attraction toward nucleic acids and proteins is not an optimal property by today's standards. Nevertheless, the relative success of the alkylating agents in gaining therapeutic responses to diseases that are difficult to treat continues to serve as an impetus to use alkylating moieties as a means to kill cells. Some novel agents are presently in development. Cyclophosphamide and ifosfamide were prodrugs synthesized in the hope that high levels of phosphoamidase in epithelial tumors would selectively activate the drugs.[27] Other efforts to improve selectivity have centered on the synthesis of antibody–enzyme conjugates that bind to tumor-specific surface antigens. Enzymes frequently associated with the cell surface include peptidases, nitroreductases, and γ-glutamyl transpeptidase; to some degree, each has been targeted to cleave circulating alkylating prodrugs, thereby in a localized fashion releasing active alkylating species. Antibody-directed enzyme prodrug therapy is exemplified by the use of an antibody linked to the peptidase carboxypeptidase G-2, which releases an active alkylator from an inactive γ-glutamyl conjugate.[54] Linkage of the peptidase to any antibody that localizes selectively to a tumor cell membrane is a viable option. Expression of the peptidase on the cell surface then leads to prodrug activation and cell kill. Such approaches have had limited clinical impact to this time; however, their development does continue.

A further rationale for enhancing tumor-specific delivery takes advantage of the observation that glutathione-S-transferase *pi* (GSTP1-1) is preferentially expressed in a number of solid tumors and some lymphomas. In this case, the prodrug consists of an unusual alkylating agent conjugated to a substituted glutathione peptidomimetic. GSTP initiates the cleavage, thereby creating a cytotoxic alkylating species.[55] The initial canfosfamide design strategy relied on the principle that proton-abstracting sites at the active site of GST could initiate a cleavage reaction that would convert an inactive prodrug into a cytotoxic species. The presence of a histidine residue in proximity to the G binding site was integral to the removal of the sulfhydryl proton from the GSH cosubstrate, resulting in the generation of a nucleophilic sulfide anion. This moiety would be more reactive with electrophiles in the absence of GSH. Unlike other standard nitrogen mustard drugs, canfosfamide contains a tetrakis (chloroethyl) phosphorodiamidate moiety. Other compounds bearing this structure have been shown to be more cytotoxic than a similar structure with a single bis-(chloroethyl) amine group.[56]

As in other nitrogen mustards, the chlorines can act as leaving groups, thus creating aziridinium ions with electrophilic characteristics. Although the exact temporal or sequential formation of the four possible chlorine leaving events is not known, the assumption is that these species possess cytotoxic properties through their capacity to alkylate target nucleophiles, such as DNA bases. Tetrafunctionality could result in the formation of cross-links with bonding distances greater than for bifunctional agents. However, a number of caveats apply to this interpretation. For example, alkylating agents, whether mono-, bi-, or putatively tetrafunctional, generally lead to some form of myelosuppression. A number of clinical trials with canfosfamide have now been completed. These include, phase 1,[57] phase 1/2a,[58] phase 2,[59] and phase 3.[60] The phase 3 study was in platinum refractory ovarian cancer patients and proved negative for enhanced survival. Nevertheless, additional trials are still in progress.

Another targeting approach delivers the gene for a cytochrome P-450 isoenzyme to tumors by viral vector, thereby enhancing specific tumor cell activation of cyclophosphamide.[61] Because this therapy has its base in gene delivery technologies, successful development in humans will await further advances in this arena.

Laromustine is in the sulfonylhydrazine class of alkylating agents. It is presently in clinical development for the treatment of malignancies such as acute myelogenous leukemia (AML).[62] Similar to nitrosoureas, laromustine is a prodrug that yields a chloroethylating and a carbamoylating (methyl isocyanate) species. As with nitrosoureas, the cytotoxicity of laromustine is attributed primarily to the chloroethylating-mediated alkylation of DNA and subsequent interstrand cross-links.[63] The carbamoylating species can inhibit DNA repair and other cellular enzyme systems. Phase 1 trials in patients with solid tumors indicated the expected myelosuppression, although few extramedullary toxicities were observed, indicating potential efficacy in the treatment of hematologic malignancies. Phase 2 trials have been completed in patients with untreated AML, high-risk myelodysplastic syndrome, and relapsed AML. The most encouraging results have been found in patients older than 60 years with poor-risk, de novo AML for which no standard treatment exists. Laromustine is currently in phase 2/3 trials for AML and phase 2 trials for myelodysplastic syndrome and solid tumors.[64] Laromustine appears to be a promising agent in elderly patients who do not respond to or are not fit for intensive chemotherapy.

Although not a new drug, bendamustine is a unique cytotoxic agent with structural similarities to alkylating agents and antimetabolites, but it lacks cross-resistance with other established alkylating agents both in vitro and in the clinic.[65] Its mechanism of action is similar to other mustards in causing DNA intra- and interstrand cross-links. In comparison with other more commonly used alkylating agents, such as cyclophosphamide or phenylalanine mustard, more DNA double-strand breaks are formed at equitoxic dosages. Treatment with bendamustine induces a concentration-dependent apoptosis as evidenced by changes in Bcl-2 and Bax expression profiles in chronic B-cell lymphocytic leukemia.[66] DNA damage produced by bendamustine is repaired via base-excision repair mechanisms, implicating an unusual mode of action, which was recently confirmed through gene expression profiling analyses. This also provided an explanation for the lack of cross-resistance with other alkylating agents, as observed in vitro with anthracycline-resistant breast cancer and cisplatin-resistant ovarian cancer.[66,67]

Clinical studies conducted in Germany more than 30 years ago suggested activity in indolent non-Hodgkin lymphoma. Subsequent American trials showed responses in more than 70% of patients with drug refractory disease, with the implication that bendamustine may be the most effective drug in this patient population. Combinations of bendamustine and rituximab elicited response rates of 90% to 92%, with complete remission in 55% to 60% in follicular and mantle cell lymphoma. Superiority over chlorambucil in previously untreated patients with chronic lymphocytic leukemia (CLL) led to its recent approval for this disease

in the United States. Bendamustine is approved in Germany for the treatment of patients with indolent non-Hodgkin lymphoma, CLL, and multiple myeloma. Activity has also been noted in patients with breast cancer and non–small-cell lung cancer.

Bendamustine has been used both as a single agent and in combination with other agents, including etoposide, fludarabine, mitoxantrone, methotrexate, prednisone, rituximab, and vincristine. A multicenter phase 2 trial in lymphomas had an overall response rate of 89%; (35% complete response and 54% partial response). In previously treated patients. the overall response rate was 76% (38% complete response and 38% partial response). The estimated median progression-free survival was 19 months.[67] In CLL patients, the drug is administered at 100 mg/m^2 intravenously over 30 minutes on days 1 and 2 of a 28-day cycle, for up to six cycles. Efficacy relative to first-line therapies other than chlorambucil has not been established. It is also indicated for the treatment of patients with indolent B-cell non-Hodgkin lymphoma that has progressed during, or within, 6 months of treatment with rituximab or rituximab-containing regimens. As with most alkylating agents, the primary dose-limiting toxicity is myelosuppression; nonhematologic toxicities were mild and included fatigue, nausea, loss of appetite, and vomiting. The optimization of dose and schedule, particularly relative to other drugs, and the management of toxicities has allowed its use in combination with a range of other chemotherapeutic agents, including prednisone, methotrexate, fludarabine, etoposide, mitoxantrone, vinca alkaloids, and rituximab. The availability of bendamustine provides another effective treatment option for patients with lymphoid malignancies, frequently reducing the side effects of the more standard cyclophosphamide, hydroxy doxorubicin, Oncovin, and prednisone (CHOP) regimen.[68] Recent approval by the U.S. Food and Drug Administration has allowed Cephalon, Inc. to market bendamustine under the trade name Treanda and, in combination with mitoxantrone and rituximab, it is now standard of care in indolent lymphomas. Trial results released in 2013 indicated that this combination more than doubled the progression-free survival in this disease[69] and there is early evidence that there may be utility in relapsed or refractory multiple myeloma.[70]

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18 Platinum Analogs

Peter J. O'Dwyer and A. Hilary Calvert

INTRODUCTION

The platinum drugs represent a unique and important class of antitumor compounds. Alone or in combination with other chemotherapeutic agents, *cis*-diamminedichloroplatinum (II) (cisplatin) and its analogs have made a significant impact on the treatment of a variety of solid tumors for nearly 40 years. The unique activity and toxicity profile observed with cisplatin in early clinical trials fueled the development of platinum analogs that are less toxic and more active against a variety of tumor types, including those that have developed resistance to cisplatin. In addition to cisplatin, two other platinum complexes are currently approved for use in the United States: *cis*-diamminecyclobutanedicarboxylate platinum (II) (carboplatin) and 1,2-diaminocyclohexaneoxalato platinum (II) (oxaliplatin). Several other analogs with unique activities are in various stages of clinical development, and nedaplatin (Japan) and lobaplatin (China) are locally registered. Progress in the development of superior analogs requires a thorough understanding of the chemical, biologic, pharmacokinetic, and pharmacodynamic properties of this important class of drugs.

HISTORY

The realization that platinum complexes exhibited antitumor activity began serendipitously in a series of experiments to investigate the effect of electromagnetic radiation on the growth of bacteria, carried out by Dr. Barnett Rosenberg and colleagues beginning in 1961.[1,2] Exposure of the bacteria to an electric field resulted in a profound change in their morphology; this effect was found not to be from the electric field, but from electrolysis products produced by the platinum electrodes. An analysis of these products resulted in the identification of the cis-isomer of a platinum coordination complex as the active compound. Tests of *cis*-diamminedichloroplatinum (II) in mice bearing several model tumor types indicated that cisplatin exhibited a broad spectrum of antitumor activity. Although early clinical trials demonstrated responses in several tumor types, particularly testicular cancers, the severe renal and gastrointestinal toxicity caused by the drug nearly led to its abandonment. Work at Memorial Sloan-Kettering[3,4] showed that these effects could be ameliorated, in part, by aggressive prehydration, which rekindled interest in its clinical use. Currently, cisplatin is curative in testicular cancer and significantly prolongs survival in combination regimens for ovarian, lung, head and neck, bladder, and upper gastrointestinal (GI) cancers. Its role is being reexamined in other tumors, too, and especially breast cancer.

PLATINUM CHEMISTRY

Platinum exists primarily in either a 2+ or 4+ oxidation state. These oxidation states dictate the stereochemistry of the ligands surrounding the platinum atom. Platinum (II) compounds exhibit a square planar geometry, in which the ammine ligands (also called carrier groups) are relatively stable, whereas the opposite, more polar ligands (leaving groups) are more easily displaced and so confer reactivity toward charged macromolecules, including DNA.[5] The stereochemistry of platinum complexes is critical to their antitumor activity as evidenced by the significantly reduced efficacy observed with *trans*-diamminedichloroplatinum (II).

In an aqueous solution, the chloride leaving groups of cisplatin are subject to mono- and diaqua substitution, particularly at chloride concentrations below 100 mmol, which characterize the intracellular environment. The administration of cisplatin in high chloride solutions (normal saline usually), therefore, contributes to stability. Intracellular formation of partially and fully aquated complexes creates the chloroaqua and hydroxoaqua cisplatin species that bind DNA.[6]

PLATINUM COMPLEXES AFTER CISPLATIN

Early in the clinical development of cisplatin, it became clear that its toxicity was a limitation to its therapeutic effectiveness, and that its activity, although striking in certain diseases, did not extend to all cancers. These observations then motivated a search for structural analogs with less toxicity and a different profile of antitumor activity. In addition, the side effects of cisplatin stimulated the development of antiemetics and other supportive care measures for use with chemotherapy. Progress in understanding the chemistry and pharmacokinetics of cisplatin has guided the development of new analogs. In general, modification of the chloride leaving groups of cisplatin results in compounds with different pharmacokinetics and reactivity towards DNA, whereas modification of the carrier ligands alters the activity of the resulting complex. The features of the more important platinum analogs that have been developed are shown in Figure 18.1.

Carboplatin

The carboplatin molecule has the same ammine carrier ligands as cisplatin. Using a murine screen for nephrotoxicity, Harrap and Calvert discovered that substituting a cyclobutanedicarboxylate moiety for the two chloride ligands of cisplatin resulted in a complex with reduced renal toxicity. This observation was translated to the clinic in the form of carboplatin, a more stable and pharmacokinetically predictable analog.[7,8] The results in humans were accurately predicted by the animal models, and marrow toxicity rather than nephrotoxicity was the principal side effect. At effective doses, carboplatin produced less nausea, vomiting, nephrotoxicity, and neurotoxicity than cisplatin. Furthermore, the myelosuppression was closely associated with the pharmacokinetics. The work of Calvert et al.[9] and Egorin and colleagues[10] showed that toxicity can be made more predictable and dose intensity less variable by dosing strategies based on the exposure. Carboplatin was shown to be indistinguishable from cisplatin in its clinical activity in all but a handful of tumor types and is the most frequently used form of platinum in current use. Cisplatin and carboplatin have almost superimposable profiles of activity in the NCI60 cell line screen, which further emphasizes the dependence of spectrum of activity on the carrier ligand.

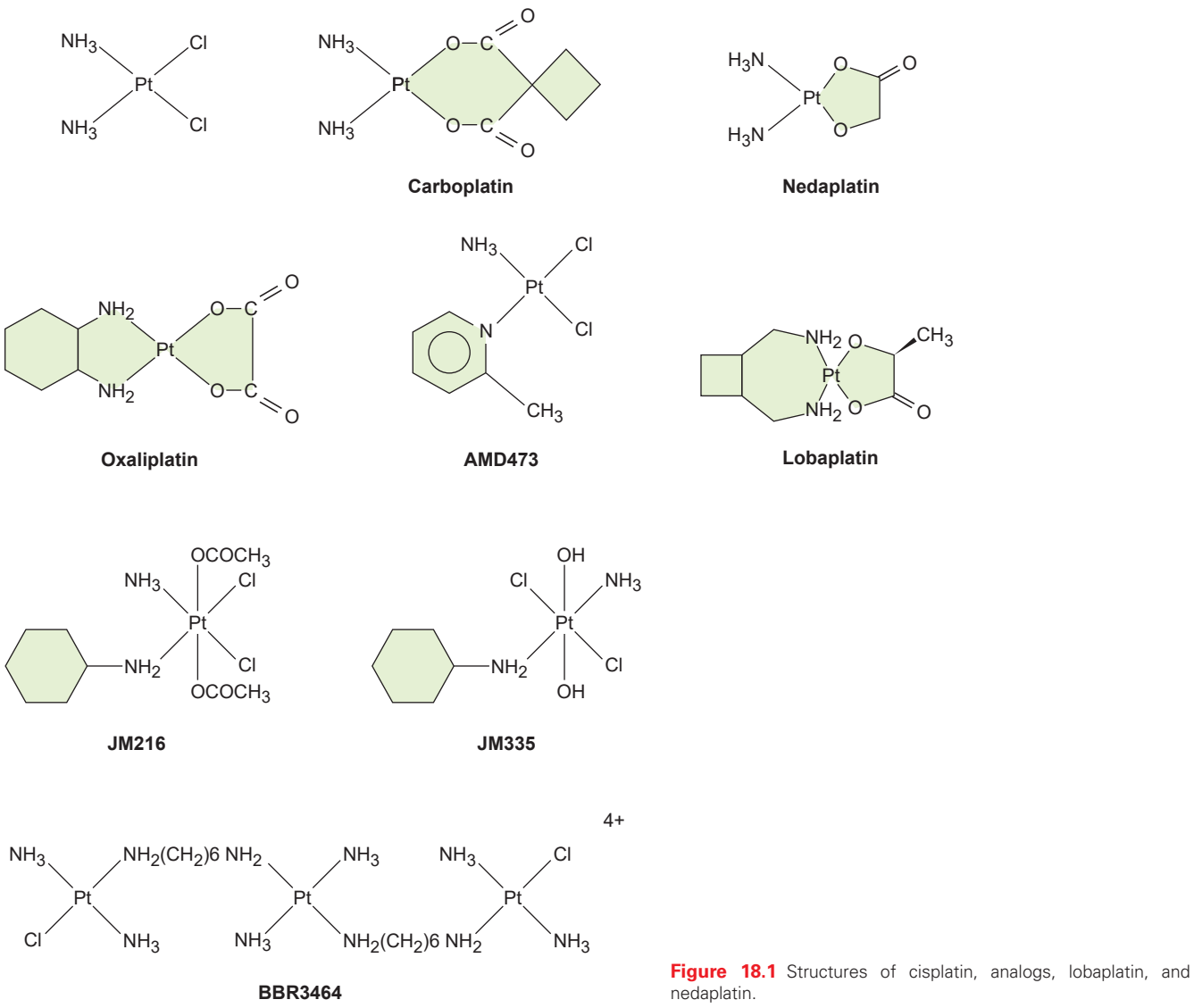

Figure 18.1 Structures of cisplatin, analogs, lobaplatin, and nedaplatin.

Oxaliplatin

Compounds with activity in cisplatin-resistant models emerged from modifications to the carrier group (see left side of the analogs in Fig. 18.1). Connors, in the late 1960s, synthesized platinum coordination compounds with varying physicochemical characteristics and found that the series that possessed a diaminocyclohexane (DACH) carrier group was active in models of cancer in vitro[11] and in vivo.[12] Subsequent studies supported the idea that DACH-based platinum complexes were non–cross-resistant with cisplatin, and DACH derivatives exhibited a unique cytotoxicity profile compared to cisplatin and carboplatin in the National Cancer Institute 60 cell line screen.[13–15] After a number of delays, a DACH analog that had been synthesized by Kidani and colleagues in the early 1970s, was developed in the clinic.[13] Oxaliplatin, a coordination compound of a DACH carrier group and an oxalato leaving group, was active in cisplatin-resistant tumor models. Like cisplatin, oxaliplatin preferentially forms adducts at the N7 position of guanine and, to a lesser extent, adenine. However, there is evidence that the three-dimensional structure of the DNA adducts and biologic response(s) they elicit are different from those of cisplatin. Oxaliplatin demonstrated activity in combination with 5-fluorouracil and leucovorin in colon cancer, a disease that is unresponsive to cisplatin. This finding validated the focus on cisplatin-resistant preclinical models to identify new active molecules. Oxaliplatin is approved for the treatment of advanced colorectal cancer, and enhances cure rates in the adjuvant setting. The therapeutic role of oxaliplatin has been found to extend to pancreatic, gastric, and esophageal cancers, in all of which it is the more active platinum derivative.

Nedaplatin and Lobaplatin

Nedaplatin is cis-diammineglycolatoplatinum, developed as a less nephrotoxic second-generation platinum analog, has been shown to be active in a range of tumors similar to that of cisplatin and carboplatin.[16] As a diammine structure, nedaplatin would fall among the cisplatin analogs analyzed in the NCI60 cell line screen,[17] and this activity is therefore anticipated. Lobaplatin is a platinum (II) complex in which the leaving group is lactic acid and the stable ammine ligand is 1,2-bis(aminomethyl)cyclobutane. In a similar way to oxaliplatin the stable ammine ligand may convey some non–cross-resistance compared to cisplatin or carboplatin. It is licensed in China for the breast cancer, small-cell lung cancer, and chronic myelogenous leukemia. It is unique among the platinum drugs for its approval for breast cancer, but there are few published clinical data and no randomized trials. It has not achieved approval in the United States or Europe.

Newer Platinum Structures

The octahedral stereochemistry adopted by platinum (IV) compounds has led investigators to speculate that they may exhibit a different spectrum of activity than that of platinum (II) drugs. Two compounds that were tested clinically without much success are ormaplatin and iproplatin. Two other platinum (IV) compounds that exhibit novel structural features, satraplatin (previously JM216) and JM335 (*trans*-ammine[cyclohexylamine]dichlorodihydroxo platinum [IV]), underwent more limited development. Satraplatin was the first orally active platinum compound, and showed some activity in lung and ovarian cancers, but despite promising activity in prostate cancer, a phase III trial was not successful.[18,19]

An approach based on the chemistry of the platinum-DNA interaction led to design and synthesis by Farrell et al.[20] of a novel class of compounds containing multiple platinum atoms (see Fig. 18.1). These bi- and trinuclear structures form adducts that span greater distances across the minor groove of DNA and have a profile of cell kill that differs from that of the small molecules. These compounds are unique in that their interaction with DNA is considerably different from that of cisplatin, particularly in the abundance of interstrand cross-links formed. Clinical development of candidate compounds is at a preliminary stage.

Efforts have been made to design novel platinum analogs that can circumvent putative cisplatin resistance mechanisms. An example is *cis*-amminedichloro(2-methylpyridine) platinum (II) (also known as *AMD473* and *ZD0473*). This compound is a sterically hindered platinum complex that was designed to have minimal reactivity with thiols and thus avoid inactivation by molecules such as glutathione.[21,22] Responses were identified with its use in the clinic, but development was curtailed based on low levels of activity. The recent description of a monofunctional platinum (II) analog, phenanthriplatin, from the lab of Lippard is potentially of great interest, based on both potency in vitro and a mechanistic profile different from existing analogs.[23] A renewed appreciation that chemotherapeutic drugs have a continuing role in managing cancer is likely to prompt additional clinical development of novel platinum structures.

MECHANISM OF ACTION

DNA Adduct Formation

DNA has long been thought to be the major therapeutic target for platinum compounds. The cytotoxic effects are determined, in part, by the structure and relative amount of DNA adducts formed. Cisplatin and its analogs react preferentially at the N7 position of guanine and adenine residues to form a variety of monofunctional and bifunctional adducts.[24] The monoadducts may form intrastrand or interstrand cross-links. The predominant lesions that are formed when platinum compounds bind DNA are d(GpG)Pt intrastrand cross-links. Cisplatin also forms interstrand cross-links between guanine residues located on opposite strands, and these account for less than 5% of the total DNA-bound platinum. The formation of adducts and cross-links has been associated with therapeutic efficacy.[25,26] These adducts may contribute to the drug's cytotoxicity because they impede certain cellular processes that require the separation of both DNA strands, such as replication and transcription. The adducts formed in the reaction between carboplatin and DNA in cultured cells are essentially the same as those of cisplatin; however, higher concentrations of carboplatin are required (20- to 40-fold for cells) to obtain equivalent total platinum-DNA adduct levels due to its slower rate of aquation.[27] Oxaliplatin intrastrand adducts form even more slowly due to a slower rate of conversion from monoadducts; however, they are formed at similar DNA sequences and regions as cisplatin adducts. At equitoxic doses, oxaliplatin forms fewer DNA adducts than does cisplatin. This has been interpreted to mean that oxaliplatin lesions are more cytotoxic than those formed by cisplatin.

The differences observed in cytotoxicity between the diammine (e.g., cisplatin, carboplatin) and DACH platinum compounds may not depend on the type and relative amounts of the adducts formed, but on the overall three-dimensional structure of the adduct and its recognition by various cellular proteins. The major difference between them is the protrusion of the DACH moiety of oxaliplatin into the major groove of DNA, which thus produces a bulkier adduct than that of cisplatin. This bulkier, more hydrophobic adduct seems to be recognized differently by cellular proteins involved in sensing DNA damage.[28] The functional consequences are twofold: Proteins such as polymerases that recognize and participate in reactions on DNA under normal circumstances may be perturbed, whereas processes that are controlled by proteins that recognize damaged DNA may become activated (the DNA damage response). The latter group of proteins function both in the DNA repair process and in cellular signaling toward cell survival/death decisions.

DNA Interstrand Cross-Links

Although the DNA adducts are well-recognized to result in G-G interstrand cross-links, like classical alkylating agents, platinum drugs have the capacity to form intrastrand cross-links, albeit to a lesser degree. By blocking essential aspects of DNA metabolism, such as replication and transcription, intrastrand cross-links are highly cytotoxic. Recent studies have drawn attention both to the cytotoxicity of these lesions, and their differing mechanisms of repair, both replication dependent and independent.[29,30] These studies may have clinical implications in selecting patients for therapy based on the repair competence of tumors.

CELLULAR RESPONSES TO PLATINUM-INDUCED DNA DAMAGE

Multiple cellular outcomes may follow the formation of platinum-DNA adducts, including cell death by apoptosis, necrosis, or mitotic catastrophe, or cell survival by activation of various protective mechanisms including DNA repair, DNA damage signaling pathways, cell cycle arrest, and autophagy (the last may have a dual role, possibly context dependent).

Cell Fate

The cellular effects following DNA binding by platinum drugs have been analyzed. The studies of Sorenson and Eastman,[31] using DNA repair-deficient Chinese hamster ovary (CHO) cells, indicated that passage through the S phase is necessary for G2 arrest and cell death, which suggests that DNA replication on a damaged template may result in the accumulation of further damage. An aberrant mitosis was observed before apoptosis in this model.

DNA Damage Recognition

Among the initiation events that ultimately result in platinum drug–induced cell death are the binding of platinum-DNA damage recognition proteins, which then seed the accumulation of a large protein complex capable both of DNA damage signaling (as to cell cycle proteins to halt replication) and repair of the damaged DNA. Among the DNA-binding proteins are the high-mobility group proteins HMG1 and HMG2.[32–34] These proteins are capable of bending DNA as well as recognizing bent DNA structures, such as that produced by cisplatin, and different specificities for cisplatin and for oxaliplatin adducts are observed in structural studies.[35,36] Other candidate platinum-DNA damage recognition proteins include histone H1, RNA polymerase I transcription upstream binding factor (hUBF), the TATA binding protein (TBP), and proteins

involved in mismatch repair (MMR). The MMR complex has been implicated in cisplatin sensitivity.[37] Studies have shown that the MSH2 and MLH1 proteins participate in the recognition of DNA adducts formed by cisplatin, but not oxaliplatin, which could contribute to differences in the cytotoxicity profiles observed between these two platinum complexes.

DNA Damage Signaling

A number of signaling events have been shown to occur after treatment of cells with platinum drugs.[38] For example, the ATM- and Rad3-related (ATR) proteins that are involved in cell-cycle checkpoint activation are activated by cisplatin. These kinases phosphorylate and activate several downstream effectors that regulate cell cycle, DNA repair, cell survival, and apoptosis, including p53, CHK2, and members of the mitogen-activated protein kinase (MAPK) pathway (extracellular signal-related kinase [ERK], c-Jun amino-terminal kinase [JNK], and p38 kinase). Recent data especially implicate signaling through the JNK pathway, and inhibition at the level of JNK seems especially relevant to platinum drug cytotoxicity in vitro and in vivo.[39,40] The pleiotropic nature of this stress response only grows, because each of these molecules subsequently controls the activity and expression of many more proteins. As a result of this complexity, acting in the context of variable genomic tumor aberrations, therapeutic strategies directed to these pathways have been slow to emerge. However, clinical trials to investigate specific inhibitors of DNA damage responses are underway and hold promise. It is also relevant to point out that these signaling pathways affect not just the tumor cell, but also may communicate to cells in the microenvironment, the responses of which may also determine the effectiveness of therapy.

IS DNA THE ONLY TARGET?

Early analyses of the action of cytotoxic drugs included a probe of whether effects on DNA were sufficient to explain drug effects. A pioneer in this field was Tritton,[41] who proposed that effects of DNA-intercalating agents on the plasma membrane could underlie the cytotoxicity of the drug. More recently, enucleated cells were shown to be susceptible to cisplatin, and a seminal paper from Voest and colleagues showed that platinum sensitivity was determined not solely by the accumulation of DNA damage in the tumor cell.[42] In analyzing the contribution of cells in the microenvironment of tumors, he showed that tumor infiltration with mesenchymal stem cells could confer drug resistance. A search for secreted factors defined platinum-induced fatty acids, metabolic products in the thromboxane synthetase, and cyclooxygenase-1 pathways as determining the effectiveness of drug therapy. A proteomic study in cisplatin-sensitive and -resistant cells confirmed the substantial effects of drug exposure on lipid metabolites and their relation to susceptibility. A current focus on therapies directed to the microenvironment, including immunologic and anti-inflammatory interventions,[43] has the potential to expand our ability to apply platinum drugs in the clinic.

MECHANISMS OF RESISTANCE

The major limitation to the successful treatment of solid tumors with platinum-based chemotherapy is the emergence of drug-resistant tumor cells.[44] Developments in tumor biology have advanced our thinking with regard to how and when these cells emerge; heterogeneity within a tumor even at its earliest diagnosis reflects the emergence of treatment-resistant clones even in advance of selection pressure and the realization that resistance may not be specific to the DNA-damaging drug. Indeed, this may be reflected clinically in the finding that after progression on initial chemotherapy, the use of second-line therapy is usually associated with a shorter duration of response.

Currently described mechanisms of platinum drug resistance (Fig. 18.2) include reduced cellular accumulation, intracellular detoxification, repair of Pt-DNA lesions, increased damage tolerance, and the activation of cellular defense mechanisms such as autophagy. In addition, we have already alluded to exogenous influences on mechanism, as may be mediated by other cells, metabolites, of physicochemical conditions (such as hypoxia) in the tumor microenvironment. It must be acknowledged, however, that our insights are very limited as to why some tumors respond and others do not to platinum chemotherapy. As genome sequencing yields increasing and often surprising revelations about the genes that drive cancers and the complexity inherent in cancers of a single histologic type, it is likely that when associated with outcomes in large patient populations, patterns will emerge to guide selection of therapies.

Reduced Accumulation

Platinum uptake in cells occurs by simple diffusion and by carrier-mediated mechanisms. Inhibition of transport mechanisms has a marked effect on intracellular platinum accumulation, and Howell's group has shown the importance of the copper transporters CTR-1 and CTR-2 in regulating the influx of various platinum analogs in eukaryotic cells.[45,46] The contribution of these mechanisms to clinical platinum drug resistance is being explored.[47] Accumulation may also be influenced by enhanced efflux, and various transport proteins are upregulated in cell lines selected for acquired resistance, and in platinum-resistant ovarian cancers.

Inactivation

Platinum complexes are highly reactive molecules and bind rapidly to multiple cellular macromolecules. Protection from such chemicals in the environment is afforded by cellular thiols, including

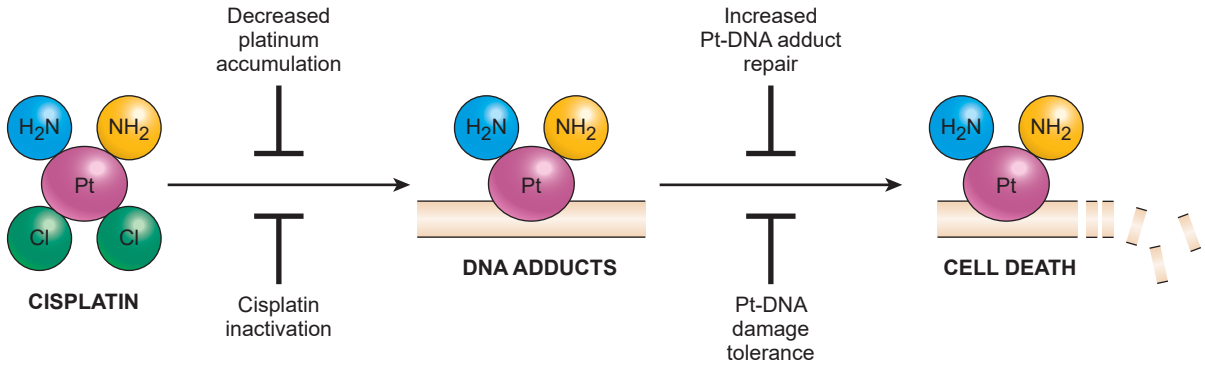

Figure 18.2 Cellular mechanisms of cisplatin resistance.

small peptides such as glutathione (GSH) and larger proteins as exemplified by metallothionein (MT). There are many reports of an association between platinum drug sensitivity and glutathione levels[48–50]; however, reducing intracellular glutathione levels with drugs such as buthionine sulfoximine has resulted in only low to modest potentiation of cisplatin sensitivity.[51] Buthionine sulfoximine was developed for clinical use, and some impact on GSH content of tumors and normal tissues was demonstrated. However, the depletion of GSH was not consistent, and ultimately, the cost of producing the active stereoisomer of the drug was judged prohibitive. Inactivation of the platinum drugs may also occur through binding to the MTs, a family of sulfhydryl-rich, low–molecular-weight proteins that participate in heavy metal binding and detoxification; however, the contribution of MT to clinical platinum drug resistance is unclear, and a therapeutic role has not emerged.

Increased DNA Repair

Once platinum-DNA adducts are formed, cells must either repair or tolerate the damage to survive. In general, the capacity to repair DNA damage seems to play a role in determining a tumor cell's sensitivity to platinum drugs and other DNA-damaging agents. For example, tumors that are unusually sensitive to cisplatin, such as testicular nonseminomatous germ cell tumors, may be deficient in their ability to repair platinum-DNA adducts.[52] The increased repair of platinum-DNA lesions in cisplatin-resistant cell lines as compared to their sensitive counterparts has been shown in several human cancer cell lines, but translation of these observations to the clinic has been difficult. The repair of platinum-DNA adducts appears to occur predominantly by nucleotide excision repair (NER), with a role for MMR under certain circumstances.[53] The molecular basis for the increased repair activity observed in cisplatin-resistant cells is not known precisely, but formation of the ERCC1/XPF protein complex may be a key step. Selvakumaran et al.[54] showed that the downregulation of ERCC-1 using an antisense approach sensitized a platinum-resistant cell line to cisplatin both in vitro and in vivo. There is substantial clinical evidence that implicates *ERCC1* expression in increased NER and cisplatin resistance, and high expression of ERCC1 has been demonstrated to confer a worse outcome after cisplatin treatment in several resistant tumors. The most extensive study of this as a marker has been in non–small-cell lung cancer, results in which were summarized and analyzed by Hubner et al.[55] In gastric cancer also, high levels of ERCC1 are associated with resistance to cisplatin treatment.[56–58] However, a recent reevaluation of discrepant results questioned the reliability of the assays of ERCC1 and their relationship to function.[59] These data suggest that there is a relationship between ERCC1 expression and treatment, but that the lag in marker development precludes implementation of a predictive assay until additional studies have been performed.

Perhaps the most striking evidence that DNA repair is a determinant of platinum drug responses is that breast and ovarian cancers occurring in BRCA1 or BRCA2 mutation carriers are particularly responsive to cisplatin or carboplatin. These cancers are also sensitive to inhibitors of poly(ADP-ribose)polymerase (PARPi), several of which are currently in clinical development. The mechanism of the sensitivity to PARPi has been elucidated. Both the BRCA1 and 2 proteins for part of the homologous recombination repair (HR) system that achieves error-free repair of double strand breaks. Carriers are heterozygous and, therefore, have normal repair function, but loss of the second allele leads to the use of error-prone backup systems and is therefore oncogenic. The cancers that arise are unable to perform HR and, therefore, are sensitive to drugs that induce single strand breaks, such as PARPi.[60,61] A mechanism of resistance to PARPi has been described, which is due to reactivation of the function of the BRCA2 leading to restoration of HR and sensitivity to PARPi.[62] This reactivation is accomplished by an intragenic deletion and the restoration of an open reading frame. It has further been shown that such revertant cells are resistant to cisplatin as well as PARPi. Finally, recurrent cancers in BRCA2 mutation carriers, which have acquired platinum resistance, have been shown to have undergone reversion of the BRCA2 mutation.[63] This clearly shows that the HR system can be one cause of cisplatin resistance. However, not all cisplatin-resistant patients are also resistant to PARPi,[64] showing that there are multiple other causes of cis/carboplatin resistance.

Combinations of platinum drugs with PARPi are being actively pursued in patients with BRCA-related tumors and also in patients whose tumors are likely to have acquired loss of HR function (poorly differentiated serous ovarian cancer and triple negative breast cancer).

Autophagy

After platinum-DNA adduct formation, the cell detects the DNA damage and initiates signaling through multiple pathways, the effects of which include mobilization of repair proteins; arrest of the cell cycle; altered transcriptional programs; redirection of energy production and consumption; activation of cell death pathways and, simultaneously, of pathways that would counter a cell death decision, and so to permit survival. A process recently characterized to perform the last function is autophagy. Initially described as a mechanism of cell death, autophagy represents a regulated dissolution of cellular elements into a characteristic set of subcellular organelles detectable by electron microscopy and linked by a particular profile of gene expression changes.[65] Multiple stimuli precipitate these changes and have in common scarcity of nutrients that are required for survival, from oxygen and glucose withdrawal to less specific calorie deprivation, and inhibition of metabolic pathways. Autophagy is also a consequence of cytotoxic drug treatment and, more recently, has been appreciated as a means by which cells might survive the stress of cellular insults, and so become resistant to treatment.[66] Amaravadi and colleagues[67] demonstrated that autophagy reversal can sensitize tumors to cytotoxic drugs and several trials of platinum compounds along with the autophagy inhibitor hydroxychloroquine are in progress.

Increased DNA Damage Tolerance

The net result of DNA damage signaling in a sensitive tumor cell is engagement of cell death pathways, including apoptosis, and therapeutic benefit. In a resistant tumor cell, the cell survives as a consequence of one or many of these mechanisms, and this can result in platinum-DNA damage tolerance or multidrug resistance phenotype, or both. Contributors to the tolerance might include deficient DNA MMR (which could excise the adduct if NER failed), enhanced replicative bypass (which essentially ignores the adduct, allowing the cell to survive, but could contribute to the increase in mutation frequency observed in chemotherapy-treated cancers), and altered signaling through stress-related kinases such as JNK, which can both alter transcriptional programs and activate autophagy. Indeed JNK, by phosphorylating Bcl-2 or Bcl-XL, and releasing beclin-1 from inhibition, acts as a key switch to turn on autophagy. The enhanced DNA damage tolerance, in addition to permitting persistence of the cancer cell, may have an additional deleterious effect by fostering further mutagenesis within the tumor, facilitating its evolution to a more malignant phenotype.

CLINICAL PHARMACOLOGY

Pharmacokinetics

The pharmacokinetic differences observed between platinum drugs may be attributed to the structure of their leaving groups. Platinum complexes containing leaving groups that are less easily displaced exhibit reduced plasma protein binding, longer plasma half-lives, and higher rates of renal clearance. These features are

TABLE 18.1

Comparative Parmacokinetics of Platinum Analogs After Bolus or Short Intravenous Infusion

	Cisplatin	Carboplatin	Oxaliplatin
$T_{1/2}\alpha$			
Total platinum	14–49 min	12–98 min	26 min
Ultrafiltrate	9–30 min	8–87 min	21 min
$T_{1/2}\beta$			
Total platinum	0.7–4.6 h	1.3–1.7 h	—
Ultrafiltrate	0.7–0.8 h	1.7–5.9 h	—
$T_{1/2}\gamma$			
Total platinum	24–127 h	8.2–40.0 h	38–47 h
Ultrafiltrate	—	—	24–27 h
Protein binding	>90%	24%–50%	85%
Urinary excretion	23%–50%	54%–82%	>50%

$T_{1/2}\alpha$, half-life of first phase; $T_{1/2}\beta$, half-life of second phase; $T_{1/2}\gamma$, half-life of terminal phase.

evident in the pharmacokinetic properties of cisplatin, carboplatin, and oxaliplatin, which are summarized in Table 18.1. Platinum drug pharmacokinetics have been reviewed.[68]

Cisplatin

After intravenous infusion, cisplatin rapidly diffuses into tissues and is covalently bound to plasma protein. More than 90% of platinum is bound to plasma protein at 4 hours after infusion. The disappearance of ultrafilterable platinum is rapid and occurs in a biphasic fashion. Half-lives of 10 to 30 minutes and 0.7 to 0.8 hours have been reported for the initial and terminal phases, respectively. Cisplatin excretion is dependent on renal function, which accounts for the majority of its elimination. The percentage of platinum excreted in the urine has been reported to be between 23% and 40% at 24 hours after infusion. Only a small percentage of the total platinum is excreted in the bile.

Carboplatin

The differences in pharmacokinetics observed between cisplatin and carboplatin depend primarily on the slower rate of conversion of carboplatin to a reactive species. Thus, the stability of carboplatin results in a low incidence of nephrotoxicity. Carboplatin diffuses rapidly into tissues after infusion; however, it is considerably more stable in plasma. Only 24% of a dose was bound to plasma protein at 4 hours after infusion. The disappearance of platinum from plasma after short intravenous infusions of carboplatin has been reported to occur in a biphasic or triphasic manner. The initial half-lives for total platinum, which vary considerably among several studies, are listed in Table 18.1. The half-lives for total platinum range from 12 to 98 minutes during the first phase ($T_{1/2}\alpha$) and from 1.3 to 1.7 hours during the second phase ($T_{1/2}\beta$). Half-lives reported for the terminal phase range from 8.2 to 40 hours. The disappearance of ultrafilterable platinum is biphasic with $T_{1/2}\alpha$ and $T_{1/2}\beta$ values ranging from 7.6 to 87 minutes and 1.7 to 5.9 hours, respectively. Carboplatin is excreted predominantly by the kidneys, and cumulative urinary excretion of platinum is 54% to 82%, most as unmodified carboplatin. The renal clearance of carboplatin is closely correlated with the glomerular filtration rate (GFR).[69] This observation enabled Calvert et al.[9] to design a carboplatin-dosing formula based on the individual patient's GFR.

Oxaliplatin

After oxaliplatin infusion, platinum accumulates into three compartments: plasma-bound platinum, ultrafilterable platinum, and platinum associated with erythrocytes. When specific and sensitive mass spectrometric techniques are used, oxaliplatin itself is undetectable in plasma, even at end infusion.[70] The active forms of the drug have not been extensively characterized. Approximately 85% of the total platinum is bound to plasma protein at 2 to 5 hours after infusion.[71] Plasma elimination of total platinum and ultrafilterates is biphasic. The half-lives for the initial and terminal phases are 26 minutes and 38.7 hours, respectively, for total platinum and 21 minutes and 24.2 hours, respectively, for ultrafilterable platinum (see Table 18.1).[72] Thus, as with carboplatin, substantial differences between total and free platinum kinetics are not observed. As with cisplatin, a prolonged retention of oxaliplatin is observed in red blood cells. However, unlike cisplatin, oxaliplatin does not accumulate to any significant level after multiple courses of treatment.[71] This may explain why neurotoxicity associated with oxaliplatin is reversible. Oxaliplatin is eliminated predominantly by the kidneys, with more than 50% of the platinum being excreted in the urine at 48 hours.

Pharmacodynamics

Pharmacodynamics relates pharmacokinetic indices of drug exposure to biologic measures of drug effect, usually toxicity to normal tissues or tumor cell kill. Two issues to be addressed in such studies are whether the effectiveness of the drug can be enhanced and whether the toxicity can be attenuated by knowledge of the platinum pharmacokinetics in an individual. These questions are appropriate to the use of cytotoxic agents with relatively narrow therapeutic indices. Toxicity to normal tissues can be quantitated as a continuous variable when the drug causes myelosuppression. Thus, the early studies of carboplatin demonstrated a close relationship of changes in platelet counts to the area under the concentration-time curve (AUC) in the individual. The AUC was itself closely related to renal function, which was determined as creatinine clearance. Based on these observations, Egorin et al.,[10] Calvert et al.,[9] and Chatelut and colleagues[73] derived formulas based on creatinine clearance to predict either the percentage change in platelet count or a target AUC. Application of pharmacodynamically guided dosing algorithms for carboplatin has been widely adopted as a means of avoiding overdosage (by producing acceptable nadir platelet counts) and of maximizing dose intensity in the individual. There is good evidence that this approach can decrease the risk of unacceptable toxicity. Accordingly, a dosing strategy based on renal function is recommended for the use of carboplatin.

A key question is whether maximizing carboplatin exposure in an individual can measurably increase the probability of tumor regression or survival. In an analysis by Jodrell et al.,[74] carboplatin AUC was a predictor of response, thrombocytopenia, and leukopenia. The likelihood of a tumor response increased with increasing AUC up to a level of 5 to 7 mg × hour per milliliter, after which a plateau was reached. Similar results were obtained with carboplatin in combination with cyclophosphamide, and neither response rate nor survival was determined by the carboplatin AUC in a cohort of ovarian cancer patients.[75] As a result, most carboplatin recommended doses are based on an AUC in this range (for every 3 to 4 week schedules), and modifications of these are used for more frequent administration (as in combined chemoradiotherapy regimens).

The relationship of pharmacokinetics to response has been sought by investigating the cellular pharmacology of these agents.[76] The formation and repair of the platinum-DNA adducts in human cells are not easily measured. Schellens and colleagues[77,78] analyzed the pharmacokinetic and pharmacodynamic interactions of cisplatin administered as a single agent. In a series of patients with head and neck cancer, they found that cisplatin exposure (measured as the AUC) closely correlated with both the peak DNA adduct content in leukocytes and the area under the DNA-adduct

time curve. These measures were important predictors of response, both individually and in logistic regression analysis. However, as an approach to determine who should or should not be treated with platinum drugs, it seems more likely that genomic analyses will provide guidance in the near future.

Pharmacogenomics

Variability in pharmacokinetics and pharmacodynamics of cytotoxic drugs is an important determinant of therapeutic index. This interindividual variation may be attributed in part to genetic differences among patients. Targeted analyses of germ-line DNA and, increasingly, Genome-wide association studies (GWAS) approaches, have yielded genotypic features associated with results of therapy. Detoxification pathways and DNA repair have emerged as having markers attributable to response of lack of it in response to platinum drugs. Single nucleotide polymorphisms (SNP) in genes related to glutathione metabolism and in several DNA repair genes have been identified in lung cancer, breast cancer, and various GI cancers. A concern is that larger trials have not always confirmed early findings. As yet, informative SNPs that could be used to define therapeutic strategies for individual patients have not yet been defined.

FORMULATION AND ADMINISTRATION

Cisplatin (Platinol)

Cisplatin is administered in a chloride-containing solution intravenously over 0.5 to 2.0 hours. To minimize the risk of nephrotoxicity, patients are prehydrated with at least 500 mL of salt-containing fluid. Immediately before cisplatin administration, mannitol (12.5 to 25.0 g) is given parenterally to maximize urine flow. A diuretic such as furosemide may be used also, along with parenteral antiemetics. These currently include dexamethasone together with a 5-hydroxytryptamine (5-HT_3) antagonist. A minimum of 1 L of posthydration fluid is usually given. The intensity of hydration varies somewhat with the dose of cisplatin. High-dose cisplatin (up to 200 mg/m^2 per course) may be administered in a formulation containing 3% sodium chloride, but this method is no longer widely used. Cisplatin may also be administered regionally to increase local drug exposure and diminish side effects. Its intraperitoneal use was defined by Ozols et al.[79] and by Howell and colleagues.[80] Measured drug exposure in the peritoneal cavity is some 50-fold higher compared to levels achieved with intravenous administration. At standard dosages in ovarian cancer patients with low-volume disease, a randomized intergroup trial suggested that intraperitoneal administration is superior to intravenous cisplatin in combination with intravenous cyclophosphamide.[81] The development of combinations of carboplatin and paclitaxel has, however, superseded this technique in the treatment of ovarian cancer, and the intraperitoneal route is now infrequently used. Regional uses also include intra-arterial delivery (as for hepatic tumors, melanoma, and glioblastoma), but none have been adopted as a standard method of treatment. There is growing interest in chemoembolization for the treatment of tumors confined to the liver, and cisplatin is a component of many popular regimens.[82]

Carboplatin (Paraplatin)

Cisplatin treatment over 3 to 6 hours is burdensome for clinical resources and tiring for cancer patients. Previously given as an in-hospital treatment, it is now usually administered in the outpatient setting. The exigencies of the modern health-care environment have contributed to the expanding use of carboplatin as an alternative to cisplatin except in circumstances in which cisplatin is clearly the superior agent. Carboplatin is substantially easier to administer. Extensive hydration is not required because of the lack of nephrotoxicity at standard dosages. Carboplatin is reconstituted in chloride-free solutions (unlike cisplatin, because chloride can displace the leaving groups) and administered over 30 minutes as a rapid intravenous infusion.

Oxaliplatin (Eloxatin)

Oxaliplatin is also uncomplicated in its clinical administration. For bolus infusion, the required dose is administered in 500 mL of chloride-free diluent over a period of 2 hours. Oxaliplatin is most frequently given as a single dose every 2 weeks (85 mg/m^2) or every 3 weeks (130 mg/m^2), alone or with other active agents. It is common to pretreat patients with active antiemetics, such as a 5-HT_3 antagonist, but the nausea is not as severe as with cisplatin. No prehydration is required. Besides a relatively low incidence of myelosuppression, the predominant toxicity of oxaliplatin is cumulative neurotoxicity. The development of an oropharyngeal dysesthesia, often precipitated by exposure to cold, may require prolonging the duration of administration to 6 hours. On occasion, the occurrence of hypersensitivity also requires slowing the infusion.

TOXICITY

A substantial body of literature documents the side effects of platinum compounds. As noted in the section titled History, earlier in this chapter, the toxicity of cisplatin was a driving force both in the search for less toxic analogs and for more effective treatments for its side effects, especially nausea and vomiting. The toxicities associated with cisplatin, carboplatin, and oxaliplatin are described in detail in the following sections and summarized in Table 18.2. Please review the package inserts for these drugs for full prescribing information and delineation of toxic effects.

Cisplatin

The side effects associated with cisplatin (at single doses of more than 50 mg/m^2) include nausea and vomiting, nephrotoxicity, ototoxicity, neuropathy, and myelosuppression. Rare effects include visual impairment, seizures, arrhythmias, acute ischemic vascular events, glucose intolerance, and pancreatitis. The nausea and vomiting stimulated a search for new antiemetics. These effects are currently best managed with 5-HT_3 antagonists, usually given with a glucocorticoid, although other combinations of agents are still widely used. In the weeks after treatment, continuous antiemetic therapy may be required. Nephrotoxicity is ameliorated but not completely prevented by hydration. The renal damage to both glomeruli and tubules is cumulative, and after cisplatin treatment, serum creatinine levels are no longer a reliable guide to GFR. An acute elevation of serum creatinine level may follow a cisplatin dose, but this index returns to normal with time. Tubule damage may be reflected in a salt-losing syndrome that also resolves with time.

TABLE 18.2
Toxicity Profiles of Platinum Analogs in Clinical Use

Toxicity	Cisplatin	Carboplatin	Oxaliplatin
Myelosuppression		X	
Nephrotoxicity	X		
Neurotoxicity	X		X
Ototoxicity	X		
Nausea and vomiting	X	X	X

Ototoxicity is a cumulative and irreversible side effect of cisplatin treatment that results from damage to the inner ear. The initial audiographic manifestation is loss of high-frequency acuity (4,000 to 8,000 Hz). When acuity is affected in the range of speech, cisplatin should be discontinued under most circumstances and carboplatin substituted where appropriate. Peripheral neuropathy is also cumulative, although less common than with agents such as vinca alkaloids. This neuropathy is usually reversible, although recovery is often slow. A number of agents with the potential for protection from neuropathy have been developed, but none is yet used widely.

Carboplatin

Myelosuppression, which is not usually severe with cisplatin, is the dose-limiting toxicity of carboplatin. The drug is most toxic to the platelet precursors, but neutropenia and anemia are frequently observed. The lowest platelet counts after a single dose of carboplatin are observed 17 to 21 days later, and recovery usually occurs by day 28. The effect is dose dependent, but individuals vary widely in their susceptibility. As shown by Egorin et al.[10] and Calvert et al.,[9] the severity of platelet toxicity is best accounted for by a measure of the drug exposure in an individual, the AUC. Both groups derived pharmacologically based formulas to predict toxicity and guide carboplatin dosing. That of Calvert and colleagues targets a particular exposure to carboplatin:

$$\text{Dose (mg)} = \text{target AUC (mg} \cdot \text{min/mL)} \times (\text{GFR mL/min} + 25)$$

This formula has been widely used to individualize carboplatin dosing and permits targeting an acceptable level of toxicity. Patients who are elderly, have a poor performance status, or have a history of extensive pretreatment have a higher risk of toxicity even when dosage is calculated with these methods, but the safety of drug administration has been enhanced. In the combination of carboplatin and paclitaxel, AUC-based dosing has helped to maximize the dose intensity of carboplatin. Dosages some 30% higher than those using a dosing strategy based solely on body surface area may safely be used. A determination of whether this approach to dosing improves outcomes will require a randomized trial.

The other toxicities of carboplatin are generally milder and better tolerated than those of cisplatin. Nausea and vomiting, although frequent, are less severe, shorter in duration, and more easily controlled with standard antiemetics (i.e., prochlorperazine [Compazine]), dexamethasone, lorazepam) than that after cisplatin treatment. Renal impairment is infrequent, although alopecia is common, especially with the paclitaxel-containing combinations. Neurotoxicity is also less common than with cisplatin, although it is observed more frequently with the increasing use of high-dose regimens. Ototoxicity is also less common.

Oxaliplatin

The dose-limiting toxicity of oxaliplatin is sensory neuropathy, a characteristic of all DACH-containing platinum derivatives. This side effect takes two forms. First, a tingling of the extremities, which may also involve the perioral region, that occurs early and usually resolves within a few days. With repeated dosing, symptoms may last longer between cycles, but do not appear to be cumulative or of long duration. Laryngopharyngeal spasms and cold dysesthesias have also been reported but are not associated with significant respiratory symptoms and can be prevented by prolonging the duration of infusion. A second neuropathy, more typical of that seen with cisplatin, affects the extremities and increases with repeated doses. Definitive physiologic characterization of oxaliplatin-induced neuropathy has proven difficult in large studies. Electromyograms performed in six patients treated by Extra et al.[83] revealed an axonal sensory neuropathy, but nerve conduction velocities were unchanged. Specimens from peripheral nerve biopsies performed in this study showed decreased myelination and replacement with collagen pockets. The neurologic effects of oxaliplatin appear to be cumulative in that they become more pronounced and of greater duration with successive cycles; however, unlike those of cisplatin, they are reversible with drug cessation. In a review of 682 patient experiences, Brienza et al.[84] reported that 82% of patients who experienced grade 2 neurotoxicity or higher had their symptoms regress within 4 to 6 months. In a larger adjuvant trial, de Gramont et al.[85] reported that 12% of patients had grade 3 toxicity at the end of a 6-month treatment period and that the majority of these patients had relief, but not always complete resolution of the symptoms, by 1 year later. The persistence of the neurotoxicity has led to approaches to ameliorate it, including the use of protective agents. The use of calcium and magnesium salts intravenously before and after each infusion has been shown to be ineffective. Ototoxicity is not observed with oxaliplatin. Nausea and vomiting do occur and generally respond to 5-HT_3 antagonists. Myelosuppression is uncommon and is not severe with oxaliplatin as a single agent, but it is a feature of combinations including this drug. Oxaliplatin therapy is not associated with nephrotoxicity.

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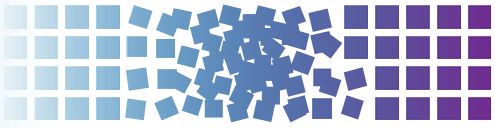
19 Antimetabolites



M. Wasif Saif and Edward Chu

ANTIFOLATES

Reduced folates play a key role in one-carbon metabolism, and they are essential for the biosynthesis of purines, thymidylate, and protein biosynthesis. Aminopterin was the first antimetabolite with documented clinical activity in the treatment of children with acute leukemia in the 1940s. This antifolate analog was subsequently replaced by methotrexate (MTX), the 4-amino, 10-methyl analog of folic acid, which remains the most widely used antifolate analog, with activity against a wide range of cancers (Table 19.1), including hematologic malignancies (acute lymphoblastic leukemia and non-Hodgkin's lymphoma) and many solid tumors (breast cancer, head and neck cancer, osteogenic sarcoma, bladder cancer, and gestational trophoblastic cancer).

Pemetrexed is a pyrrolopyrimidine, multitargeted antifolate analog that targets multiple enzymes involved in folate metabolism, including thymidylate synthase (TS), dihydrofolate reductase (DHFR), glycinamide ribonucleotide (GAR) formyltransferase, and aminoimidazole carboxamide (AICAR) formyltransferase.[1,2] This agent has broad-spectrum activity against solid tumors, including malignant mesothelioma and breast, pancreatic, head and neck, non–small-cell lung, colon, gastric, cervical, and bladder cancers.[3–5]

The third antifolate compound to have entered clinical practice is pralatrexate (10-propargyl-10-deazaaminopterin), a 10-deazaaminopterin antifolate that was rationally designed to bind with higher affinity to the reduced folate carrier (RFC)-1 transport protein, when compared with MTX, leading to enhanced membrane transport into tumor cells. It is also an improved substrate for the enzyme folylpolyglutamyl synthetase (FPGS), resulting in enhanced formation of cytotoxic polyglutamate metabolites.[6,7] When compared with MTX, this analog is a more potent inhibitor of multiple enzymes involved in folate metabolism, including TS, DHFR, and GAR and AICAR formyltransferases. This agent is presently approved for the treatment of relapsed or refractory peripheral T-cell lymphomas.[8]

Mechanism of Action

The antifolate compounds are tight-binding inhibitors of DHFR, a key enzyme in folate metabolism.[1] DHFR plays a pivotal role in maintaining the intracellular folate pools in their fully reduced form as tetrahydrofolates, and these compounds serve as one-carbon carriers required for the synthesis of thymidylate, purine nucleotides, and certain amino acids.

The cytotoxic effects of MTX, pemetrexed, and pralatrexate are mediated by their respective polyglutamate metabolites, with up to 5 to 7 glutamyl groups in a γ-peptide linkage. These polyglutamate metabolites exhibit prolonged intracellular half-lives, thereby allowing for prolonged drug action in tumor cells. Moreover, these polyglutamate metabolites are potent, direct inhibitors of several folate-dependent enzymes, including DHFR, TS, AICAR formyltransferase, and GAR formyltransferase.[1]

Mechanisms of Resistance

The development of cellular resistance to antifolates remains a major obstacle to its clinical efficacy.[9,10] In experimental systems, resistance to antifolates arises from several mechanisms, including an alteration in antifolate transport because of either a defect in the reduced folate carrier or folate receptor systems, decreased capacity to polyglutamate the antifolate parent compound through either decreased expression of FPGS or increased expression of the catabolic enzyme γ-glutamyl hydrolase, and alterations in the target enzymes DHFR and/or TS through increased expression of wild-type protein or overexpression of a mutant protein with reduced binding affinity for the antifolate. Gene amplification is a common resistance mechanism observed in various experimental systems, including tumor samples from patients. In in vitro and in vivo experimental model systems, the levels of DHFR and/or TS protein acutely increase after exposure to MTX and other antifolate compounds. This acute induction of target protein in response to drug exposure is mediated, in part, by a translational regulatory mechanism, which may represent a clinically relevant mechanism for the acute development of cellular drug resistance.

Clinical Pharmacology

The oral bioavailability of MTX is saturable and erratic at doses greater than 25 mg/m^2. MTX is completely absorbed from parenteral routes of administration, and peak serum levels are achieved within 30 to 60 minutes of administration.

The distribution of MTX into third-space fluid collections, such as pleural effusions and ascitic fluid, can substantially alter MTX pharmacokinetics. The slow release of accumulated MTX from these third spaces over time prolongs the terminal half-life of the drug, leading to potentially increased clinical toxicity. It is advisable to evacuate these fluid collections before treatment and monitor plasma drug concentrations closely.

Renal excretion is the main route of drug elimination, and this process is mediated by glomerular filtration and tubular secretion. About 80% to 90% of an administered dose is eliminated unchanged in the urine. Doses of MTX, therefore, should be reduced in proportion to reductions in creatinine clearance. Renal excretion of MTX is inhibited by probenecid, penicillins, cephalosporins, aspirin, and nonsteroidal anti-inflammatory drugs.

Pemetrexed enters the cell via the RFC system and, to a lesser extent, by the folate receptor protein. As with MTX, it undergoes polyglutamation within the cell to the pentaglutamate form, which is at least 60-fold more potent than the parent compound. This agent is mainly cleared by renal excretion, and in the setting of renal dysfunction, the terminal drug half-life is significantly prolonged to up to 20 hours. Pemetrexed, therefore, should be used with caution in patients with renal dysfunction. In addition, renal excretion is inhibited in the presence of other agents including probenecid, penicillins, cephalosporins, aspirin, and nonsteroidal anti-inflammatory drugs.

TABLE 19.1

Antimetabolites: Indications, Doses and Schedules, and Toxicities

Drug	Main Therapeutic Uses	Main Doses and Schedule	Major Toxicities
Methotrexate	Non-Hodgkin's lymphoma Primary CNS lymphoma Acute lymphoblastic leukemia Breast cancer Bladder cancer Osteogenic sarcoma Gestational trophoblastic cancer	Low dose: 10–50 mg/m^2 IV every 3–4 weeks Low dose weekly: 25 mg/m^2 IV weekly Moderate dose: 100–500 m/m^2 IV every 2–3 weeks High dose: 1–12 gm/m^2 IV over a 3- to 24-hour period every 1–3 weeks Intrathecal (IT): 10–15 mg IT 2 times weekly until CSF is clear, then weekly dose for 2–6 weeks, followed by monthly dose	Mucositis, diarrhea, myelosuppression, acute renal failure, transient elevations in serum transaminases and bilirubin, pneumonitis, neurologic toxicity
Pemetrexed	Mesothelioma Non–small-cell lung cancer	500 mg/m^2 IV, every 3 weeks	Myelosuppression, skin rash, mucositis, diarrhea, fatigue
Pralatrexate	Peripheral T-cell lymphoma	30 mg/m^2 IV, weekly for 6 weeks; cycles repeated every 7 weeks	Myelosuppression, skin rash, mucositis, diarrhea, elevation of serum transaminases and bilirubin, mild nausea/vomiting
5-Fluorouracil	Breast cancer Colorectal cancer Anal cancer Gastroesophageal cancer Hepatocellular cancer Pancreatic cancer Head and neck cancer	Bolus monthly schedule: 425–450 mg/m^2 IV on days 1–5 every 28 days Bolus weekly schedule: 500–600 mg/m^2 IV every week for 6 weeks every 8 weeks Infusion schedule: 2,400–3,000 mg/m^2 IV over 46 hours every 2 weeks 120-hour infusion: 1,000 mg/m^2/d IV on days 1–5 every 21–28 d Protracted continuous infusion: 200–400 mg/m^2/d IV	Nausea/vomiting, diarrhea, mucositis, myelosuppression, neurotoxicity, coronary artery vasospasm, conjunctivitis
Capecitabine	Breast cancer Colorectal cancer Gastroesophageal cancer Hepatocellular cancer Pancreatic cancer	Recommended dose for monotherapy is 1,250 mg/m^2 PO bid for 2 weeks with 1 wk rest May decrease dose of capecitabine to 850–1,000 mg/m^2 bid on days 1–14 to reduce risk of toxicity without compromising efficacy An alternative dosing schedule for monotherapy is 1,250–1,500 mg/m^2 PO bid for 1 week on and 1 week off; this schedule appears to be well tolerated, with no compromise in clinical efficacy Capecitabine should be used at lower doses (850–1,000 mg/m^2 bid on days 1–14) when used in combination with other cytotoxic agents, such as oxaliplatin and lapatinib	Diarrhea, hand-foot syndrome, myelosuppression, mucositis, nausea/vomiting, neurologic toxicity, coronary artery vasospasm
Cytarabine	Hodgkin's lymphoma Non-Hodgkin's lymphoma Acute myelogenous leukemia Acute lymphoblastic leukemia	Standard dose: 100 mg/m^2/day IV on days 1–7 as a continuous IV infusion, in combination with an anthracycline as induction chemotherapy for acute myelogenous leukemia High-dose: 1.5–3.0 gm/m^2 IV q 12 hours for 3 days as a high dose, intensification regimen for acute myelogenous leukemia SC: 20 mg/m^2 SC for 10 days per month for 6 months, associated with IFN-α for treatment of chronic myelogenous leukemia IT: 10–30 mg IT up to 3 times weekly in the treatment of leptomeningeal carcinomatosis secondary to leukemia or lymphoma.	Nausea/vomiting, myelosuppression, cerebellar ataxia, lethargy, confusion, acute pancreatitis, drug infusion reaction, hand-foot syndrome High-dose therapy: noncardiogenic pulmonary edema, acute respiratory distress and *Streptococcus viridans* pneumonia, conjunctivitis, and keratitis
Gemcitabine	Pancreatic cancer Non–small-cell lung cancer Breast cancer Bladder cancer Hodgkin's lymphoma Ovarian cancer Soft tissue sarcoma	Pancreatic cancer: 1,000 mg/m^2 IV every week for 7 weeks with 1 week rest Treatment then continues weekly for 3 weeks followed by 1 week off Bladder cancer: 1,000 mg/m^2 IV on days 1, 8, and 15 every 28 days Non–small-cell lung cancer: 1,000-1,200 mg/m^2 IV on days 1 and 8 every 21 days	Nausea/vomiting, myelosuppression, flulike syndrome, elevation of serum transaminases and bilirubin, pneumonitis, infusion reaction, mild proteinuria, and rarely, hemolytic-uremic syndrome and thrombotic thrombocytopenic purpura

(continued)

TABLE 19.1

Antimetabolites: Indications, Doses and Schedules, and Toxicities *(continued)*

Drug	Main Therapeutic Uses	Main Doses and Schedule	Major Toxicities
6-Mercaptopurine	Acute lymphoblastic leukemia	Induction therapy: 2.5 mg/kg PO daily Maintenance therapy: 1.5–2.5 mg/kg PO daily	Myelosuppression, nausea/vomiting, mucositis and diarrhea, hepatotoxicity, immunosuppression
6-Thioguanine	Acute myelogenous leukemia Acute lymphoblastic leukemia	Induction: 100 mg/m^2 PO every 12 hours on days 1–5, usually in combination with cytarabine Maintenance: 100 mg/m^2 PO every 12 hours on days 1–5, every 4 weeks, usually in combination with other agents Single agent: 1–3 mg/kg PO daily	Myelosuppression, nausea/vomiting, mucositis and diarrhea, hepatotoxicity, immunosuppression
Fludarabine	Chronic lymphocytic leukemia Non-Hodgkin's lymphoma	25 mg/m^2 IV on days 1–5 every 28 days For oral usage, the recommended dose is 40 mg/m^2 PO on days 1–5 every 28 days	Myelosuppression, immunosuppression with increased risk of opportunistic infections, mild nausea/vomiting, hypersensitivity reaction
Cladribine	Hairy cell leukemia Chronic lymphocytic leukemia Non-Hodgkin's lymphoma	Usual dose is 0.09 mg/kg/d IV via continuous infusion for 7 days; one course is usually administered	Myelosuppression, immunosuppression, mild nausea/vomiting, fever
Clofarabine	Acute lymphoblastic leukemia	52 mg/m^2 IV daily for 5 days every 2–6 weeks	Myelosuppression nausea/vomiting, diarrhea, systemic inflammatory response syndrome, increased risk of opportunistic infections, renal toxicity

CNS, central nervous system; IV, intravenously; CSF, cerebrospinal fluid; PO, by mouth; bid, twice daily; SC, subcutaneously; IFN-α, interferon alpha.

As with other antifolate analogs, pralatrexate is transported into the cell by the RFC carrier protein and then metabolized by FPGS to form longer chain polyglutamates, with up to four additional glutamate residues attached to the parent molecule. About 34% of the parent drug is cleared in the urine during the first 24 hours after drug administration. As such, caution is advised when using pralatrexate in patients with renal dysfunction. As with MTX and pemetrexed, the concomitant administration of other agents such as probenecid, penicillins, cephalosporins, aspirin, and nonsteroidal anti-inflammatory drugs, may inhibit renal clearance.

Toxicity

The main side effects of MTX are myelosuppression and gastrointestinal (GI) toxicity, which are usually completely reversed within 14 days, unless drug-elimination mechanisms are impaired. In patients with compromised renal function, even small doses of MTX may result in serious toxicity. MTX-induced nephrotoxicity is thought to result from the intratubular precipitation of MTX and its metabolites in acidic urine. Antifolates may also exert a direct toxic effect on the renal tubules. Vigorous hydration and urinary alkalinization have greatly reduced the incidence of renal failure in patients on high-dose regimens. Acute elevations in hepatic enzyme levels and hyperbilirubinemia are often observed during high-dose therapy, but these levels usually return to normal within 10 days. Methotrexate given concomitantly with radiotherapy may increase the risk of soft tissue necrosis and osteonecrosis.

The original rationale for high-dose MTX therapy was based on the concept of selective rescue of normal tissues by the reduced folate leucovorin (LV). However, recent data suggest that high-dose MTX may also overcome resistance mechanisms caused by impaired active transport, decreased affinity of DHFR for MTX, increased levels of DHFR resulting from gene amplification, and/or decreased polyglutamation of MTX.

The main toxicities of pemetrexed and pralatrexate include dose-limiting myelosuppression, mucositis, and skin rash, usually in the form of the hand-foot syndrome (HFS). Other toxicities include reversible transaminasemia, anorexia and fatigue syndrome, and GI toxicity. These side effects are reduced by supplementation with folic acid (350 μg orally daily) and vitamin B_{12} (1,000 mg subcutaneously given at least 1 week before starting therapy, and then repeated every three cycles). To date, there is no evidence to suggest that vitamin supplementation adversely affects the clinical efficacy of pemetrexed or pralatrexate.

5-FLUOROPYRIMIDINES

The fluoropyrimidine, 5-fluorouracil (5-FU) was synthesized by Charles Heidelberger in the mid 1950s. Uracil is a normal component of RNA; as such, the rationale leading to the development of the drug was that cancer cells might be more sensitive to *decoy* molecules that mimic the natural compound than normal cells. 5-FU and its derivatives are an integral part of treatment for a broad range of solid tumors (see Table 19.1), including GI malignancies (esophageal, gastric, pancreatic, colorectal, anal, and hepatocellular cancers), breast, head and neck, and skin cancers.[11] It continues to serve as the main backbone for combination regimens used to treat metastatic colorectal cancer (mCRC) and as adjuvant therapy of early-stage colon cancer.

Mechanism of Action

5-FU enters cells via the facilitated uracil base transport mechanism and is then anabolized to various cytotoxic nucleotide forms

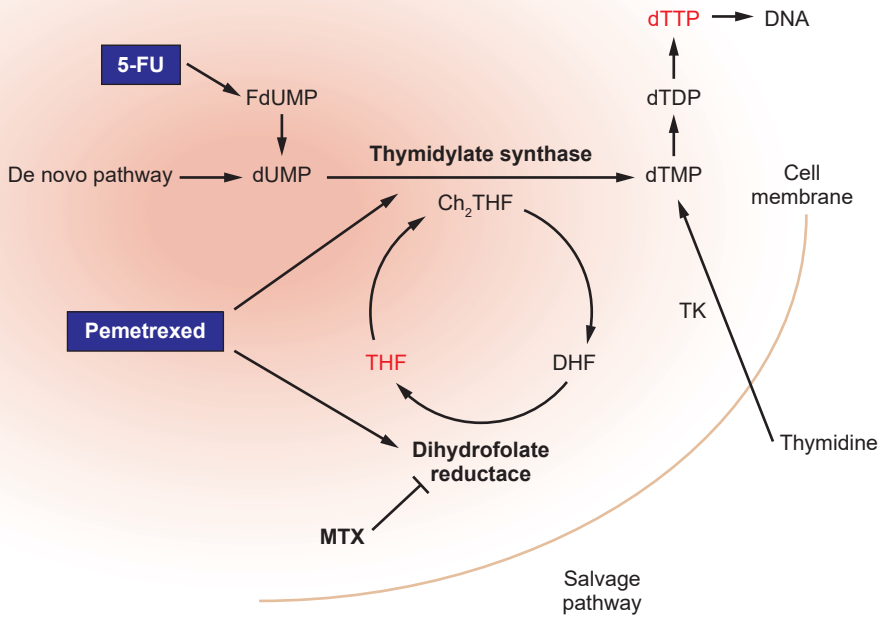


Figure 19.1 Antifolates and 5-fluorouracil (5-FU) sites of action. FdUMP, fluorodeoxyuridine monophosphate; dUMP, deoxyuridine monophosphate; dTTP, deoxythymidine triphosphate; dTDP, deoxyuridine diphosphate; dTMP, deoxythymidine monophosphate; TK, thymidine kinase; CH_2THF, 5,10-methylenetetrahydrofolate; THF, tetrahydrofolate; DHF, dihydrofolate.

by several biochemical pathways. It is thought that 5-FU exerts its cytotoxic effects through various mechanisms, including (1) the inhibition of TS, (2) incorporation into RNA, and (3) incorporation into DNA (Fig. 19.1). In addition to these mechanisms, the genotoxic stress resulting from TS inhibition may also activate programmed cell-death pathways in susceptible cells, which leads to the induction of parental DNA fragmentation.

Mechanisms of Resistance

Several resistance mechanisms to 5-FU have been identified in experimental and clinical settings. Alterations in the target enzyme TS represent the most commonly described mechanism of resistance. In vitro, in vivo, and clinical studies have documented a strong correlation between the levels of TS enzyme activity/TS protein and chemosensitivity to 5-FU. In this regard, cell lines and tumors with higher levels of TS are relatively more resistant to 5-FU. Mutations in the TS protein have been identified that lead to reduced binding affinity of the 5-FU metabolite fluorodeoxyuridine monophosphate (FdUMP) to the TS protein. Reduced expression and/or diminished activity of key activating enzymes may interfere with the formation of cytotoxic 5-FU metabolites. Decreased expression of mismatch repair enzymes, such as human mutL homolog 1 (hMLH1) and human mutS homolog 2 (hMSH2), and increased expression of the catabolic enzyme dihydropyrimidine dehydrogenase (DPD) are associated with fluoropyrimidine resistance. At this time, the relative contribution of each of these mechanisms in the development of cellular resistance to 5-FU in the actual clinical setting remains unclear.

Clinical Pharmacology

5-FU is not orally administered, given its erratic bioavailability resulting from high levels of the catabolic enzyme DPD present in the gut mucosa. After intravenous bolus doses, metabolic elimination is rapid, with a half-life of 8 to 14 minutes. More than 85% of an administered dose of 5-FU is enzymatically inactivated by DPD, the rate-limiting enzyme in the catabolism of 5-FU.

A pharmacogenetic syndrome has been identified in which partial or compete deficiency in the DPD enzyme is present in 3% to 5% and 0.1% of the general population, respectively. As DPD catalyzes the rate-limiting step in the catabolic pathway of 5-FU, a deficiency of DPD can result in a clinically dangerous increase in the anabolic products of 5-FU. Unfortunately, patients with DPD deficiency do not manifest a phenotype only until they are treated with 5-FU, and in that setting, they can develop severe GI toxicity in the form of mucositis and/or diarrhea, myelosuppression, neurologic toxicity, and in rare cases, death. In patients being treated with 5-FU or any other fluoropyrimidine, it is important to consider DPD deficiency in patients who present with excessive, severe toxicity.[12] It is now increasingly appreciated that DPD mutations are unable to account for all of the observed cases of excessive 5-FU toxicity, because up to 50% of patients who experience 5-FU toxicity will have no documented alterations in the *DPD* gene. Moreover, individuals with normal DPD enzyme activity may be diagnosed with high plasma levels of 5-FU, resulting in increased toxicity. Although DPD enzyme activity can be assayed from peripheral blood mononuclear cells in a specialized laboratory, routine phenotypic and genotypic screenings for DPD deficiency prior to 5-FU therapy are not yet available.

Biomodulation of 5-FU

Significant efforts have focused on enhancing the antitumor activity of 5-FU through biochemical modulation in which 5-FU is combined with various agents, including leucovorin, MTX, N-phosphonacetyl-L-aspartic acid, interferon-α, interferon-γ, and

TABLE 19.2
Toxicities of Different Forms of 5-FU

Route	Schedule	Dose	DLT
IV	Daily × 5, bolus	400–500 (mg/m^2/d)	⇓ BM D M
IV	Weekly bolus	450–500 (mg/m^2/d)	⇓ BM
IV	Daily × 5, CI	750–1,000 (mg/m^2/d)	M D
IV	PCI	200–400 (mg/m^2/d)	M HFS
HAI	Daily × 14–21, CI	750–1,000 (mg/m^2/d)	M D
IP	32–120 hr	5 nM	M D
Oral (Xeloda)	14–21 d	2,000–2,500 (mg/m^2/d)	HFS

DLT, dose limiting toxicity; IV, intravenous; BM, bone marrow; D, diarrhea; M, mucositis; CI, continuous infusion; PCI, protracted continuous infusion; HFS, hand-foot syndrome; HAI, hepatic artery infusion; IP, intraperitoneal.

a whole host of other agents.[13] For the past 20 to 25 years, the reduced folate LV has been the main biochemical modulator of 5-FU. An alternative approach has been to alter the schedule of 5-FU administration. Given the S-phase specificity of this agent, prolonged exposure of tumor cells to 5-FU would increase the fraction of cells being exposed to the drug. Overall response rates are significantly higher in patients treated with infusional schedules of 5-FU than in those treated with bolus 5-FU, and this improvement in response rate has translated into an improved progression-free survival. Moreover, the overall safety profile is improved with infusional regimens. A hybrid schedule of bolus and infusional 5-FU was originally developed in France, and this regimen has shown superior clinical activity compared with bolus 5-FU schedules. This hybrid schedule has now been simplified by using only the 46-hour infusion of 5-FU and completely eliminating the 5-FU bolus doses.

Toxicity

The spectrum of 5-FU toxicity is dose- and schedule-dependent (Table 19.2). The main side effects are diarrhea, mucositis, and myelosuppression. The dermatologic HFS is more commonly observed with infusional 5-FU therapy. Acute neurologic symptoms have also been reported, and they include somnolence, cerebellar ataxia, and upper motor signs. Treatment with 5-FU can, on rare occasions, cause coronary vasospasm, resulting in a syndrome of chest pain, cardiac enzyme elevations, and electrocardiographic changes. Cardiac toxicity seems to be related more to infusional 5-FU than bolus administration.[14]

CAPECITABINE

Capecitabine is an oral fluoropyrimidine carbamate that was rationally designed to allow for selective 5-FU activation in tumor tissue.[15] This oral agent was initially approved in anthracycline- and taxane-resistant breast cancer and subsequently approved for use in combination with docetaxel as second-line therapy in metastatic breast cancer and in combination with lapatinib, a tyrosine-kinase inhibitor of human epidermal growth factor receptor type 2 (HER2) and epidermal growth factor receptor (EGFR) in women with HER2-positive metastatic breast cancer following progression on trastuzumab-based therapy.[16] This agent is also approved by the U.S. Food and Drug Administration (FDA) for the first-line treatment of mCRC and as adjuvant therapy for stage III colon cancer when fluoropyrimidine therapy alone is preferred.[17] In Europe and throughout much of the world, the combination of capecitabine plus oxaliplatin (XELOX) is approved for the treatment of mCRC as well as for the adjuvant therapy of stage III colon cancer.[18] In addition, recent studies have documented the noninferiority of capecitabine to 5-FU when combined with cisplatin in the treatment of metastatic gastric cancer.

Clinical Pharmacology

Capecitabine is rapidly and extensively absorbed by the gut mucosa, with nearly 80% oral bioavailability. It is inactive in its parent form and undergoes enzymatic conversion via three successive steps. Of note, the third and final step occurs in tumor tissue and involves the conversion of 5′-deoxy-5-fluorouridine to 5-FU by the enzyme thymidine phosphorylase (TP), which is expressed at much higher levels in tumors when compared with corresponding normal tissue. Capecitabine and capecitabine metabolites are primarily excreted by the kidneys, and in contrast to 5-FU, caution must be taken in the presence of renal dysfunction, with appropriate dose modification. The use of capecitabine is absolutely contraindicated in patients whose creatinine clearance is less than 30 mL per minute. The FDA and Roche have added a black box warning and strengthened the precautions section on the capecitabine label about the drug–drug interaction between warfarin and capecitabine-based chemotherapy. It is generally recommended to do weekly monitoring of the coagulation parameters (prothrombin time/international normalized ratio [PT/INR]) for all patients receiving concomitant warfarin and capecitabine, with an appropriate adjustment of warfarin dose.

Toxicity

Similar to what is observed with infusional 5-FU, the main side effects of capecitabine include diarrhea and HFS. Of note, the incidence of myelosuppression, neutropenic fever, mucositis, alopecia, and nausea/vomiting is lower with capecitabine when compared with 5-FU. Elevations in indirect serum bilirubin can be observed, but are usually transient and clinically asymptomatic. Patients in the United States appear to be unable to tolerate as high doses of capecitabine as European patients, either as monotherapy or in combination with other cytotoxic chemotherapy.[19] Although the underlying reasons for this discrepancy are not known, it may in part be related to the increased fortification of the US diet with folate and the increased focus on vitamin and folic acid supplementation.

S-1

S-1 is an oral fluoropyrimidine that consists of tegafur (FT), a prodrug of 5-FU, combined with two 5-FU biochemical modulators: 5-chloro-2,4-dihydroxypyridine (gimeracil or CDHP), a competitive inhibitor of DPD, and oteracil potassium, which inhibits phosphorylation of 5-flurouracil in the GI tract, thereby decreasing serious GI toxicities such as nausea/vomiting, mucositis, and diarrhea.[20] As with other oral agents, S-1 offers several advantages over 5-FU, including ease of administration, no risks associated with use of central venous access such as infection, thrombosis, etc., and reduced toxicities, especially neurotoxicity. Although S-1 has yet to be approved by the FDA, it has been approved for the treatment of gastric cancer, head and neck, colorectal cancer (CRC), non–small-cell lung, breast, pancreatic, and biliary tract cancers in several countries in Asia and for the treatment of advanced gastric cancer in combination with cisplatin in a large number of European countries.

Clinical Pharmacology

S-1 was designed to provide continuous 5-FU plasma exposure comparable to the intravenous (IV) infusion. FT, the 5-FU prodrug, is absorbed in the small intestine and converted to 5-FU through the liver microsomal P-450 metabolizing enzyme system (CYP2A6). Most of the 5-FU is degraded (85%) by DPD, leading to the formation of fluoro-beta-alanine (FBAL).[21] CDHP inhibits DPD, thus allowing higher concentrations of 5-FU to enter the anabolic pathway and enhance its therapeutic effect. Additionally, the inhibition of DPD leads to a decreased amount of FBAL formation, which presumably leads to reduced neurotoxicity. Oteracil is the final component of the S-1 formulation, and it inhibits orotate phosphoribosyltransferase in the GI mucosa, which prevents the formation of fluorouridine monophosphate (FUMP), thereby decreasing GI toxicity.

The maximum tolerated dose was established at 80 mg/m^2 in two divided doses for a Japanese population and 25 mg/m^2 twice a day for a Caucasian population. This interethnic variability of S-1 pharmacokinetics and pharmacodynamics has been attributed to differences in the CYP2A6 genotypes.[22] Studies have demonstrated a high frequency of allelic variants CYP2A6*4, *7, and *9 in East Asians than in Caucasians, which might be associated with reduced enzymatic activity and decreased activation of FT. On the other hand, higher FT metabolism is seen in Caucasian patients due to higher CYP2A6 activity. However, investigators have established similar 5-FU exposure between these two ethnic groups. These findings were explained by higher CDHP exposure in Asians, resulting in increased DPD inhibition and slower catabolism of 5-FU, despite having low CYP2A6 activity, whereas Caucasians had higher CYP2A6 activity but faster 5-FU clearance.

Clinical Toxicity

Clinical studies have shown that the GI toxicities associated with S-1, such as diarrhea, nausea, vomiting, and hyperbilirubinemia, are more prominent in Western patients, whereas hematologic toxicities are more prevalent in Japanese patients. The difference in safety profile cannot be explained by differences in 5-FU exposure, because pharmacokinetic studies have shown that overall drug exposures are similar. A potential explanation might involve interethnic variations in TS promoter enhancer region polymorphisms, which are more frequently seen in Asians or in Caucasians on a higher folate diet.

CYTARABINE

Cytarabine (ara-C) is a deoxycytidine nucleoside analog isolated from the sponge *Cryptotethya crypta*, and it differs from its physiologic counterpart by virtue of a stereotypic inversion of the 2′-hydroxyl group of the sugar moiety.[23] A regimen of ara-C, combined with an anthracycline and given as a 5- or 7-day continuous infusion, is considered the standard induction treatment for acute myeloid leukemia (AML). Ara-C is active against other hematologic malignancies, such as non-Hodgkin's lymphoma, chronic myelogenous leukemia, and acute lymphocytic leukemia (see Table 19.1). However, this agent has absolutely no activity against solid tumors.

Mechanism of Action

Ara-C enters cells via nucleoside transport proteins, the most important one being the equilibrative inhibitor-sensitive (ES) receptor. Once inside the cell, ara-C requires activation for its cytotoxic effects.[23,24] The first metabolic step is the conversion of ara-C to the monophosphate form ara-cytidine monophosphate (ara-CMP) by the enzyme deoxycytidine kinase (dCK) with subsequent phosphorylation to the di- and triphosphate metabolites, respectively. Ara-cytidine triphosphate (ara-CTP) is a potent inhibitor of DNA polymerases α, β, and γ, which in turn interferes with DNA chain elongation, DNA synthesis, and DNA repair. Ara-CTP is also incorporated directly into DNA and functions as a DNA chain terminator, interfering with chain elongation. Catabolism of ara-C involves two key enzymes, cytidine deaminase and deoxycytidylate deaminase. These breakdown enzymes convert ara-C and ara-CMP into the inactive metabolites, ara-uridine (ara-U) and ara-uridine monophosphate (ara-UMP), respectively. The balance between intracellular activation and degradation is critical in determining the amount of drug that is ultimately converted to ara-CTP and, thus, its subsequent cytotoxic and antitumor activity.

Mechanisms of Resistance

Several resistance mechanisms to ara-C have been described. An impaired transmembrane transport, a decreased rate of anabolism, and an increased rate of catabolism may result in the development of ara-C resistance.[23,25,26] The level of cytidine deaminase enzyme activity has been shown to correlate with clinical response in patients with AML undergoing induction chemotherapy with ara-C–containing regimens.

Clinical Pharmacology

Ara-C has poor oral bioavailability given its extensive deamination within the GI tract. Thus, ara-C is administered intravenously via continuous infusion. After administration, ara-C undergoes extensive metabolism in the liver, plasma, and peripheral tissues. Within 24 hours, up to 80% of drug is recovered in the urine as the ara-U metabolite. Ara-C crosses the blood–brain barrier when used at high doses, with cerebrospinal fluid levels between 7% and 14% of plasma levels and reaching peak levels of up to 10 μM.

Toxicity

The toxicity profile of ara-C is highly dependent on the dose and schedule of administration. Myelosuppression is dose-limiting with a standard 7-day regimen. Leukopenia and thrombocytopenia are observed most frequently, with nadirs occurring between days 7 and 14 after drug administration. GI toxicity commonly manifests as a mild-to-moderate degree of anorexia, nausea, and vomiting along with mucositis, diarrhea, and abdominal pain. In rare cases, acute pancreatitis has been observed. The ara-C syndrome has been described in pediatric patients with hematologic malignancies, usually begins within 12 hours after the start of drug infusion, and is characterized by fever, myalgia, bone pain, maculopapular rash, conjunctivitis, malaise, and occasional chest pain.

The administration of ara-C at high doses (2 to 3 g/m^2 with each dose) is associated with profound myelosuppression.[27] Severe GI toxicity in the form of mucositis and/or diarrhea is also observed. Neurologic toxicity is significantly more common with high-dose ara-C than with standard doses, and presents with seizures, cerebral and cerebellar dysfunction, and peripheral neuropathy. Clinical signs of cerebellar dysfunction occur in up to 15% of patients and include dysarthria, dysmetria, and ataxia. Change in alertness and cognitive ability, memory loss, and frontal lobe release signs reflect cerebral toxicity. Despite discontinuation of therapy, clinical recovery is incomplete in up to 30% of affected patients. Pulmonary complications may include noncardiogenic pulmonary edema, acute respiratory distress, and pneumonia, resulting from *Streptococcus viridans* infection. Other side effects associated with high-dose ara-C include conjunctivitis (often responsive to topical corticosteroids), a painful HFS, and rarely, anaphylactic reactions.

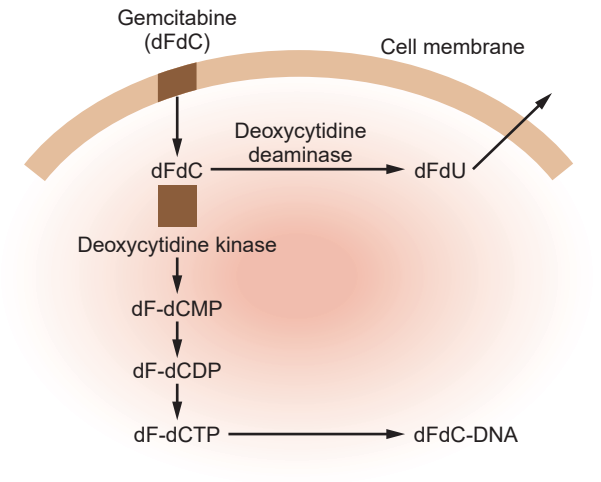


Figure 19.2 Transport and metabolism of gemcitabine. dFdC, gemcitabine; dFdU, 2′,2′-difluorodeoxyuridine; dF-dCMP, gemcitabine monophosphate; dF-dCDP, gemcitabine diphosphate; dF-dCTP, gemcitabine triphosphate.

GEMCITABINE

Gemcitabine (2′,2′-difluorodeoxycytidine) is a difluorinated deoxycytidine analog. Despite its similarity in structure, metabolism, and mechanism of action to ara-C, the spectrum of antitumor activity of gemcitabine is much broader.[23,28] This compound has significant clinical activity against several human solid tumors, including pancreatic, bile duct, gall bladder, small cell and non–small-cell lung, bladder, ovary, and breast cancers as well as hematologic malignancies, namely Hodgkin's and non-Hodgkin's lymphoma (see Table 19.1).

Mechanism of Action

The transport of gemcitabine into cells requires the nucleoside transporter system. Gemcitabine is inactive in its parent form and requires intracellular activation for its cytotoxic effects. The steps involved in the metabolic activation of gemcitabine are similar to those observed with ara-C, with both drugs being activated by the same enzymatic machinery to the active triphosphate metabolite (see Fig. 19.2). Gemcitabine triphosphate is then incorporated into DNA, resulting in chain termination and the inhibition of DNA synthesis and function, or the triphosphate form can directly inhibit DNA polymerases α, β, and γ, which in turn, interferes with DNA chain elongation, DNA synthesis, and DNA repair. The triphosphate metabolite is also a potent inhibitor of ribonucleotide reductase, which further mediates inhibition of DNA biosynthesis by reducing the levels of key deoxynucleotide pools.[29]

Mechanisms of Resistance

Several mechanisms of resistance to gemcitabine have been described in various preclinical experimental models.[30] Gemcitabine is a polar nucleoside analog that requires the activity of human equilibrative nucleoside transporter 1 (hENT1) to enter cells and exert its cytotoxic effects. Preclinical data in human pancreatic cancer cell lines showed that gemcitabine resistance is negatively correlated with hENT1 expression and can be induced by specific inhibitors of hENT1.[31] Clinical data also support the concept that a lack of hENT1 may be predictive of resistance to gemcitabine. CO-101, a lipid-drug conjugate of gemcitabine, was rationally designed to enter cells independently of hENT1. Unfortunately, two studies in pancreatic cancer failed to show any benefit of CO-101.

Additionally, several enzymes involved in the intracellular metabolism of gemcitabine have been implicated in the development of cellular drug resistance, including reduced expression and/or deficiency in dCK enzyme activity as well as increased expression and/or activity of the catabolic enzymes cytidine deaminase and dCMP deaminase. Recent studies have also identified a subset of CD44-positive cancer stem cells within pancreatic tumors that sustain tumor formation and growth, and are resistant to gemcitabine therapy.[33]

Clinical Pharmacology

Gemcitabine is administered via the intravenous route, typically over a 30-minute intravenous infusion, and it undergoes extensive metabolism by deamination to the catabolic metabolite, difluorodeoxyuridine (dFdU), with more than 90% of the metabolized drug being recovered in urine. Plasma clearance is about 30% lower in women and in elderly patients, and this pharmacokinetic difference may result in an increased risk of toxicity in these respective patient populations. The initial findings from pilot pharmacokinetic studies suggested that gemcitabine, when given at a fixed dose rate (FDR) intravenous infusion of 10 mg/m^2 per minute, produced the highest accumulation of active dFdCTP metabolites in peripheral blood mononuclear cells, which led to a randomized phase II trial that compared gemcitabine 1,500 mg/m^2 by FDR or 2,200 mg/m^2 of gemcitabine over 30 minutes. Although this phase II study suggested an improved overall survival with FDR, a subsequent phase III trial failed to confirm the survival advantage of gemcitabine by FDR over its conventional administration schedule.[34]

Toxicity

Gemcitabine is a relatively well-tolerated drug when used as a single agent. The main dose-limiting toxicity is myelosuppression, with neutropenia more commonly experienced than thrombocytopenia. Toxicity is schedule dependent, with longer infusions producing greater hematologic toxicity. Transient flulike symptoms, including fever, headache, arthralgias, and myalgias, occur in 45% of patients. Asthenia and transient transaminasemia may occur. Renal microangiopathy syndromes, including hemolytic-uremic syndrome and thrombotic thrombocytopenic purpura, have been reported rarely.

6-THIOPURINES

The development of the purine analogs in cancer chemotherapy began in the early 1950s with the synthesis of the thiopurines, 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG). 6-MP has an important role in maintenance therapy for acute lymphoblastic leukemia, whereas 6-TG is active in remission induction and in maintenance therapy for AML (see Table 19.1).

Mechanism of Action

The thiopurines, 6-MP and 6-TG, act similarly with respect to their cellular biochemistry.[34] In their respective monophosphate nucleotide forms, they inhibit enzymes involved in de novo purine synthesis and purine interconversion reactions. The triphosphate nucleotide forms can get directly incorporated into either cellular RNA or DNA, leading to the inhibition of RNA and DNA synthesis and function, respectively.

Mechanisms of Resistance

The development of cellular resistance to 6-thiopurines results from a decreased level of key cytotoxic nucleotide metabolites,

either through decreased formation or increased breakdown. Resistant cells have been identified that express either complete or partial deficiency of the activating enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT). In clinical samples derived from patients with AML, drug resistance has been associated with increased concentrations of a membrane-bound alkaline phosphatase or a conjugating enzyme, 6-thiopurine methyltransferase (TPMT), the end-result being reduced formation of cytotoxic thiopurine nucleotides. Finally, the decreased expression of mismatch repair enzymes, including hMLH1 and hMSH2, has been associated with cellular drug resistance.

Clinical Pharmacology

Oral absorption of 6-MP is highly erratic, and the relatively poor oral bioavailability is mainly related to rapid first-pass metabolism in the liver. The major route of drug elimination is via metabolism by several enzymatic pathways. 6-MP is oxidized to the inactive metabolite 6-thiouric acid by xanthine oxidase. Enhanced 6-MP toxicity may result from the concomitant administration of 6-MP and the xanthine oxidase inhibitor allopurinol. In patients receiving both 6-MP and allopurinol, the 6-MP dose must be reduced by at least 50% to 75%. 6-MP also undergoes S-methylation by the enzyme TPMT to yield 6-methylmercaptopurine.[35]

6-TG is administered orally in the treatment of AML. Its oral bioavailability is erratic, with peak plasma levels occurring 2 to 4 hours after ingestion. The catabolism of 6-TG differs from 6-MP in that it is not a direct substrate for xanthine oxidase.

TPMT enzyme activity may vary considerably among patients as a result of point mutations or loss of alleles of TPMT.[36] Approximately 0.3% of the Caucasian population expresses either a homozygous deletion or a mutation of both alleles of the *TPMT* gene. In these patients, grossly elevated thiopurine nucleotides concentrations, profound myelosuppression with pancytopenia, and extensive GI symptoms are observed after only a brief course of thiopurine treatment. An estimated 10% of patients may be at increased risk for toxicity because of heterozygous loss of the gene or a mutant allele coding for a less enzymatically active TPMT.

Toxicity

The major dose-related toxicities of the thiopurines are myelosuppression and GI toxicity in the form of nausea/vomiting, anorexia, diarrhea, and stomatitis.[37] In TPMT-deficient patients, dosage reduction to 5% to 25% of the standard dosage is necessary to prevent severe excessive toxicity. Thiopurine hepatotoxicity occurs in up to 30% of adult patients and presents mainly as cholestatic jaundice, although elevations of hepatic transaminases may also be seen. Combinations of thiopurines with other known hepatotoxic agents should be avoided, and liver function should be closely monitored. The thiopurines are also potent suppressors of cell-mediated immunity, and prolonged therapy results in an increased predisposition to bacterial and parasitic infections.

FLUDARABINE

Fludarabine (9-β-D-arabinosyl-2-fluoroadenine monophosphate, F-ara-AMP) is an active agent in the treatment of chronic lymphocytic leukemia (CLL) (see Table 19.1).[38,39] It is also active against indolent non-Hodgkin's lymphoma, prolymphocytic leukemia, cutaneous T-cell lymphoma, and Waldenström macroglobulinemia. This agent has also shown promising activity in mantle cell lymphoma. In contrast to its activity in hematologic malignancies, this compound has virtually no activity against solid tumors.

Mechanism of Action

The active cytotoxic metabolite is the triphosphate metabolite F-ara-ATP, which competes with deoxyadenosine triphosphate (dATP) for incorporation into DNA and serves as a highly effective chain terminator. In addition, F-ara-ATP directly inhibits enzymes involved in DNA replication, including DNA polymerases, DNA primase, DNA ligase I, and ribonucleotide reductase.[37] F-ara-ATP is also incorporated into RNA, causing the inhibition of RNA function, processing, and mRNA translation. In contrast to other antimetabolites, fludarabine is active against nondividing cells. In fact, the primary effect of fludarabine may result from activation of apoptosis, through an as yet ill-defined mechanisms.[39] This finding may explain the activity of fludarabine in indolent lymphoproliferative diseases with relatively low growth fractions.

Mechanisms of Resistance

The decreased expression of the activating enzyme dCK resulting in diminished intracellular formation of F-ara-AMP is one of the main resistance mechanisms identified in preclinical models.[38] A high degree of cross-resistance develops to multiple nucleoside analogs, requiring activation by dCK, including cytarabine, gemcitabine, cladribine, and clofarabine. Reduced cellular transport of drug has also been identified as a resistance mechanism.

Clinical Pharmacology

Peak concentrations of F-ara-A are reached 3 to 4 hours after intravenous administration.[40] The main route of elimination is via the kidneys, with about 25% of a given dose of drug being excreted unchanged in the urine.

Toxicity

Myelosuppression and immunosuppression are the major side effects of fludarabine as highlighted by dose-limiting and possibly cumulative lymphopenia and thrombocytopenia. Suppression of the immune system affects T-cell function more than B-cell function. Fevers, often in the setting of neutropenia, occur in 20% to 30% of patients. Lymphocyte counts, specifically CD4-positive cells, decrease rapidly after the initiation of therapy, and recovery of CD4-positive cells to normal levels may take longer than 1 year. Common opportunistic pathogens include the varicella-zoster virus, *Candida*, and *Pneumocystis carinii*. In general, patients are empirically placed on sulfamethoxazole trimethoprim prophylaxis to prevent the development of *P. carinii* infection.

CLADRIBINE

Cladribine (2-CdA) is a purine deoxyadenosine analog, and it is the drug of choice for hairy cell leukemia with activity in low-grade lymphoproliferative disorders (see Table 19.1).[41,42] Salvage treatment of patients previously treated with interferon-α or splenectomy is as effective as first-line treatment. Retreatment with cladribine results in a complete response in up to 60% of relapsing patients. In addition, this agent has promising activity in patients with CLL and non-Hodgkin's lymphoma.

Mechanism of Action

Upon entry into the cell, 2-CdA undergoes an initial conversion to cladribine-monophosphate (Cd-AMP) via the reaction catalyzed by dCK, and Cd-AMP is subsequently metabolized to the active metabolite, cladribine-triphosphate. The triphosphate metabolite competitively inhibits incorporation of the normal dATP

nucleotide into DNA, a process that results in the termination of chain elongation.[43] Progressive accumulation of the triphosphate metabolite leads to an imbalance in deoxyribonucleotide pools, thereby inhibiting further DNA synthesis and repair. Finally, the triphosphate metabolite is a potent inhibitor of ribonucleotide reductase, which further facilitates the inhibition of DNA biosynthesis.

Mechanisms of Resistance

Resistance to 2-CdA has been attributed to altered intracellular drug metabolism. A reduction in the activity of dCK, the enzyme responsible for generating cytotoxic nucleotide metabolites, is a major determinant of acquired resistance. The monophosphate and triphosphate metabolites are dephosphorylated by the cytoplasmic enzyme 5′-nucleotidase. Interestingly, resistant cells derived from a patient with CLL exhibited both low levels of dCK expression and high levels of 5′-nucleotidase.

Clinical Pharmacology

2-CdA is orally bioavailable, with 50% of an administered dose orally absorbed. Approximately 50% of an administered dose of drug is cleared by the kidneys, and 20% to 35% of the drug is excreted unchanged in the urine. Of note, this nucleoside can cross the blood–brain barrier with penetration into the cerebrospinal fluid.

Toxicity

At conventional doses, myelosuppression is dose limiting. After a single course of drug, recovery from thrombocytopenia usually occurs within 2 to 4 weeks, whereas recovery from neutropenia takes place in 3 to 5 weeks. GI toxicities are generally mild, with nausea/vomiting and diarrhea. Mild-to-moderate neurotoxicity occurs in 15% of patients and is at least partly reversible with discontinuation of the drug. Immunosuppression accounts for the late morbidity observed in 2-CdA–treated patients. Lymphocyte counts, particularly CD4-positive cells, decrease within 1 to 4 weeks of drug administration and may remain depressed for several years.[44] After discontinuation of 2-CdA, a median time of up to 40 months may be required for complete recovery of normal CD4-positive counts. Although opportunistic infections occur, they do so less frequently than with fludarabine therapy. Infectious complications correlate with decreases in the CD4-positive count, and they include herpes zoster, *Candida*, *Pneumocystis*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Cryptococcus neoformans*, *Aspergillus*, *P. carinii*, and cytomegalovirus.

CLOFARABINE

Clofarabine is a purine deoxyadenosine nucleoside analog, and it is approved for the treatment of pediatric patients with relapsed or refractory acute lymphoblastic leukemia (see Table 19.1).[45] Ongoing studies are exploring the benefit of clofarabine alone and in combination with other agents in less heavily pretreated patients and in the use of different dose schedules for other hematologic malignancies.[46]

Mechanism of Action

Clofarabine is inactive in its parent form and, like other purine analogs, it requires intracellular activation by dCK to form the monophosphate nucleotide, which undergoes further metabolism to the cytotoxic triphosphate metabolite. Clofarabine triphosphate is then incorporated into DNA, resulting in chain termination, and inhibition of DNA synthesis and function or the triphosphate form can directly inhibit DNA polymerases α, β, and γ, which in turn, interferes with DNA chain elongation, DNA synthesis, and DNA repair. The triphosphate metabolite is also a potent inhibitor of ribonucleotide reductase, further mediating the inhibition of DNA biosynthesis by reducing the levels of key deoxyribonucleotide pools.

Mechanisms of Resistance

Several resistance mechanisms have been identified in various preclinical systems, and they include decreased activation of the drug through the reduced expression of the anabolic enzyme deoxycytidine kinase, the decreased transport of drug into cells via the nucleoside transporter protein, and the increased expression of CTP synthetase activity resulting in increased concentrations of competing physiologic nucleotide substrate dCTP. To date, the precise resistance mechanism(s) that are relevant in the clinical setting remain to be determined.

Clinical Pharmacology

Approximately 50% to 60% of an administered dose of drug is excreted unchanged in the urine, and the terminal half-life is on the order of 5 hours. To date, the pathways for nonrenal elimination have not been well defined. Caution should be exercised in patients with abnormal renal function, and concomitant use of medications known to cause renal toxicity should be avoided during drug treatment.

Toxicity

Myelosuppression is dose limiting with neutropenia, anemia, and thrombocytopenia. The capillary leak syndrome (systemic inflammatory response syndrome) presents with tachypnea, tachycardia, pulmonary edema, and hypotension.[47] In essence, this adverse event is part of the tumor lysis syndrome and results from rapid cytoreduction of peripheral leukemic cells following treatment.[47] Other side effects may include nausea/vomiting, reversible liver dysfunction (hyperbilirubinemia and elevated serum transaminases), renal dysfunction (approximately 10%), and cardiac toxicity in the form of tachycardia and acute pump dysfunction.

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20 Topoisomerase Interactive Agents

Khanh T. Do, Shivaani Kummar, James H. Doroshow, and Yves Pommier

CLASSIFICATION, BIOCHEMICAL, AND BIOLOGIC FUNCTIONS OF TOPOISOMERASES

Nucleic acids (DNA and RNA) being long polymers, topoisomerases fulfill the need for cellular DNA to be densely packaged in the cell nucleus, transcribed, replicated, and evenly distributed between daughter cells following replication without tangles. Topoisomerases are ubiquitous and essential for all organisms as they prevent and resolve DNA and RNA entanglements and resolve DNA supercoiling during transcription and replication. This chapter first summarizes the basic elements necessary to understand the mechanism of action of topoisomerases and their inhibitors. More detailed information can be found in recent reviews[1–7] and two recent books.[8,9] The second part of the chapter summarizes the use of topoisomerase inhibitors as anticancer drugs.

Classification of Topoisomerases

Human cells contain six topoisomerase genes (Table 20.1), which have been numbered historically. The commonly used abbreviations are Top1 for topoisomerases I (Top1mt being the mitochondrial topoisomerase whose gene is encoded in the cell nucleus),[10] Top2 for topoisomerases II, and Top3 for topoisomerases III. Top1 was the first eukaryotic topoisomerase discovered by Champoux and Dulbecco.[11] Topoisomerases solve DNA topologic problems by cutting the DNA backbone and religating without the assistance of any additional ligase. Top1 and Top3 act by cleaving/religating a single strand of the DNA duplex, whereas Top2 enzymes cleave and religate both strands, making a four–base pair reversible staggered cut (Fig. 20.1). It is convenient to remember that odd-numbered topoisomerases (Top1 and Top3) cleave and religate one strand, whereas the even numbered topoisomerases (Top2s) cleave and religate both strands.

Biochemical Characteristics and Cleavage Complexes of the Different Topoisomerases

The DNA cutting/relegation mechanism is common to all topoisomerases and utilizes an enzyme catalytic tyrosine residue acting as a nucleophile and becoming covalently attached to the end of the broken DNA. These catalytic intermediates are referred to as cleavage complexes (see Fig. 20.1B, E). The reverse religation reaction is carried out by the attack of the ribose hydroxyl ends toward the tyrosyl-DNA bond.

Top1 (and Top1mt) attaches to the 3′-end of the break, whereas the other topoisomerases (Top2 and Top3) have opposite polarity and covalently attach to the 5′-end of the breaks (see Table 20.1 [second column] and Fig. 20.1B, E). Topoisomerases have distinct biochemical requirements. Top1 and Top1mt are the simplest, nicking/closing, and relaxing DNA as monomers in the absence of cofactor, and even at ice temperature. Top2 enzymes, on the other hand, are the most complex topoisomerases working as dimers, requiring ATP binding and hydrolysis, and a divalent metal (Mg^{2+}) for catalysis. Top3 enzymes also require Mg^{2+} for catalysis but function as monomers without ATP requirement. Notably, the DNA substrates differ for Top3 enzymes. Whereas both Top1 and Top2 process double-stranded DNA, the Top3 substrates need to be single-stranded nucleic acids (DNA for Top3α and DNA or RNA for Top3β).[10,12,13]

Differential Topoisomerization Mechanisms: Swiveling Versus Strand Passage, DNA Versus RNA Topoisomerases

Topoisomerases use two main mechanisms to change nucleic topology. The first is by "untwisting" the DNA duplex. This mechanism is unique to Top1, which, by an enzyme-associated single-strand break, allows the broken strand to rotate around the intact strand (see Fig. 20.1B) until DNA supercoiling is dissipated. At this point, the stacking energy of adjacent DNA bases realigns the broken ends, and the 5′-hydroxyl end attacks the 3′-phosphotyrosyl end, thereby relegating the DNA. A remarkable feature of this Top1 untwisting mechanism is its extreme efficiency with a rotation speed around 6,000 rpm and relative independence from torque, thereby allowing full relaxation of DNA supercoiling.[14]

The second topologic mechanism is by "strand passage." This mechanism allows the passage of a double- or a single-stranded DNA (or RNA) through the cleavage complexes. Top2α and Top2β both act by allowing the passage of an intact DNA duplex through the DNA double-strand break generated by the enzymes. After which, Top2 religates the broken duplex. Such reactions permit DNA decatenation, unknotting, and relaxation of supercoils.[3] Top3 enzymes also act by strand passage but only pass one nucleic acid strand through the single-strand break generated by the enzymes. In the case of Top3α, the substrate is a single-stranded DNA segment (such as a double-Holliday junction), whereas in the case of Top3β, the substrate can be a single-stranded RNA segment, with Top3β acting as a RNA topoisomerase.[13,15]

TOPOISOMERASE INHIBITORS AS INTERFACIAL POISONS

Topoisomerase Inhibitors Act as Interfacial Inhibitors by Binding at the Topoisomerase–DNA Interface and Trapping Topoisomerase Cleavage Complexes

Relegation of the cleavage complexes is dependent on the structure of the ends of the broken DNA (i.e., the realignment of the broken ends). Binding the drugs at the enzyme–DNA interface misaligns the ends of the DNA and precludes relegation, resulting in the stabilization of the topoisomerase cleavage complexes (Top1cc and Top2cc). Crystal structures of drug-bound cleavage complexes have firmly established this mechanism for both Top1- and Top2-targeted drugs.[16]

TABLE 20.1

Classification of Human Topoisomerases and Topoisomerase Inhibitors

Type	Polarity	Mechanism	Genes	Proteins	Main Functions	Drugs
IB	3′-PY	Rotation/ swiveling	*TOP1*	Top1	DNA supercoiling relaxation, replication, and transcription	Camptothecins, noncamptothecins
			TOP1MT	Top1mt		
IIA	5′-PY	Strand passage ATPase	*TOP2A*	Top2α	Decatenation/replication	Anthracyclines, anthracenediones, epipodophyllotoxins
			TOP2B	Top2β	Transcription	
IA	5′-PY	Strand passage	*TOP3A*	Top3α	DNA replication with BLM	None
			TOP3B	Top3β	RNA topoisomerase	

Top1mt, mitochondrial DNA topoisomerase; BLM, Bloom's syndrome helicare.

It is critical to understand that the cytotoxic mechanism of topoisomerase inhibitors requires the drugs to trap the topoisomerase cleavage complexes rather than block catalytic activity. This sets apart topoisomerase inhibitors from classical enzyme inhibitors such as antifolates. Indeed, knocking out Top1 renders yeast cells totally immune to camptothecin,[17,18] and reducing enzyme levels in cancer cells confers drug resistance. Conversely, in breast cancers, amplification of TOP2A, which is on the same locus as HER2, contributes to the efficacy of doxorubicin.[19] Also, cellular mutations of Top1 and Top2 that renders cells insensitive to the trapping of topoisomerase cleavage complexes produce high resistance to Top1 or Top2 inhibitors. Based on this trapping of cleavage complexes mechanism, we refer to topoisomerase inhibitors as topoisomerase cleavage complex-targeted drugs.

Top1cc-Targeted Drugs (Camptothecin and Noncamptothecin Derivatives) Kill Cancer Cells by Replication Collisions

Top1cc are cytotoxic by their conversion into DNA damage by replication and transcription fork collisions. This explains why cytotoxicity is directly related to drug exposure and why arresting DNA replication protects cells from camptothecin.[20,21] The collisions arise from the fact that the drugs, by slowing down the nicking/closing activity of Top1, uncouple the kinetics of Top1 with the polymerases and helicases, which lead polymerases to collide into Top1cc (Fig. 20.2A). Such collisions have two consequences. They generate double-strand breaks (replication and transcription runoff) and irreversible Top1–DNA adducts (see Fig. 20.2B). The replication double-strand breaks are repaired by homologous recombination, which explains the hypersensitivity of BRCA-deficient cancer cells to Top1cc-targeted drugs.[22] The Top1-covalent complexes can be removed by two pathways, the excision pathway centered around tyrosyl-DNA-phosphodiesterase 1 (TDP1)[23] and the endonuclease pathway involving 3′-flap endonucleases such as XPF-ERCC1.[24] It is also possible that drug-trapped Top1cc directly generate DNA double-strand breaks when they are within 10 base pairs on opposite strands of the DNA duplex or when they occur next to a preexisting single-strand break on the opposite strand. Finally, it is not excluded that topologic defects contribute to the cytotoxicity of Top1cc-targeted drugs (the accumulation of supercoils[25] and the formation of alternative structures such as R-loops) (see Fig. 20.2D).[26]

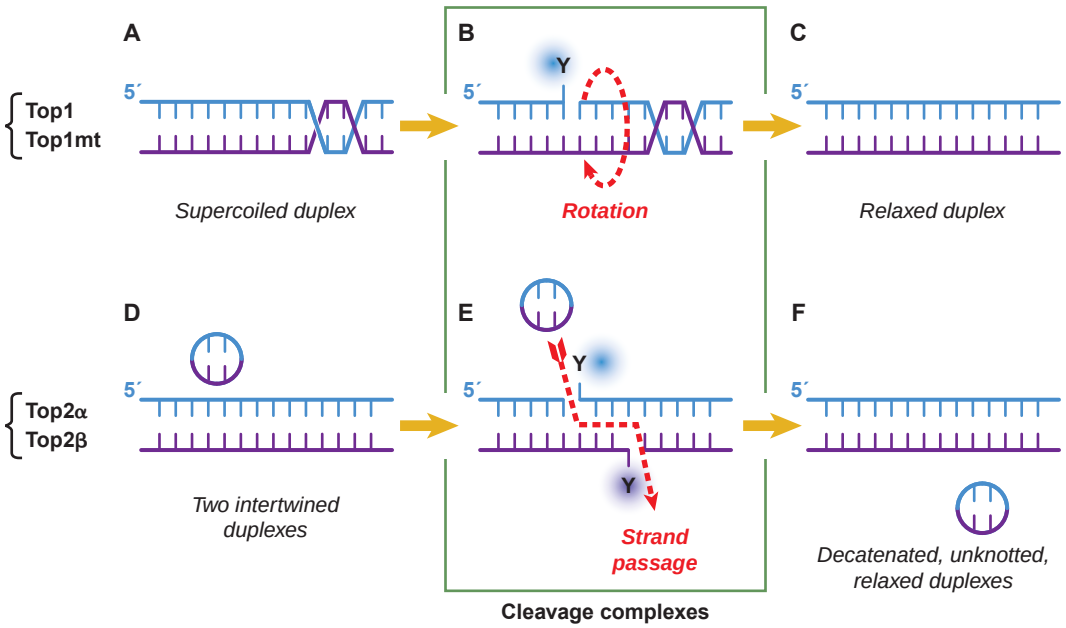

Figure 20.1 Mechanisms of action of topoisomerases. **(A–C)** Topoisomerases I (Top1 for nuclear DNA and Top1mt for mitochondrial DNA) relax supercoiled DNA **(A)** by reversibly cleaving one DNA strand, forming a covalent bond between the enzyme catalytic tyrosine and the 3′ end of the nicked DNA (the Top1 cleavage complex [Top1cc]) **(B).** This reaction allows the swiveling of the broken strand around the intact strand. Rapid religation allows the dissociation of Top1. **(D–F)** Topoisomerases II (Top2α and Top2β) act on two DNA duplexes **(A).** They act as homodimers, cleaving both strands and forming a covalent bond between their catalytic tyrosine and the 5′ end of the DNA break (Top2cc) **(E).** This reaction allows the passage of the intact duplex through the Top2 homodimer *(red dotted arrow)* **(E).** Top2 inhibitors trap the Top2cc and prevent the normal religation **(F).**

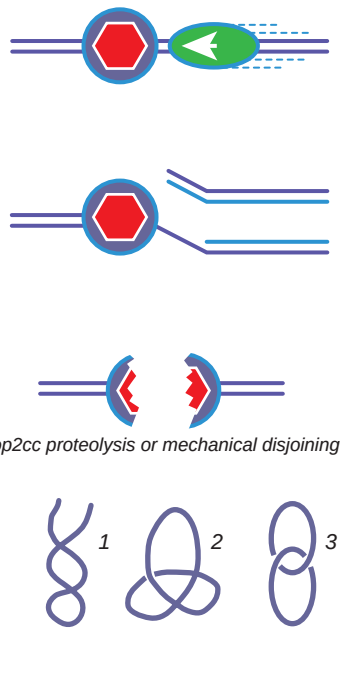

Figure 20.2 Mechanisms of action of topoisomerase inhibitors beyond the trapping of topoisomerase cleavage complexes. **(A)** Stalled or slow cleavage complexes lead to collisions with replication and transcription complexes. **(B)** Collisions of replication complexes with Top1cc on the leading strand for DNA synthesis generate DNA double-strand breaks by replication runoff. Top1cc can also form DNA double-strand breaks (DSBs) when they occur opposite to another Top1cc or preexisting nick. **(C)** Top2cc, which are normally held together by Top2 homodimers, can be converted to free DSBs upon Top2cc proteolysis or dimer disjunction. **(D)** Topologic defects resulting from functional topoisomerase deficiencies play a minor role in the anticancer activity of topoisomerase cleavage complex targeted drugs.

Cytotoxic Mechanisms of Top2cc-Targeted Drugs (Intercalators and Demethyl Epipodophyllotoxins)

Contrary to camptothecins, Top2 inhibitors kill cancer cells without requiring DNA replication fork collisions. Indeed, even after a 30-minute exposure, doxorubicin and other Top2cc-targeted drugs can kill over 99% of the cells, which is in vast excess of the fraction of S-phase cells in tissue culture (generally less than 50%).[27,28] The collision mechanism in the case of Top2cc-targeted drugs (see Fig. 20.2A) appears to involve transcription and proteolysis of both Top2 and RNA polymerase II.[29] Such situation would then lead to DNA double-strand breaks by disruption of the Top2 dimer interface (see Fig. 20.2C). Alternatively, the Top2 homodimer interface could be disjoined by mechanical tension (see Fig. 20.2C). Yet, it is important to bear in mind that 90% of Top2cc trapped by etoposide are not concerted and, therefore, consist in single-strand breaks,[3,30,31] which is different from doxorubicin, which traps both Top2 monomers and produces a majority of DNA double-strand breaks.[32] Finally, it is not excluded that topologic defects resulting from Top2 sequestration by the drug-induced cleavage complexes could contribute to the cytotoxicity of Top2cc-targeted drugs (see Fig. 20.2D). Such topologic defects would include persistent DNA knots and catenanes, potentially leading to chromosome breaks during mitosis.

TOPOISOMERASE I INHIBITORS: CAMPTOTHECINS AND BEYOND

Camptothecin is an alkaloid identified in the 1960s by Wall and Wani[33] in a screen of plant extracts for antineoplastic drugs. The two water-soluble derivatives of camptothecin containing the active lactone form are topotecan and irinotecan, which are approved by the U.S. Food and Drug Administration (FDA) for the treatment of several cancers. In addition, several Top1cc-targeting drugs are in clinical development, including camptothecin derivatives and formulations (including high–molecular-weight conjugates or liposomal formulations), as well as noncamptothecin compounds that exhibit greater potency or noncross resistance to irinotecan and topotecan in preclinical cancer models.[31,34–36]

Irinotecan

Irinotecan, a prodrug containing a bulky dipiperidine side chain at C-10 (Fig. 20.3), is cleaved by a carboxylesterase-converting enzyme in the liver and other tissues to generate the active metabolite, SN-38. Irinotecan is FDA approved for the treatment of colorectal cancer in the metastatic setting as first-line treatment in combination with 5-fluorouracil/leucovorin (5-FU/LV) and as a single agent in the second-line treatment of progressive colorectal cancer after 5-FU–based therapy (see Table 20.1).[37,38] Newer therapeutic uses of irinotecan include a combination with oxaliplatin and 5-FU as first-line treatment in pancreatic cancer.[39] Irinotecan is additionally used in combination with cisplatin or carboplatin in extensive-stage small-cell lung cancer[40,41] as well as refractory esophageal and gastroesophageal junction (GEJ) cancers, gastric cancer, cervical cancer, anaplastic gliomas and glioblastomas, and non–small-cell lung cancer (Table 20.2). Irinotecan is usually administered intravenously at a dose of 125 mg/m^2 for 4 weeks with a 2-week rest period in combination with bolus 5-FU/LV, 180 mg/m^2 every 2 weeks in combination with an infusion of 5-FU/LV, or 350 mg/m^2 every 3 weeks as a single agent.

Diarrhea and myelosuppression are the most common toxicities associated with irinotecan administration. Two mechanisms explain irinotecan-induced diarrhea. Acute cholinergic effects resulting in abdominal cramping and diarrhea occur within 24 hours of drug administration are the result of acetylcholinesterase inhibition by the prodrug, and can be treated with the administration of atropine. Direct mucosal cytotoxicity with diarrhea is typically observed after 24 hours and can result in significant morbidity. Symptoms are managed with loperamide. Hepatic metabolism and biliary excretion accounts for >70% of the elimination of the administered dose, with renal excretion accounting for the remainder of the dose. SN-38 is glucuronidated in the liver by UGT1A1, and deficiencies in this pathway increase the risk of diarrhea and myelosuppression. Dose reductions are recommended for patients who are homozygous for the UGT1A1*28 allele, for which an FDA-approved test for detection of the UGT1A1*28 allele in patients is available.[42,43] Additionally, dose reductions of irinotecan are recommended for patients with hepatic dysfunction, with bilirubin greater than 1.5 mg/mL.[44]