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Precision Molecular Pathology of Prostate Cancer

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Brian D. Robinson • Juan Miguel Mosquera
Jae Y. Ro • Mukul Divatia
Editors

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Editors
Brian D. Robinson
Pathology and Laboratory Medicine
New York-Presbyterian Hospital
Weill Cornell Medical College
New York, NY
USA

Juan Miguel Mosquera
Pathology and Laboratory Medicine
New York-Presbyterian Hospital
Weill Cornell Medical College
New York, NY
USA

Jae Y. Ro
Institute for Academic Medicine
Houston Methodist Hospital Pathology
and Genomic Medicine, Weill Medical
College of Cornell University
Houston, TX
USA

Mukul Divatia
Institute for Academic Medicine
Houston Methodist Hospital Pathology
and Genomic Medicine, Weill Medical
College of Cornell University
Houston, TX
USA

ISSN 1935-987X ISSN 1935-9888 (electronic)
Molecular Pathology Library
ISBN 978-3-319-64094-5 ISBN 978-3-319-64096-9 (eBook)
https://doi.org/10.1007/978-3-319-64096-9

Library of Congress Control Number: 2017962624

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Printed on acid-free paper

This Springer imprint is published by the registered company Springer International Publishing AG part of Springer Nature
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Contents

Editors and Contributors

Editors

Juan Miguel Mosquera, M.D., M.Sc Department of Pathology and Laboratory Medicine, New York-Presbyterian Hospital, Weill Cornell Medical College, New York, NY, USA

Englander Institute for Precision Medicine, New York-Presbyterian Hospital, Weill Cornell Medical College, New York, NY, USA

Brian D. Robinson, M.D. Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, New York-Presbyterian Hospital, New York, NY, USA

Jae Y. Ro, M.D. Institute for Academic Medicine, Houston Methodist Hospital Pathology and Genomic Medicine, Weill Medical College of Cornell University, Houston, TX, USA

Mukul Divatia, M.D. Institute for Academic Medicine, Houston Methodist Hospital Pathology and Genomic Medicine, Weill Medical College of Cornell University, Houston, TX, USA

Contributors

Benjamin Abbott, B.Sc., M.Res. Manchester Academic Health Sciences Centre, Faculty of Biology, Medicine and Health, Division of Cancer Sciences, Manchester Cancer Research Centre, University of Manchester, Manchester, UK

John W. Babich, Ph.D. Division of Radiopharmaceutical Sciences and Molecular Imaging Innovations Institute, Department of Radiology, Weill Cornell Medical College of Cornell University, New York, NY, USA

Shaham Beg, M.D. Department of Pathology and Laboratory Medicine, Weill Cornell Medicine, New York, NY, USA

Adeline Berger, Ph.D. Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, New York, NY, USA

Jennifer L. Bishop, Ph.D. Department of Urologic Sciences, Vancouver Prostate Centre, Vancouver, BC, Canada

Fadi Brimo, M.D., F.R.C.P.(C) Department of Pathology, McGill University Health Center, Montreal, QC, Canada

Simone de Brot, D.V.M., Dip. E.C.V.P. Faculty of Medicine and Health Sciences, School of Veterinary Medicine and Health Science, University of Nottingham, Nottingham, UK

Michael D. Brown, B.Sc., Ph.D. Manchester Academic Health Sciences Centre, Faculty of Biology, Medicine and Health, Division of Cancer Sciences, Manchester Cancer Research Centre, University of Manchester, Manchester, UK

Stefano Cacciatore, Ph.D. Department of Oncologic Pathology, Dana-Farber Cancer Institute, Boston, MA, USA

Institute of Reproductive and Developmental Biology, Imperial College, London, England, UK

Lingwu Chen, M.D., Ph.D. Department of Urology, First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China

Noel W. Clarke, M.B.B.S., Ch.M., F.R.C.S.(Urol). The Christie and Salford Royal NHS Foundation Trusts, Manchester, UK

Alastair H. Davies, Ph.D. Department of Urologic Sciences, Vancouver Prostate Centre, Vancouver, BC, Canada

Olivier Elemento, Ph.D. Caryl and Israel Englander Institute for Precision Medicine, Institute for Computational Biomedicine, Department of Physiology and Biophysics, Weill Cornell Medicine, New York, NY, USA

Bishoy M. Faltas, M.D. Division of Hematology and Medical Oncology, Department of Medicine, Weill Cornell Medical College, New York Presbyterian Hospital, New York, NY, USA

Stephen P. Finn, M.B., B.Ch., B.A.O., Ph.D. Department of Histopathology, St. James's Hospital and Trinity College Dublin, Dublin, Ireland

Jacqueline Fontugne, M.D. Department of Pathology and Laboratory Medicine, Englander Institute for Precision Medicine, Weill Cornell Medical College and New York-Presbyterian, New York, NY, USA

Giuseppe Galletti, M.D., Ph.D. Hematology-Medical Oncology, Department of Medicine, Weill Cornell Medical College, New York, NY, USA

Paraskevi (Evi) Giannakakou, Ph.D. Hematology-Medical Oncology, Department of Medicine, Weill Cornell Medical College, New York, NY, USA

John P. Greene, M.B., B.Ch., B.A.O Department of Histopathology, St. James's Hospital and Trinity College Dublin, Dublin, Ireland

Jiaoti Huang, M.D., Ph.D. Department of Pathology, Duke University School of Medicine, Durham, NC, USA

Michael Ittmann, M.D., Ph.D. Department of Pathology and Immunology, Baylor College of Medicine, Houston, TX, USA

Thomas R. Jackson, B.Sc., M.Sc., Ph.D. Manchester Academic Health Sciences Centre, Faculty of Biology, Medicine and Health, Division of Cancer Sciences, Manchester Cancer Research Centre, University of Manchester, Manchester, UK

Zhenyu Jia, Ph.D. Department of Botany and Plant Sciences, University of Califorania, Riverside, Department of Pathology and Laboratory Medicine, University of California Irvine, Irvine, CA, USA

Heather Johnson, Ph.D. Olympia Diagnostics, Inc., Sunnydale, CA, USA

Institute of Basic Medical Sciences, Beijing, China

Beerinder S. Karir, M.B.B.S. Division of Hematology and Medical Oncology, Department of Medicine, Weill Cornell Medical College, New York Presbyterian Hospital, New York, NY, USA

Francesca Khani, M.D. Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, New York, NY, USA

Robert J. Klein, M.Sc., Ph.D. Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, USA

Samantha E.T. Larkin, M.Sc., Ph.D. Cancer Sciences Unit, Faculty of Medicine, University of Southampton, Southampton, UK

Chung Lee, Ph.D. Department of Botany and Plant Sciences, University of Califorania, Riverside, Department of Pathology and Laboratory Medicine, University of California at Irvine, Irvine, CA, USA

Yanjing Li, B.S. Department of Pathology, Duke University School of Medicine, Durham, NC, USA

Massimo Loda, M.D. Department of Oncologic Pathology, Dana-Farber Cancer Institute, Boston, MA, USA

Department of Pathology, Brigham and Women's Hospital, Boston, MA, USA

The Broad Institute, Boston, MA, USA

Division of Cancer Studies, King's College London, London, England, UK

Tamara L. Lotan, M.D. Johns Hopkins School of Medicine, Baltimore, MA, USA

Michael McClelland, Ph.D. Department of Microbiology and Molecular Genetics, University of California, Irvine, CA, USA

Dan Mercola, M.D., Ph.D. Department of Botany and Plant Sciences, University of Califorania, Riverside, Department of Pathology and Laboratory Medicine, University of California at Irvine, Irvine, CA, USA

Ana M. Molina, M.D. Division of Hematology and Medical Oncology, Department of Medicine, Meyer Cancer Center, New York Presbyterian Hospital, Weill Cornell Medical College, New York, NY, USA

Nigel P. Mongan, B.Sc (Hons), Ph.D., F.R.CPath Faculty of Medicine and Health Sciences, School of Veterinary Medicine and Health Science, University of Nottingham, Nottingham, UK

Department of Pharmacology, Weill Cornell Medicine, New York, NY, USA

Anne Offermann, M.D. Pathology of the University Hospital Schleswig-Holstein, Campus Luebeck and Research Center Borstel, Leibniz Center for Medicine and Biosciences, Luebeck and Borstel, Germany

Padraic O'Malley, M.Sc., M.D., F.R.C.S.C Department of Urology, Queen Elizabeth II Health Sciences Centre, Dalhousie University, Halifax, Nova Scotia, Canada

Marcia Paddock, M.D., Ph.D. Division of Hematology and Medical Oncology, Department of Medicine, Meyer Cancer Center, New York Presbyterian Hospital, Weill Cornell Medical College, New York, NY, USA

Sven Perner, M.D., Ph.D. Pathology of the University Hospital Schleswig-Holstein, Campus Luebeck and Research Center Borstel, Leibniz Center for Medicine and Biosciences, Luebeck and Borstel, Germany

Jenny L. Persson, Ph.D. Department of Translational Medicine, Clinical Research Centre, Lund University, Malmö, Sweden

Department of Molecular Biology, Umeå University, Umeå, Sweden

Berna D. Pollack, M.D. Division of Radiopharmaceutical Sciences and Molecular Imaging Innovations Institute, Department of Radiology, Weill Cornell Medical College of Cornell University, New York, NY, USA

David S. Rickman, Ph.D. Department of Pathology and Laboratory Medicine, Weill Cornell Medicine, New York, NY, USA

Meyer Cancer Center, Weill Cornell Medicine, New York, NY, USA

Englander Institute for Precision Medicine, New York-Presbyterian Hospital, Weill Cornell Medicine, New York, NY, USA

Verena Sailer, M.D. Department of Pathology and Laboratory Medicine, Weill Cornell Medicine, New York, NY, USA

Englander Institute for Precision Medicine, New York-Presbyterian, Weill Cornell Medicine, New York, NY, USA

Andrea Sboner, Ph.D. Department of Pathology and Laboratory Medicine, Institute for Computational Biomedicine, Englander Institute for Precision Medicine, Weill Cornell Medicine, New York Presbyterian Hospital, New York, NY, USA

Clare Stirzaker, B.Sc. (Honours), Ph.D. Epigenetic Deregulation Group, Genomics and Epigenetics Division, Garvan Institute of Medical Research, Sydney, Australia

St. Vincent's Clinical School, UNSW Sydney, Sydney, Australia

Scott T. Tagawa, M.D., M.S. Division of Hematology and Medical Oncology, Department of Medicine, Weill Cornell Medical College, New York Presbyterian Hospital, New York, NY, USA

Peyman Tavassoli, M.D., Ph.D., F.R.C.P.C. BC Cancer Agency Vancouver, University of British Columbia, Vancouver, BC, Canada

Scott A. Tomlins, M.D., Ph.D. Department of Pathology, University of Michigan Medical School, Ann Arbor, MI, USA

Department of Urology, University of Michigan Medical School, Ann Arbor, MI, USA

Michigan Center for Translational Pathology, Ann Arbor, MI, USA

Paul A. Townsend, B.Sc., Ph.D. Manchester Academic Health Sciences Centre, Faculty of Biology, Medicine and Health, Division of Cancer Sciences, Manchester Cancer Research Centre, University of Manchester, Manchester, UK

Fátima Valdés-Mora, B.Sc., Ph.D. Histone Variant Group. Genomics and Epigenetics Division, Garvan Institute of Medical Research, Sydney, Australia

St. Vincent's Clinical School, UNSW Sydney, Sydney, Australia

Shankar Vallabhajosula, Ph.D. Division of Radiopharmaceutical Sciences and Molecular Imaging Innovations Institute, Department of Radiology, Weill Cornell Medical College of Cornell University, New York, NY, USA

Panagiotis J. Vlachostergios, M.D., Ph.D. Division of Hematology and Medical Oncology, Department of Medicine, Meyer Cancer Center, New York Presbyterian Hospital, Weill Cornell Medical College, New York, NY, USA

Joshua I. Warrick, M.D. Department of Pathology, Penn State Milton S. Hershey Medical Center, Hershey, PA, USA

Kefeng Xiao, M.D., Ph.D. Department of Urology, The Second Clinical Medical College of Jinan University (Shenzhen People's Hospital), Shenzhen Urology Minimally Invasive Engineering Center, Shenzhen, China

Giorgia Zadra, Ph.D. Department of Oncologic Pathology, Dana-Farber Cancer Institute, Boston, MA, USA

Department of Pathology, Brigham and Women's Hospital, Boston, MA, USA

Xiaolin Zi, M.D., Ph.D. Department of Urology, University of California Irvine, Orange, CA, USA

Amina Zoubeidi, Ph.D. Department of Urologic Sciences, Vancouver Prostate Centre, Vancouver, BC, Canada

Part I
General Principles

Chapter 1
Precision Medicine in Prostate Cancer: Approach to the Patient

Beerinder S. Karir, Bishoy M. Faltas, and Scott T. Tagawa

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Introduction

The advent of genomic discoveries and decreasing cost of next-generation sequencing (NGS) technologies has ushered in a new era of precision medicine [1]. The early effects of this paradigm shift in oncology are beginning to impact patient care and thus increase the relevance of a discussion on the approach to patients with

B.S. Karir, M.B.B.S. • B.M. Faltas, M.D. • S.T. Tagawa, M.D., M.S. (✉)
Division of Hematology and Medical Oncology, Department of Medicine, Weill Cornell Medical College, New York Presbyterian Hospital, New York, NY, USA
e-mail: stt2007@med.cornell.edu

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*,
Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_1

Table 1.1 Summary: approach to patient

Precision medicine role in prostate cancer
Settings with clinical dilemma
Newly diagnosed disease:
Indolent vs. aggressive disease (importance of gene panels and other biomarkers)
Advanced disease: discovery of driver pathways and targets
Initial patient encounter
Importance of patient and family history of prostate disease
Informed consent detailing:
Patient preferences and data privacy, GINA
Nonactionable alterations and patient expectations
Turnaround time
Tissue for genomic analysis and serial biopsies
Tumor biopsy
Liquid biopsy (generally whole blood)
Organoid cultures (currently from tissue biopsies)
Understanding precision medicine reports
Communicating genomic results
Clinicians—precision medicine report and tumor board
Patients—role of genetic counselors
Issue of incidental germline mutations
May be a deterrent for some patients to get genomic sequencing done
Future directions
Annotating genomic with clinical data
PC biomarkers
Realistic expectations

prostate cancer (PC). Bringing the application of precision medicine to the clinic raises new challenges related to informed consent prior to testing, effectively communicating the results of cancer genomic testing to the patient, understanding and managing the patient's expectations, and working with the patient to select the best treatment options based on genomic tests (or not) [2, 3]. This introductory chapter will cover the approach to men with PC undergoing genomic testing of their tumors (see Table 1.1 for summary).

Precision Medicine in Prostate Cancer

Prostate cancer (PC) is the most common non-skin cancer and the second leading cause of cancer death in men in the United States [4]. This disease is on the forefront of precision medicine with multiple opportunities for benefiting from translational genomics [5]. While any given man might benefit from individualized tumor testing, two very important clinical subsets within PC that might benefit most from additional molecular analysis are patients with clinically localized "indolent" disease that are probably best served without treatment and those with more aggressive, particularly advanced disease with no curative therapy.

Newly Diagnosed Prostate Cancer

Every year more than 1,000,000 men in the United States undergo prostatic biopsies based on elevated levels of prostate-specific antigen (PSA). Among the newly diagnosed PC patients, many patients have clinically diagnosed (presumed) low-volume and low- to intermediate-risk Gleason scores (3 + 3 or 3 + 4 in a small percent of cores). This subset of PC patients presents a clinical dilemma for patients and their treating physicians. Various non-genomic biomarkers like Prostate Health Index (PHI) have been shown to better identify patients with aggressive disease, but there is still an unmet need for better biomarkers [6]. Genomic biomarkers including gene panels like the Decipher genomic classifier and Oncotype DX have demonstrated the ability to better stratify PC patients [7]. After various positive validating studies, Oncotype DX has recently been included under Medicare coverage thereby making such PC genomic-based diagnostics reimbursable [8]. In addition to gene panels, various other genomic biomarkers like urine TMPRSS2-ERG fusion transcripts and long noncoding RNAs(lncRNAs) may show promise in identifying PC patients who may need aggressive treatments [9, 10].

Advanced Prostate Cancer

On the opposite end of the spectrum, advanced PC represents another disease state that could benefit from precision medicine approaches. Prostate tumors may remain responsive to androgen deprivation therapy for years (variable among patients) until it evolves into the castration-resistant state (CRPC), which generally is still driven by the AR pathway. The median overall survival after diagnosis of CRPC is 18–32 months. Although newer-generation hormonal, cytotoxic, immunotherapeutic, and bone-targeted drugs have increased survival in CRPC patients leading to their FDA approval, the development of resistant PC disease remains inevitable. Using a combination of improved biopsy techniques and NGS technologies, molecular characterization of such advanced prostate tumors is increasingly being done. Recently, one such multi-institutional study found that 90% of advanced PC tumor harbor molecular alteration with potential targeting agent/drugs [11]. Another study that included mostly prostate cancer patients also demonstrated targetable alterations with an in-depth analysis of an “exceptional responder” based upon this mutation [12]. This highlights the importance of precision medicine for subclassification of prostate disease into molecularly defined subgroups with each subtype amenable to different targeted therapies [13].

Initial Clinical Encounter

The initial patient visit to a physician’s office generally includes comprehensive elicitation of disease history including diagnosis and initial treatments. Successful integration of precision medicine into oncology clinic will further emphasize

clinical data recording and sharing. Without accurate linkage of genomic data to clinical data, even the latest genomic technologies will have a limited impact on patient outcomes [14].

Family History in Prostate Disease

Within PC patients' histories, ethnicity is relevant as PC may sometimes harbor genetic determinants. Various single nucleotide polymorphism (SNPs) and copy number variants (CNVs) have been shown to be determinants of familial risk of prostate cancer [15]. Additionally, it is being increasingly realized that germline alterations like BRCA2, ATM, and BRCA1 mutations also play an important role in PC pathogenesis [11, 16]. So, any family history of such gene abnormalities can warrant increased level of diagnostic and therapeutic interventions.

Informed Consent

Precision medicine informed consents are important part of this new paradigm. Informed consents must delineate all the details including risks and also elaborate upon likelihood of finding somatic molecular alterations of unknown significance as well as incidental germline mutations. Pretest counseling should focus on addressing the key components of informed consent.

Patient Preferences and Data Privacy

One of the most important parts of the consent is the preferences of patients and families regarding level of detail and the scope of genetic information resulting from molecular testing, especially regarding incidental findings. This issue is discussed in detail in a separate section. Risks due to testing procedures (i.e., biopsy procedures) as well as data privacy should also be clearly detailed. Patients should also be made aware of existing legal protection against discrimination and the provisions of the Genetic Information Nondiscrimination Act (GINA). GINA protects US citizens from discrimination and restricts insurers from limiting coverage/altering premiums based on such genetic information. It prevents insurers from requiring policyholders to undergo genetic testing but could make testing a requirement for treatment [17].

Actionability of Precision Medicine Results

Due to enormous media attention generated by the precision medicine initiative started by President Obama [18], patients have high expectations from genomic profiling and its implications especially in terms of cancer cure [19]. These

expectations, especially as they relate to the "actionability" of results, should be addressed up front within the framework of the consent process. The possibilities of nonactionable genomic results, biopsy failure (poor tissue quality/tumor content), analytical validity issues, and turnaround time should be discussed with the patient during this process [20]. Realistic expectations set through early patient education lead to better patient compliance and satisfaction. When managed appropriately, the potential for personal benefit from targeted therapy raises hopes and drives enhanced participation of patients in clinical trials [3].

It is important to understand that the definition of "actionable" molecular alterations is dependent on several molecular, patient-specific, and practical factors. A recent survey of practicing oncologists who had just received their patients' cancer genome sequencing reports showed that 78% did not expect to implement any changes to the current treatment plan [21]. In this study, barriers to "actionability" included lack of local clinical trials (41%), absence of actionable mutation (33%), and good response to ongoing treatment (16%). In light of these findings, physicians need to explain to patients all the factors that could limit actionability of precision medicine test results.

Turnaround Time

Patient's expectations about the turnaround time for genomic profile results also need to be recalibrated. Presently, waiting time ranging from weeks to months is needed starting with acquisition of tumor sample plus germline sample to generation of a precision medicine report. This is acceptable to stable patients and their treating physicians, but for patients with progressive advanced cancer, such long waiting time may not be clinically useful. Though the latest NGS methodologies have significantly shortened turnaround times, bottlenecks in the process still remain. These include sample acquisition and logistics and data analysis and interpretation [20].

Tissue for Genomic Profiling and Need for Serial Biopsies

Successful application of precision medicine requires availability of tissue of origin and/or metastatic site [22]. In many cases with a distant history of prostatectomy, tissue acquisition is not feasible thus leaving only the option of metastatic site biopsy. As most common site for PC metastasis is the bone which is a difficult organ to biopsy, metastatic site biopsies in PC have been very daunting process until lately. However, advancements in biopsy technology have increased the chances of successful tissue procurement from a PC patient [11, 23].

Over time, the true success of precision medicine may hinge on our ability to get serial biopsies to see real-time genomic evolution of the prostate disease. Liquid biopsy technologies such as circulating tumor cells (CTCs) or cell-free DNA (cfDNA) are a good surrogate for tissue biopsies [24]. Though true utility of liquid

biopsies needs further validation [25], application of liquid biopsies seems immense extending to CTC-derived xenografts [26]. Another useful application of CTCs for prostate cancer patients may be in generation of patient-derived organoid cultures. Such cultures have shown to recapitulate the entire molecular diversity of prostate disease and hold promise for use in genetic and pharmacologic studies [27].

Understanding "Precision Medicine Reports"

All procedures for precision medicine outside of a research setting should be performed in CLIA-certified labs. After running the tissue sample through sequencing pipelines, genomic data is streamed through analytical/bioinformatic pipeline. The entire process needs to be standardized throughout for validity. Eventually a precision medicine report on patient's tumor-specific somatic alterations is generated and usually contains the following elements:

- Somatic alterations in clinically relevant genes—these alterations occur in genes that are potentially actionable as drug targets or confer resistance or susceptibility to treatment.
- Somatic alterations of unknown significance in known cancer genes—these alterations occur in genes that are cancer associated, but their impact on the disease is not fully understood.
- Somatic alterations of unknown significance—these alterations are not known to have any effect on the disease but are profiled in the event that, in future, progress in scientific knowledge could determine their role.

In addition to these, details on quality control metrics like depth and coverage of sequencing are often provided [12].

Communicating Genomic Information to Clinicians and Patients

A recent study done at Duke Medical Centre has found a number of challenges faced by institution when implementing genomic testing into patient care [28]. This necessitates a policy and education program to improve clinician support, enabling them to effectively deliver precision medicine care. One such problem is that precision medicine reports may or may not yield actionable somatic alterations in cancer-related genes. If such molecular alterations are found, these can either be targeted by FDA-approved drug for PC or other cancer types (i.e., "off-label" use), or there may be approved or investigational drug available as a clinical trial. Such drugs/trials are often enlisted in precision medicine reports made available to treating physician. But procuring the drugs targeting actionable genomic alteration can be a big hurdle. This can be especially problematic if PC is an off-label use of the drug [29].

For effectively communicating genomic results to patients, the treating physician needs to ensure proper patient education (starting with pretest counseling) and decision support systems are in place. This may require strong collaboration among genetic counselors, physicians, and nurses. Realistic expectations set during informed consent education can be especially helpful if sequencing results yield no obvious actionable alterations. In the case of incidental germline finding, the role of genetic counselors is very important to facilitate family communication [30] (see next section). To conclude, the proper utilization of cancer genomic medicine needs to be accompanied by careful thought about how the genetic test results will be communicated to patients in order to maximize their benefit.

Unique Issues Related to Incidental Discovery of Germline Mutations

Genome sequencing provides unprecedented opportunities to study the genomic landscapes and identify the actionable driver mutations for targeted therapy in precision medicine clinics. Because some NGS approaches rely on comparison between germline and somatic variants, germline alterations may be incidentally discovered. These alterations may be associated with inherited health risk or familial susceptibility to cancer. In the setting of cancer, some patients may find it burdensome to bear the knowledge of such inherited health risk in family [31]. This may have psychological consequences associated with the guilt of passing the inherited risk or increased cost of health care. This knowledge is perceived as an obligation to family and is difficult to refuse. Providing patients with simple summaries to share with their families and making local genetic counseling resources available at point of contact for the family can be helpful during the process [32]. Implications of reporting such incidental discoveries of germline mutations are very complex. Discussing these issues during the informed consent for sequencing highly penetrant disease genes and genetic counseling is essential to address the challenges faced in this situation [33]. Overall, the likelihood of finding incidental genetic variants does not appear to significantly discourage patients from adopting genomic profiling though the extent of incidental findings patients wish to be disclosed varies significantly [34].

Future Direction

Successful application of precision medicine approaches in the routine clinical care of patients requires not only a wider availability of next-generation sequencing technologies but also resourceful databases possessing consolidated clinical information [14]. As we proceed ahead, the missing metrics of clinical data will need to be “filled in” and clinical information annotated with genomic data. Another issue

will be to improve our ability to complete the entire process of genomic sequencing, generating reports, and matching/administering drugs targeting driver alterations within a rapid turnaround time. This may further require sophisticated rapid machine learning methods [35]. In addition to therapeutic benefits, the promise of precision medicine in prostate cancer will also lie in discovering and validating molecular biomarkers that distinguish aggressive from indolent disease and those that predict treatment resistance [36, 37]. Finally regarding our heightened expectations, we will need to be cautious in terms of seeking quick results through this new paradigm of precision medicine. As Amara's law correctly states "We tend to overestimate the effect of a technology in the short run and underestimate the effect in the long run." We may have outclassed Moore's law for NGS method cost efficacy, but regarding patient outcomes, it may take few years before we realize the full potential of precision medicine for prostate cancer patients.

Resources for Patients and Clinicians

- My Cancer Genome—http://www.mycancergenome.org/: This is a personalized cancer medicine knowledge resource for physicians, patients, caregivers, and researchers. It provides latest information on what mutations make cancers grow and related therapeutic implications, including available clinical trials.
- cBioPortal—http://www.cbioportal.org/: This portal maintained by Memorial Sloan Kettering Cancer Center stores genomic data from large-scale, integrated cancer genomic data sets. It allows explorative genomic data analysis
- COSMIC database—http://cancer.sanger.ac.uk/cosmic: COSMIC is a freely available online database of somatically acquired mutations found in human cancer. It is maintained by Sanger Institute, UK.
- National Cancer Institute Cancer Genetics Services Directory—http://www.cancer.gov/about-cancer/causes-prevention/genetics/directory: This NCI directory lists professionals who provide services related to cancer genetics (cancer risk assessment, genetic counseling, genetic susceptibility testing, and others).
- National Society of Genetic Counselors (NSGC)—http://nsgc.org/p/cm/ld/fid=164: For finding genetic counselors in a local area, United States.

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Chapter 2
Epidemiology and Etiology

Padraic O'Malley

Contents

Introduction: Incidence and Mortality

In 2016, in the USA, approximately 180,890 men are expected to be diagnosed with prostate cancer, and almost 26,120 men are expected to lose their life to prostate cancer [1]. It is the number one non-cutaneous, solid tumor in men and the second most common cause of cancer death in the USA among men [2]. The estimated number of new cases worldwide in 2012 was 1,112,000, making it the second most common cancer diagnosed in men [3]. 759,000 of these cases are estimated to be seen in developed countries and only 353,000 in developing countries [3]. The estimated cases of prostate cancer deaths were 307,500 worldwide, with 142,000 in developed countries and 165,500 in developing countries [3]. Cumulative lifetime risk for prostate cancer incidence varied markedly between developed countries at 8.8% versus only 1.7% in developing countries. However, lifetime mortality risks were less disparate at 0.8% and 0.6%, respectively [3]. The incidence of prostate cancer underwent a dramatic increase in the USA in the early 1990s (Fig. 2.1) [2] with the widespread introduction of transurethral prostatectomy and then the

P. O'Malley, M.Sc., M.D., F.R.C.S.C.
Department of Urology, Queen Elizabeth II Health Sciences Centre, Dalhousie University, Halifax, Nova Scotia, Canada
e-mail: pomalley@dal.ca

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*,
Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_2

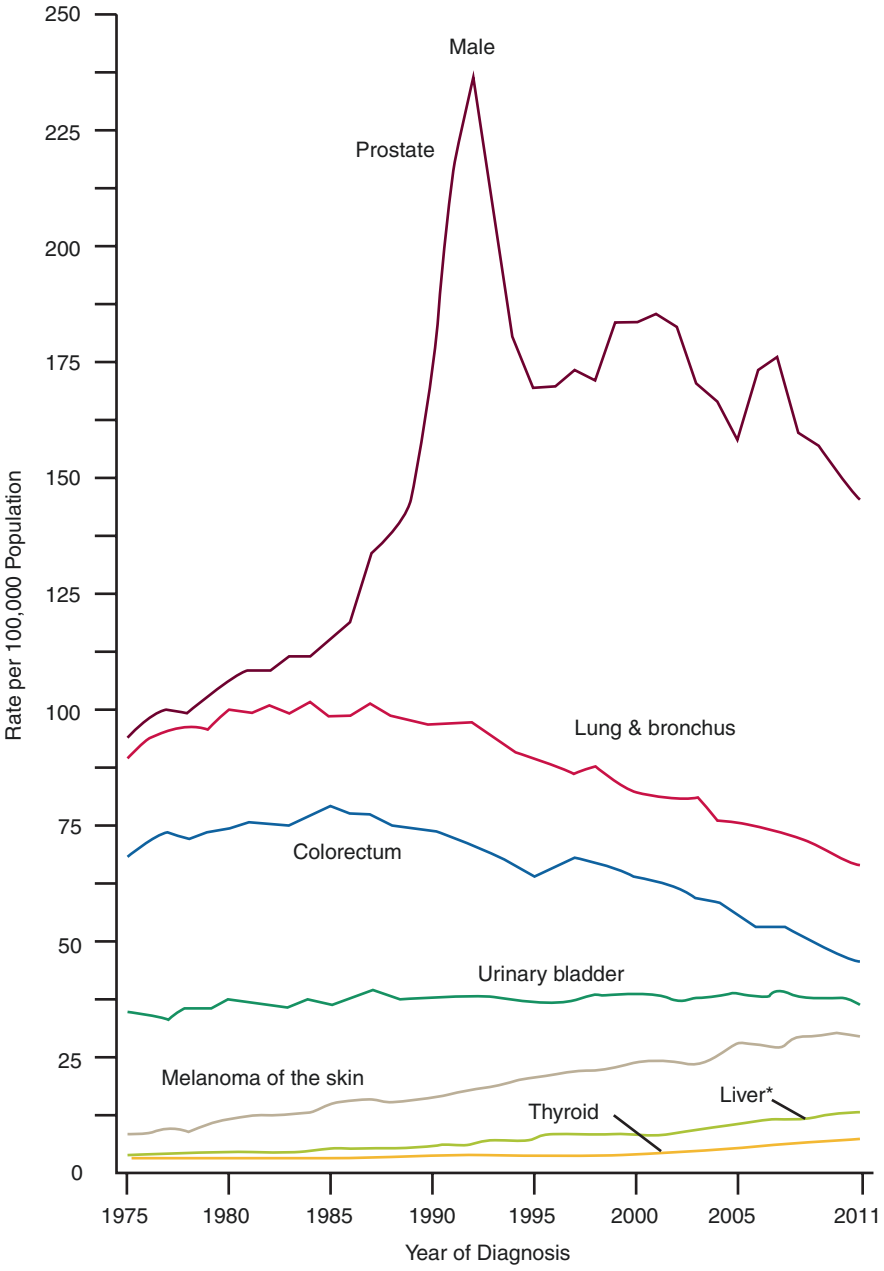

Fig. 2.1 Trends in incidence rates for selected cancers in men, USA, 1975–2011. Rates are age adjusted to the 2000 US standard population and adjusted for delays in reporting. *Asterisk* includes intrahepatic bile duct

prostate-specific antigen (PSA) test, leading to an almost threefold increase in incidence in 1975 to its peak in 1993 [2, 4, 5].

This dramatic rise was not seen in other high-income countries with less widespread adoption of PSA testing, such as those in Western Europe, and these countries demonstrated a gradual increase in incidence instead [6]. As such, due to screening and PSA utilization, as well as no doubt due to the variable risk of disease in certain population, there are marked variations, as much as 25-fold, in incidence and mortality globally (Fig. 2.2) [3, 6].

Fortunately, we have also seen a decline in mortality rates, particularly in developed countries. Beginning in 1996, the mortality rates were seen to decline after the introduction of PSA testing and continued to fall, perhaps due to this testing and/or due to improved treatment strategies, most likely in the metastatic setting [7, 8]. The effect of radical treatment on localized disease is less clear as to its overall benefit given the conflicting results of the Prostate Cancer Intervention Versus Observation Trial (PIVOT) and the Scandinavian Prostate Cancer Group 4 randomized trial [9, 10]. In the metastatic setting, however, we have seen the advent of many new therapeutics in the last 25 years including docetaxel and most recently abiraterone and enzalutamide, as well as combination therapy as seen in the recent CHAARTED and STAMPEDE studies and the benefits in overall survival these treatments have offered [11–13].

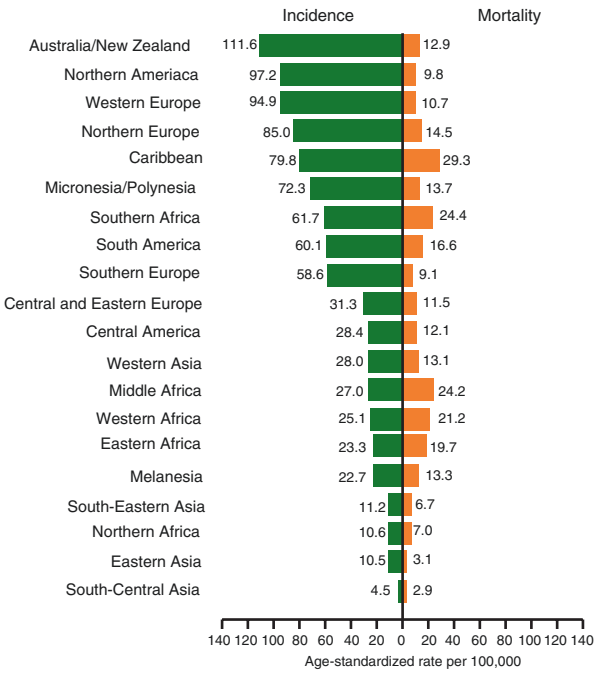

Fig. 2.2 Prostate cancer incidence and mortality rates by world area. (Reproduced from Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin. 2015 Feb 4;65(2):87–108)

Etiology and Risk Factors

Over the last few decades, we have gained an increasing understanding of prostate cancer. Although we do not know the exact etiology of prostate cancer, we have identified a number of risk factors through epidemiological studies that may provide insight into possible mechanisms that would account for it. These factors can be divided into two distinct groups as seen in Table 2.1.

To begin let us examine each category's individual risk factors and the epidemiological evidence behind their possible role in prostate cancer:

Modifiable

Age

Although several guidelines suggest screening men as young as 40 if they have high-risk features, prostate cancer is a disease of older men. Prostate cancer does have the steepest age-incidence curve of all malignancies, predominantly in the seventh decade [14–17]. The incidence of prostate cancer in men 45–54 years of age has remained fairly stable at approximately 35.7 per 100,000 in 2009–2011 [18]. There is a moderate increase in the following decade to 236 per 100,000. However, the subsequent two decades, 65–74 and 75–84, are both significantly higher at 609 and 769 per 100,000, respectively, in the modern era (Fig. 2.3). In North America, SEER data suggests approximately 0.6% of prostate cancer diagnosis are made before age 45, a further 9.7% before age 55, and 86% between the age of 55–84 years [19]. So while there has been an increase in younger men being diagnosed with prostate cancer, the lion's share of new cases is still in the seventh and eight decades of life. The role of aging in causing prostate cancer is most likely through similar mechanisms common to many malignancies. We know that the process of aging itself leads to a myriad of changes in the genome including telomere shortening, epigenetic changes including methylation and demethylation, senescence, and alterations in gene expression. Why prostate cancer is so sensitive to these influences has not yet been elucidated.

Table 2.1 Risk factors for prostate cancer by category

Modifiable		Non-modifiable
Metabolic	Obesity	Age
	Exercise	Family history and genetic changes (acquired and inherited)
	Diet	Race and ethnicity
	Diabetes	
Smoking		

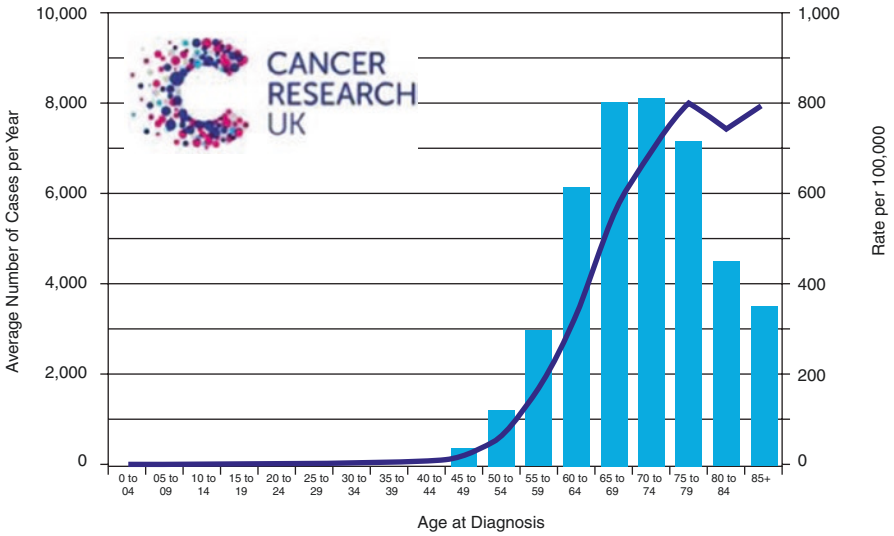

Fig. 2.3 Prostate cancer incidence rates per 100,000 men by age in the UK, 2009–2011. Data from the Cancer Research UK—UK National Statistics Office [18]. http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/prostate-cancer/incidence#heading-One

Twin studies in monozygotic twins have demonstrated epigenetic changes occur with aging and that these are a result of both environmental changes and stochastic processes as well [20]. In addition, the multifocal nature of prostate cancer demonstrates the field effect that arises from accumulation of these changes [21]. This field effect has been termed by Damaschke et al. as Age-Related Epigenetic Alterations inducing Susceptibility (AREAS) as it is related more to the field effect generated by alterations associated with age rather than alterations associated with the presence of a neoplastic nidus [22]. Specific epigenetic changes will be discussed in subsequent chapters.

Family History and Genetic Alterations (Inherited and Acquired)

Suffice it to say that there is an abundance of epidemiological evidence to support both familial and genetic components to the development of prostate cancer. As early as the mid-twentieth century, familial clustering was noted and demonstrated an increased risk for the development of prostate cancer for relatives of men with prostate cancer [23]. In more contemporary studies, this risk increases with a greater number of relatives affected, the closer the degree of relation, and younger age of the relatives at diagnosis [24]. Early age of development of aggressive prostate cancer belies a genetically driven phenotype [25]. A number of prostate cancer susceptibility genes have been identified by looking at familial prostate cancers specifically.

These and other genetic alterations both inherited and acquired will be discussed in much greater detail in this book.

The technological advancements in the last decade have also allowed us to begin performing genome-wide association studies (GWAS) to identify correlation between disease and common variants in the genome [26]. Most genetic variants discovered have been modest in their effect size, most likely because GWAS have been performed in sporadic cases. The utility of GWAS may have greater implications and utilizations in family cases or specific racial groups [27]. Replications of previously identified genetic variants were highest in African American men followed by among men with a family history. The large majority of these variants are found in chromosome 8q24 [28]. Family linkage studies have further identified variants such as HOXB13 that are associated with hereditary prostate cancer [29]. Furthermore, BRCA mutations have been clearly seen to increase the risk for developing prostate cancer and more aggressive disease [30, 31]. BRCA1 mutation carriers have roughly a 3.5-fold increased risk, while men with BRCA2 have an 8.6-fold increased risk in men younger than 65 years of age [32, 33]. Moving forward we will hope to be able to identify further genetic variants that are common to the pathway that leads to progression to cancer from a preneoplastic, benign state. Furthermore, we would like to be able to do so on a more individualized level utilizing the knowledge previously gained from the study of specific high-risk groups.

Race and Ethnicity

Along with family history, we also inherit our "forbearer" ethnic and racial identity. Clearly, certain populations have a much higher risk not only of developing but also a risk of dying from prostate cancer [6]. At particular risk are men of African American and Caribbean descent. These men have been shown to present with more aggressive disease [34] and suffer the highest prostate cancer-related death rates among all ethnic groups [35]. Clearly there are other key factors besides racial and ethnic biological variability which contribute to these outcomes including factors such as disparities in access to appropriate health care [36] and greater prevalence in this population of anterior tumors that are prone to under-sampling [37]. Important to note is that there is limited evidence to suggest black men in the USA of either African or Caribbean have any significant variability from one another in terms of prevalence of significant disease (see Table 2.2) [38–40]. In comparison to previously published studies from West Africa, the rates were similar in US-born men of West African background [38, 41–43]. This raises two important points. First, black men inherently have more biologically aggressive disease regardless of their specific ancestry. Second, the demonstration that rates of advanced disease at presentation are being essentially equivalent between a screened and an unscreened population suggests a shorter lead time in black men [38, 44].

Conversely men of Asian background have a relatively lower risk of developing prostate cancer as well as improved prostate cancer-specific and overall survival in several clinical settings [45, 46]. However, this has yet to be consolidated with several

Table 2.2 Rates of advanced[a] prostate cancer among Black racial groups of varying geography and ancestry

Study	Jamaican born and residing	Jamaican born US residing	West African born and residing	West African born US residing
Fedewa et al. [38]	—	61.1	—	58.26
Coard et al. [39]	62.1	—		
Kampel et al. [40]	—	60		
Yarney et al. [41]			56	—
Kabore et al. [42]			54.7	—
Obiorah CC and Nwosu SO [43]			58	—

[a]Defined as Gleason >7

studies that have shown worse clinical outcomes in US Asian men [47] and the higher prevalence of higher-grade disease on autopsy in Asian men [48]. Nonetheless, consideration of a patient's race and its implications on their biology are important and may become of greater importance as studies are beginning to now look at novel biomarkers within racial subgroups in the current age of personalized medicine.

Modifiable

We move on now to the modifiable drivers of prostate cancer biology, those which we can potentially manipulate and perhaps improve prognosis with. Let us begin first by examining those that center around our body's metabolism, primarily: obesity, exercise, diet, and metabolic syndrome.

Obesity, Exercise, Diet, and Diabetes (Metabolic)

Body mass index (BMI), the old standard for gauging obesity, has been correlated with colon and breast cancer risk in middle- and older-age men [49]. It was suggested as a putative risk factor for prostate cancer. Wynder proposed a role for "overnutrition" in the development of prostate cancer in 1976 [50]. In 1984, the prospectively conducted Seventh-day Adventist study by Snowdon et al. identified a higher rate of fatal prostate cancer in men with body weights greater than 130% of ideal body weight [51]. Several important studies in the last few decades have demonstrated the clear association between obesity and increased risk and death from prostate cancer [52–54]. Furthermore, there is clear evidence demonstrating that obesity is also associated with progression of low-risk cancer in men on active surveillance [55]. Although the risk of obesity may be modest, it has been shown to be consistent [54, 56, 57]. Further confounding the issue is the lower PSA as a result of obesity may lead to a detection bias when PSA is the main driving force behind biopsy utilization [58–60]. Unlike inheritable or acquired genetic alterations, obesity

more than likely drives prostate cancer risk and development via a hormonal mechanism. However, the end result on a molecular level results in a perturbation of genomic expression which leads to events such as increase tumor proliferation, reduced apoptosis, and a transition to a castrate resistant state [61]. So, if obesity promotes disease development and impacts prognosis, what can we do to alter this?

Several studies have looked at the role of exercise and diet in reducing prostate cancer incidence and prognosis [62]. Three large prospective population studies have demonstrated a decreased risk of aggressive prostate cancer [63–65]. The underlying mechanistic effects of exercise have not been examined in great detail. Of course, it is difficult to tease out the effects of exercise from those independent of its role in reducing obesity. There is some suggestion that there may be a role of altered vascular permeability and at least temporal resolution of hypoxia in the tumor microenvironment as seen in orthotopic animal models [66]. Other effects may be more endocrine related such as affecting adipokines and the insulin-like growth factor axis [67].

Many studies have examined the role of dietary components on prostate cancer susceptibility. Intake of red meat [68–71], green tea [72, 73], dairy products [74, 75], eggs [70, 76], selenium [77, 78], etc. has all been examined. However, results have invariably been inconsistent among the majority of studies. Debate has existed, for instance, whether it is the high levels of dietary branched fatty acids and the upregulation of α[alpha]-methyl-CoA racemase (AMACR) or whether the heterocyclic amines produced from cooking at high temperatures is the driving factor behind the association of red meat with prostate cancer [79, 80]. Meanwhile, soy products have been associated with a decreased risk owing to their phytoestrogens, either due to altering the level of circulating androgens, effects on the estrogen receptor directly, or apoptotic effects [81–83]. Micronutrients have also been examined extensively, in particular selenium and vitamin E. However, the largest randomized trial demonstrated initially no significant difference in prostate cancer development with the use of selenium, vitamin E, both, or placebo [84]. Furthermore, it was subsequently found that vitamin E was associated with an increased risk [85]. The idea of nutritional and metabolic effects and prevention strategies are not dead; however, as researchers have turned to metabolomics and the role of diet affecting these, in particular, much attention has been given to diabetic medications, especially metformin. There are currently a number of trials assessing the role of metformin in both prostate cancer prevention and prostate cancer progression in the active surveillance setting. Perhaps a better understanding of the role of the human body's metabolism in prostate cancer will allow us to return to dietary and lifestyle modifications which may impact significantly on the disease.

Smoking

Smoking is a well-described risk factor for a number of malignancies including primary lung and urothelial carcinoma. Its association with prostate cancer has been under increasing scrutiny in the last decade [86]. A meta-analysis by

Huncharek et al., published in 2010, examined the risk of prostate cancer from studies performed up to and including 2007 [87]. This meta-analysis demonstrated a 14% increased risk of prostate cancer death associated with current smoking and as high as 24–30% in those who were heavier smokers [87]. A more recent meta-analysis by Islami, Moreira, Boffetta, and Freedland examined prostate cancer mortality, incidence, and population attributable risk (PAR) [86]. Meta-regression analysis showed no association between smoking and prostate cancer risk ($p = 0.09$) and if anything perhaps a trend toward an inverse relationship. However, current (RR 1.24, 95% CI 1.18–1.31) and ever having smoked (RR 1.18, 95% CI 1.11–1.24) both showed a significant correlation with prostate cancer mortality. Furthermore, the total number of prostate cancer deaths attributable to cigarette smoking in the USA and Europe was approximately 10,400 deaths per year. In addition to higher prostate cancer mortality, smoking has been seen to be associated with more advanced disease at the time of surgery and subsequently higher risk of recurrence, metastasis, and death [88–92].

The possible pathways by which smoking may lead to worse outcomes in prostate cancer may be due to CpG hypermethylation of several genes which in turn leads to tumor angiogenesis [93, 94]. Other putative mechanisms include increased heme oxygenase expression (HO-1), altered adhesion molecules and extracellular matrix, and smoking-induced inflammation [95–98]. Indeed smokers have a greater degree of inflammatory changes in the prostate than non-smokers [98], and inflammation may well have a role in prostate cancer progression and/or initiation [99].

Conclusions

Clearly there are a number of risk factors, both non-modifiable and modifiable, which have significant impact on a man's risk of developing prostate cancer and risk of dying from prostate cancer. However, we have yet to derive a single genomic pathway which drives prostate cancer similar to the VHL gene in renal cell carcinoma. Furthermore, the exact mechanisms through which these risk factors impose an increased risk are relatively unknown, and most proposals of mechanisms of action are somewhat speculative. Perhaps this explains then to some degree the amazing degree of clinical heterogeneity we see in patients. Examination of genetic and genomic events in the modern era, however, may allow us to reverse engineer/discover what those mechanisms may be. The high-throughput processing and immense degree of information gained from molecular studies are rapidly opening up new areas of discovery and thought into prostate cancer biology. By understanding this biology better, we may hopefully treat these patients more efficiently and one day prevent these cancers from having the significant impact they have now on men's health and lives. Once we can understand the biology, we can truly influence the modifiable risk factor component of the equation.

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Chapter 3
High-Grade Prostatic Intraepithelial Neoplasia

Fadi Brimo

Contents

Definition, Incidence and Evidence of Preneoplastic Process

Prostatic intraepithelial neoplasia [1] is defined as a noninvasive neoplastic transformation of the lining epithelium of prostatic ducts and acini [2–6]. It is the only well-established preinvasive lesion of prostatic adenocarcinoma Prostate cancer (Pca). Although PIN was originally divided into three grades (I, II and III), the poor reproducibility and lack of clinical significance of a diagnosis of PIN I have resulted in the virtual disappearance of this diagnosis from contemporary pathology reporting [7, 8]. Currently, the term PIN is used as a synonym for high-grade PIN (HGPIN) which includes PIN II and III.

HGPIN is characterized by glands of medium to large size with an intact or fragmented basal cell layer in which a neoplastic cellular proliferation replaces the secretory epithelium. The neoplastic cells have basophilic cytoplasm, enlarged nuclei and prominent nucleoli at 200× magnification. Four main architectural patterns have been described: flat, tufting, micropapillary and cribriform. Other unusual patterns include the signet ring, small cell, mucinous, foamy and inverted patterns [4].

F. Brimo, M.D., F.R.C.P.(C)
Department of Pathology, McGill University Health Center, Montreal, QC, Canada
e-mail: fadi.brimo@mcgill.ca

© Springer International Publishing AG 2018

B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*,
Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_3

The incidence of a diagnosis of HGPIN varies significantly in the literature and ranges from 0.7 to 20% in needle biopsies and 3 to 33% in transurethral resection specimens [5, 9]. Isolated HGPIN is reported in 5–10% of needle biopsies with a mean incidence of 5.2%. Such variations are due to difference in the studied population and the inconsistent application of diagnostic criteria [4].

There is spatial, epidemiological, morphological and molecular evidence suggesting HGPIN to be the precursor of Pca. As an example, autopsy series report the incidence and extent of HGPIN to increase with age and to predate the onset of carcinoma by more than 5 years [10, 11]. The severity, multicentricity and frequency of HGPIN in prostates with cancer are also greatly increased compared with that of prostates without cancer [4, 11]. Also, similar to Pca, HGPIN preferentially involves the peripheral rather than the transition zone and occurs at a higher prevalence in African Americans compared to other races, with the lowest incidence in Asian population [10–13]. The finding of foci of HGPIN from which budding-off of rare invasive carcinoma glands occurs is further histological evidence that HGPIN is a true precursor of cancer. Such foci, referred to in the literature as HGPIN with microinvasive carcinoma, are present in 2% of high-power microscopic field of PIN and are seen in equal frequency in all architectural patterns [4, 5]. Further evidence suggesting a strong relation between HGPIN and adenocarcinoma is the fact that both lesions share similar molecular anomalies. Those molecular features are highlighted below.

Molecular Features of HGPIN

Telomere Shortening

It has been shown that telomere shortening is an early event in prostatic neoplasia that occurs frequently in HGPIN and Pca [14]. Using FISH technique applied to 6 prostatectomies with 11 HGPIN lesions and 20 needle biopsies with HGPIN without cancer, Meeker et al. [15] confirmed the presence of a significant telomere shortening in 93% of the HGPIN foci in comparison to the normal adjacent glands, those rates being similar to what has been reported in Pca. In another study by Joshua et al. [16], 68 biopsies with isolated HGPIN with histological follow-up were analysed for telomere attrition. In that study telomere shortening in the HGPIN glands and the surrounding stroma showed strong association with a subsequent diagnosis of cancer, indicating that telomere shortening is an early genomic event in the development of Pca that could affect stromal and glandular cells.

Cytogenetic Alterations

DNA aneuploidy is common to both HGPIN and Pca. In a study including 28 radical prostatectomies with Pca and associated HGPIN, DNA aneuploidy was found in 65% of HGPIN and 62% of Pca foci [17]. In that study, there was a high

correlation (75%) in ploidy and cytogenetic alterations between HGPIN and paired Pca foci with the most frequent chromosomal alteration in HGPIN being trisomy 7 (45%), followed by trisomy 8 (30%) and monosomy 8 (20%) [17]. Bostwick et al. [18] also examined allelic imbalance at 6 polymorphic microsatellite markers (on chromosomes 7q, 8p, 8q, 18q) using PCR in 84 foci of HGPIN and 95 foci of Pca from 52 completely embedded whole-mount prostates. Although the rate of allelic imbalance was higher in Pca in comparison to HGPIN (52% vs. 19%), 95% of cases showed a similar pattern of allelic imbalance of at least one markers in the matched HGPIN and Pca foci studied. A similar study evaluating 68 foci of HGPIN and 78 foci of Pca using FISH showed that chromosomal anomalies were present in 50% and 51% of HGPIN and Pca, respectively, but that the mean number of abnormal chromosomes per focus was 0.66 for HGPIN and 1.09 in Pca. The most frequent anomaly in HGPIN was a gain of chromosome 8, followed by gain of chromosome 10, 7, 12 and Y, in comparison to Pca in which the most frequent anomaly was gain of chromosome 7 and 8 [19]. Similar observations were made by Jenkins et al. [20] who reported the gain of chromosome 8 to be frequently present in HGPIN and Pca, with the latter containing more FISH anomalies than paired HGPIN foci. These results along with others' indicate that similar chromosomal anomalies are shared between HGPIN and adjacent Pca foci, although the mean overall number of alteration is higher in carcinomas. Recently, a genetic pathway for prostate carcinogenesis has been proposed by Ribiero et al. [21] who analysed the genetic profile of 51 clinically confined Pca using comparative genomic hybridization and subsequently proposed two distinct initiating events, namely, 8p and 13q losses. In that model, HGPIN does not always precede the development of Pca; while loss of 8p leads to the development of HGPIN followed by carcinoma, loss of 13q leads to Pca in the absence of HGPIN.

Overexpression of p16

Similar to Pca, HGPIN overexpresses p16INK4A, a cyclin-dependant kinase inhibitor. In one study in which HGPIN p16 immunoexpression was evaluated in 154 radical prostatectomies, overexpression of p16 was an independent predictor of disease relapse and increased risk of recurrence [22].

c-myc Amplification and Caveolin-1 Overexpression

While intermediate increase in c-myc copy number was reported to be present in similar frequencies in HGPIN and Pca (8% and 11%, respectively), substantial amplification of c-myc was not detected in HGPIN [19]. A second group reported that cribriform HGPIN harboured extra-copies of the c-myc genes in 52% of cases in comparison to 44% for Pca [20].

Similarly, overexpression of caveolin-1 which is known to be a common event in Pca was found to be present in 42% of HGPIN in 1 study including 36 radical prostatectomies [23]. In that study, positive correlation existed between caveolin-1 and c-myc in HGPIN. Furthermore, using transgenic mice with c-myc overexpression, caveolin-1 overexpression was demonstrated in mice HGPIN and prostate cancer cells and was associated with a significantly higher ratio of proliferative to apoptotic labelling in HGPIN lesions than in caveolin-1-negative HGPIN, suggesting that caveolin-1 is associated with c-myc in the development of HGPIN and Pca [23].

Dysregulation of Annexin

Several members of the annexin family show altered expression in HGPIN and Pca in comparison to benign prostate glands. In 1 study including 45 Pca with paired HGPIN foci and 14 benign prostatic gland samples, annexin-1 immunoexpression was significantly reduced in 91% and 94% of the HGPIN and Pca foci, respectively [24]. Moreover, Patton et al. [25] reported a progressive loss of annexin-1 expression in HGPIN as well as intermediate- and high-grade Pca, suggesting that loss of annexin-1 expression occurs early in prostatic tumorigenesis and becomes more prominent throughout tumour progression. Similarly, annexin-2 immunoexpression was found to be significantly reduced in HGPIN in comparison to benign lesions, including atrophy and basal cell hyperplasia [26, 27]. When evaluated, the expression of annexin A3 showed similar trends [28].

Glutathione-S-Transferase Gene Methylation

Hypermethylation of the glutathione-S-transferase gene which is considered to be a major and frequent event in prostatic carcinogenesis has also been reported in HGPIN.

In one study by Brooks et al. [1], the DNA from ten HGPIN lesions was analysed for GSTP1 CG island methylation changes using PCR technique targeting a polymorphic repeat sequence in the promoter region of the GSTP1 gene, and somatic GSTP1 methylation was detected in 70% of lesions. Another study by Bostwick et al. [29] demonstrated consistent reduction or loss of expression of all subclasses of GST (alpha, mu and pi isoenzymes) with progression of prostatic neoplasia from benign epithelium to HGPIN and Pca. Similarly, GSTP1 methylation was noted in 50% of HGPIN ($n = 10$) in comparison to 88% of Pca ($n = 24$) in a PCR-based study evaluating the methylation patterns of five genes in different prostatic lesions [30]. Interestingly, Montironi et al. [31] reported two discrete immunostaining patterns of GST pi in HGPIN: the first similar to normal glands show retention of staining in the secretory cells, and the second show complete absence of staining of the secretory cells. While the first pattern was prevalent in HGPIN of the transition zone and was not associated with Pca, the second pattern was prevalent in HGPIN of the peripheral zone and was associated with adjacent to Pca glands.

Mammalian Target of Rapamycin (mTOR) Pathway

The available data on the role of the Akt-mTOR signalling pathway in prostate carcinogenesis is conflicting. While two immunohistochemical-based studies showed significant p-mTOR decreasing expression as HGPIN progresses to Pca, Sutherland et al. [32] and Dai et al. [33] demonstrated increasing p-mTOR, p-Akt, p-4E-BP1 and p-p70S6K expression across the progression model from benign to HGPIN to Pca lesions [34, 35]. In addition, one study showed that among all prostatic lesions (benign, HGPIN and Pca of different grades), HGPIN displayed the greatest expression of p-mTOR, RAPTOR, p-p70 and pRPS6, suggesting that upregulation in the mTOR pathway may represent an early event in the transition to HGPIN [34]. Similarly, Ko et al. [36] showed significant overexpression of Akt, p-Akt, mTOR and p-mTOR in HGPIN in comparison to the benign and Pca groups, but no differences in the p-mTOR ratio between the two groups.

MicroRNA Expression Profiles

The majority of the studied miRNAs were found to be overexpressed in HGPIN compared with benign prostate tissue. Conversely, a global loss of miRNA was noted at the transition step from HGPIN to Pca [37].The most important miRNAs to be lost during this transition are miR-16 which targets Bc12, miR-21 which targets PTEN, and miR-145 which targets the insulin receptor substrate-1 [38–40].

TMPRSS2-ERG Fusion

Incidence: TMPRSS2-ERG fusion, a recurrent genetic aberration in Pca present in 40–50% of Pca, has also been found in HGPIN lesions but with rates that vary significantly among different studies [41, 42]. This variability which does not seem to be due to different detection methods (FISH or RT-PCR versus immunohistochemistry) is mostly related to both the type of specimens and the spatial distribution of HGPIN in relation to Pca. In one of the earliest studies using 34 RPs, Cerveira et al. [43] demonstrated the presence of TMPRSS2-ERG fusion transcript in 50% of Pca (17/34 cases) and 21% of paired HGPIN (4/19 cases) but its absence in benign samples ($n = 25$) using sequencing analysis and real-time PCR reaction. Similarly, using a multicolour interphase FISH assay, Perner et al. [44] reported the presence of the fusion transcript in 48.5% of clinically localized Pca (total number = 237), in comparison to 19% of intermingled HGPIN (total number = 26) and 0% of benign samples (total number = 100). Comparable rates of fusion-positive HGPIN (16%) were also reported by Mosquera et al. [42] who assessed the fusion status using a FISH assay in 143 HGPIN lesions, the majority of which originated from prostatectomy specimens and were associated with Pca. Similarly, in one study evaluating

the utility of ERG immunostaining in limited Pca in needle biopsies, 29% of HGPIN expressed the marker. Interestingly, all the ERG-positive HGPIN glands were intermingled with or immediately adjacent to ERG-positive cancer glands. In contrast, HGPIN glands away from the cancer glands were all negative [45]. Similar observations were made by Lee et al. [46] who reported 27% ERG positivity rate in HGPIN on needle biopsies, with the ERG-positive HGPIN being strongly associated with ERG-positive Pca in the same core compared with ERG-negative HGPIN.

In contrast, the incidence of TMPRSS2-ERG fusion in isolated HGPIN detected on needle biopsies is much lower with two studies showing rates of 5.3% and 11.1% for ERG immunopositivity [47, 48].

Concordance of fusion status with invasive adenocarcinoma: The available data points to an overall concordance in the TMPRSS2-ERG fusion status between HGPIN and adjacent matched Pca, especially in the presence of the fusion. In one study, Mosquera et al. [42] have shown that fusion-positive HGPIN were almost always associated with fusion-positive Pca (96%), while 32% of fusion-negative HGPIN had matching Pca that harboured the fusion. In needle biopsies, Lee et al. reported that in all cases with HGPIN and Pca on the same core, the fusion status was identical between HGPIN and the corresponding Pca [46].

Prognostic Significance: One study suggests that ERG fusion in cooperation with loss of PTEN promotes the transition from HGPIN to Pca by showing that transgenic overexpression of ERG in mouse prostate tissue results in marked acceleration and progression of HGPIN to Pca in a Pten heterozygous background [49]. However, the prognostic significance of ERG fusion in HGPIN especially in needle biopsies remains unclear due to the scarce and conflicting related literature. While He et al. [47] reported similar Pca rates following a diagnosis of ERG-negative or ERG-positive isolated HGPIN (≈40%), Park et al. [50] showed significantly higher rate of subsequent Pca in ERG-positive isolated HGPIN (53%) in comparison to ERG-negative HGPIN (35%). Also, using previously established significant cut-off of ERG rearrangement rates by FISH (≥1.6% vs. <1.6%), the group of Gao et al. has shown that isolated HGPIN with ERG rearrangement rate ≥1.6% had subsequent Pca in 94.9% of cases in comparison to only 4.9% of subsequent Pca in HGPIN with ERG rearrangement rate <1.6% [51].

Clinical Significance of HGPIN

Historically, a diagnosis of isolated HGPIN on biopsy was an indication for a routine repeat biopsy as the incidence of subsequent initially unsampled Pca was 40–60% [52–56]. However, in the era of extended biopsy techniques, the risk of detecting Pca following a diagnosis of isolated HGPIN cancer has fallen to the range of 22–25% which is not significantly different than the risk of Pca following a benign diagnosis [9, 57]. Therefore, some urologists exclude the diagnosis of isolated HGPIN as an indication for repeat biopsy but rather base their follow-up plan on the PSA-related values and the clinical/radiological index of suspicion [9, 58].

That being said, many incorporate the number of cores involved by HGPIN in their management. This approach is based on data showing that patients with multifocal HGPIN (i.e. present in more than two cores) have higher risk of harbouring Pca and should therefore be followed more aggressively than those having unifocal HGPIN [58–60].

At the molecular level, the literature is scarce and has focused mainly on the prognostic significance of TMPRSS2-ERG fusion status/ERG immunoexpression in isolated HGPIN, which yields conflicting results as previously mentioned.

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Chapter 4
Pathology of Prostate Cancer

Shaham Beg, Francesca Khani, and Brian D. Robinson

Contents

S. Beg, M.D.
Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, New York, NY, USA

Englander Institute for Precision Medicine, New York-Presbyterian Hospital, Weill Cornell Medicine, New York, NY, USA

F. Khani, M.D.
Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, New York, NY, USA

B.D. Robinson, M.D. (✉)
Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, New York-Presbyterian Hospital, New York, NY, USA
e-mail: brr2006@med.cornell.edu

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*, Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_4

Introduction

Prostate cancer is the most common non-cutaneous cancer occurring in males, and recent statistics show that it is the second most common cause of cancer mortality in males in the United States after lung cancer [1]. Geographically, the distribution in the incidence of prostate cancer is quite heterogeneous. Some of the highest incidences are reported in North America and Northern Europe and lowest incidences in Asia and the Middle East [2]. Both genetic and environmental factors have been implicated in this difference in incidences. This chapter will focus on the pathologic aspects of the disease, including updates published with the latest edition (2016) of the World Health Organization's *Classification of Tumours of the Urinary System and Male Genital Organs* [3].

Diagnosis of Prostatic Adenocarcinoma

Approximately 95% of malignant prostate cancers are adenocarcinoma, also referred to as "conventional" acinar type, and arise from prostatic epithelial cells [4]. Histopathological diagnosis of prostate carcinoma is based on a constellation of features rather than any single criterion alone. Major criteria used in—and required for–the diagnosis of prostatic adenocarcinoma are highlighted in Fig. 4.1 and include the following:

Abnormal Architecture or Pattern of Glands

Malignant cells are generally arranged into small glands (microacini), clusters, or as single cells, which are crowded and/or haphazardly arranged with infiltration of benign glands. Three features which have never been reported in benign glands and are considered diagnostic of cancer include mucinous fibroplasia, perineural invasion, and glomerulations. Mucinous fibroplasia (collagenous micronodules) consists of paucicellular areas of loose hyalinized tissue with an ingrowth of fibroblast often associated with abundant mucin production. Perineural invasion is the presence of malignant glands in perineural space that tracks along a nerve or encircling a nerve to various degrees. Of note, perineural invasion should only be diagnostic of adenocarcinoma when there is complete (360°) circumferential involvement. Glomerulations consist of intraglandular collections of tumor cells attached to one side of the gland that mimics fetal glomeruli.

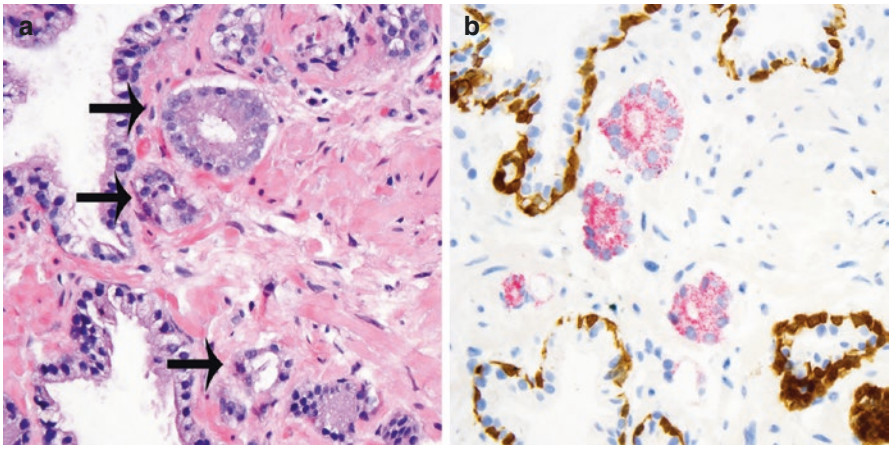

Fig. 4.1 Morphology of prostate cancer. (**a**) Prostate cancer is characterized histologically by both cytologic and architectural atypia. The small, round glands indicated by the arrows represent the cancer glands. They have more abundant and more amphophilic cytoplasm as well as prominent nucleoli, and these features cytologically distinguish the cancer cells from the adjacent benign epithelial cells. Architecturally, the cancer glands are smaller than the surrounding benign glands, infiltrate between them, and have sharp luminal borders. (**b**) This image shows the same tissue in panel **a** that has been immunohistochemically stained using a cocktail of antibodies (high-molecular cytokeratin and p63, brown; p504s, *red*). The cancer glands show overexpression of alpha-methylacyl-CoA racemase [positive p504s (*red staining*)] as well as loss of the basal cell layer [negative high-molecular-weight cytokeratin and p63 (lack of brown staining)]. These immunohistochemical findings support the H&E impression of adenocarcinoma. [**a**, hematoxylin and eosin stain; **b**, PIN-4 immunostain (original magnification for both—400×)]

Loss of Basal Cell Layer

Absence of basal cells is another major criterion. Basal cells are typically seen as round cells with scant cytoplasm that sit against the stromal compartment. The nuclei may be somewhat hyperchromatic or more open and silvery, and nucleoli may be present. On routine hematoxylin and eosin-stained (H&E) sections, it is often difficult to distinguish basal cells from periglandular stromal cells. Moreover, in thick-cut sections or tangentially cut section, carcinoma cells may simulate basal cells. In these cases, immunostains for basal cell markers, such as p63 and high-molecular-weight cytokeratin, can be of great help.

Nuclear Atypia

Nuclear atypia in the form of nuclear enlargement, hyperchromasia, and prominent nucleoli is the last major criterion. The presence of macronucleoli is probably the most important of these nuclear features. However, sometimes foamy gland variants

and other variants may lack prominent nucleoli. In addition, prominent nucleoli may be seen in various mimickers of cancer.

Minor Criteria That Are Helpful—But Not Required—In the Diagnosis of Prostatic Adenocarcinoma

Intraluminal Blue-Tinged Mucin

Blue mucin, which is an acidic mucin, may be seen within tumor lumina and occasionally extravasated into the stroma in the mucinous variant of carcinoma. It can be seen in some mimickers, and it is not present in all cancer, hence its designation as a helpful but not required criterion.

Amorphous Pink Secretions

Acellular, dense pink and often somewhat granular sections are more often seen in malignant glands. This secretion is not specific to cancer, though, and should be distinguished from corpora amylacea, which are circumscribed round-to-oval structures with concentric lamellar rings, and the usual bright-pink and relatively "smooth" secretions seen in benign glands.

Crystalloids

These are intraluminal, brightly eosinophilic, crystal-like structures that appear in various geometric shapes. They are frequently seen in association with the dense pink secretions described above.

Cytoplasmic Changes

Amphophilic cytoplasm with straight luminal borders in large glands is also a feature more common to malignant glands. Benign glands tend to have paler-pink cytoplasmic with apical blebs or snouts, papillary infoldings, and luminal undulations.

Grading of Prostatic Adenocarcinoma

The Gleason grading system remains the fundamental system for determining tumor differentiation, and the Gleason score continues to be one of the most significant prognostic factors for prostate cancer despite continued advancement in the clinical

and molecular understanding of this disease. The system has evolved over the years to improve its interobserver variability, adjust to our better understanding of the biology of the disease, and adapt to changes in clinical management of the disease; however, the fundamentals of Dr. Gleason's system remain [5].

This grading system is based solely on the architectural pattern of tumor seen at low to intermediate power (40× and 100× total magnification). The system was originally provided for grades that ranged from 1 to 5, where grade 1 corresponded to the most well-differentiated tumor with the cancer resembling normal glands and grade 5 meant the tumor was the most poorly differentiated with the cancer essentially losing all gland formation. The Gleason score is then defined as the sum of the two most common grade patterns (primary and secondary). If the tumor has only one pattern, then that pattern/grade is included twice (e.g., a tumor with only pattern 3 would be given a score of 3 + 3 = 6). Scores originally ranged from 2 (1 + 1) to 10 (5 + 5). In high-grade tumors, if a lower-grade pattern is <5% of tumor, then the low-grade component is not reported. On the contrary, if a higher-grade component is present, irrespective of the amount, it is always included in the score. Of note, Gleason scores <6 are no longer assigned on needle core biopsies and only rarely in other specimen types. In addition, Gleason pattern 1 is now recognized to be adenosis, a benign condition, so it should no longer ever be assigned. Figure 4.2 shows some examples of Gleason patterns 3, 4, and 5.

Pattern 1 was originally described as cancers that were completely circumscribed nodules of tightly packed glands that were relatively uniform in size and distribution. As mentioned above, this is now recognized as adenosis and therefore should not be used in grading.

Pattern 2 consists of a circumscribed nodule of small acini with some variation in size and minimal peripheral stromal invasion. Since the edges of tumors are not completely evaluable on needle biopsy, this pattern is no longer assigned on core biopsies.

Pattern 3 is comprised of variable-sized individual glands that are well-formed and discrete units. Tumor cells often form a single layer around to make a gland. If a few poorly formed glands are seen at higher magnification, they usually represent tangential sectioning of adjacent small well-formed glands and should still be considered pattern 3.

Pattern 4 is characterized by cribriform growth, fused glands, or glomeruloid structures. If a significant number of poorly formed glands are present in a cluster, such that tangential sectioning cannot explain their presence, then this can also be assigned pattern/grade 4.

Pattern 5 is the highest grade and consists of malignant cells in sheets, cords, or as individual single cells. Additionally, the presence of comedonecrosis also is considered pattern 5.

In needle biopsies, the score is assigned using the most common pattern first followed by next highest grade regardless of the quantity of the highest tumor grade. For example, a tumor with a majority of pattern 3, lesser amount of pattern 4, and minimal amount of pattern 5 on needle core biopsy should be reported as 3 + 5 = 8. In radical prostatectomy specimens, the highest Gleason pattern can be listed as a tertiary pattern if it represents less than 5% of the tumor volume. For instance, in the

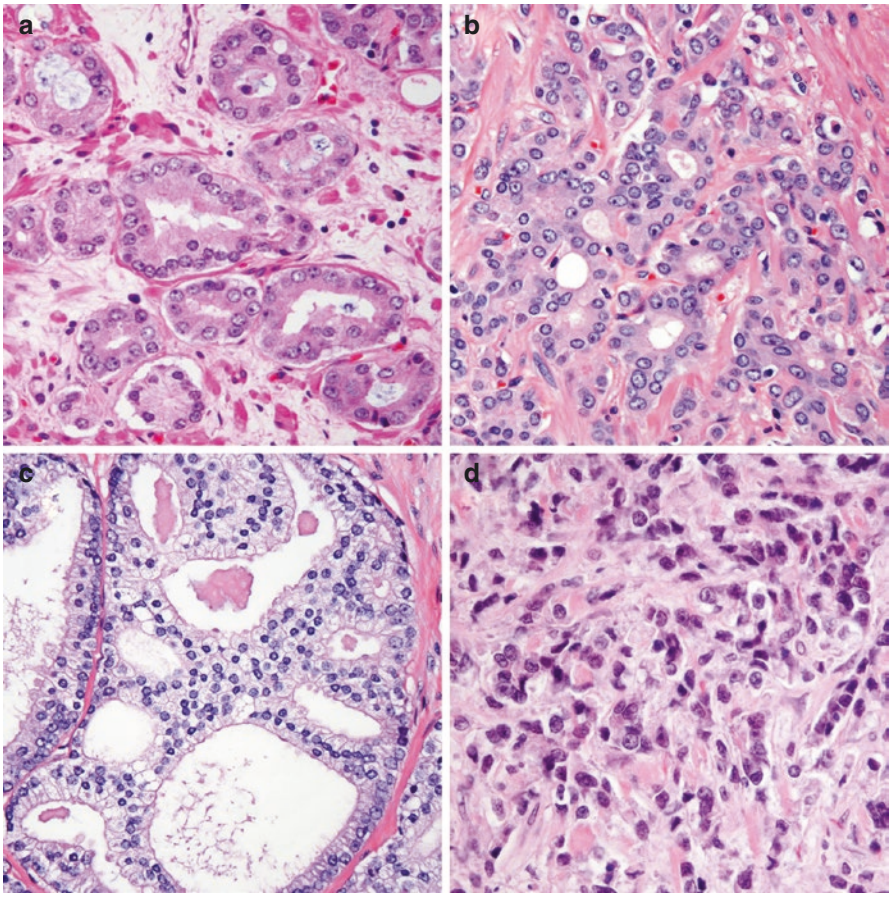

Fig. 4.2 Examples of Gleason patterns. (**a**) Gleason pattern 3 consists of individual, round glands with a single lumen. At least a thin amount of stroma separates each well-formed gland. (**b**) When glands begin to fuse, such as in the center of this image, stroma no longer separates individual glands. This pattern is considered Gleason grade 4. At the periphery of this image, clusters of cells without a well-formed lumen (i.e., poorly formed glands) are present, and this pattern is also considered Gleason grade 4. (**c**) Cribriform growth pattern is another pattern of growth seen in Gleason grade 4. (**d**) When tumor cells no longer attempt any gland formation (i.e., single, individual cancer cells or linear, single-file arrangement of cancer cells), Gleason grade 5 is assigned. The presence of comedonecrosis (not shown) is also considered Gleason grade 5 (**a**–**d**, hematoxylin and eosin stain; original magnification for all—400×)

previous example on needle core biopsy, the tumor at radical prostatectomy may be reported as 3 + 4 = 7 with tertiary Gleason pattern 5.

One limitation of the modern Gleason scoring system was that, despite being scored on a scale from 2 to 10, the lowest score assigned in practice is now a 6 since scores 2–5 are no longer reported on biopsies. This can lead to the patient potentially misunderstanding that he had an intermediate or moderately aggressive tumor when in actuality it was the most well-differentiated and least aggressive tumor possible. This creates unnecessary confusion and complexities between the patient and clinician. Secondly,

patients having a Gleason score of 7 (3 + 4 or 4 + 3) were not adequately understanding that one (4 + 3 = 7) was significantly worse than the other, even though both were a sum score of 7 [6–9]. Third, many cases graded as Gleason score 6 previously are now reclassified as Gleason score 7 in the current scoring system, hence modern Gleason 6 tumors have a better prognosis compared to previously reported Gleason score 6 cases. Tumors with pure Gleason 6 score have been shown to have very rare risk of progression and virtually no recurrence after radical prostatectomy [10, 11].

To address these limitations, a new system of grade grouping was adopted to improve the understanding of the current Gleason scoring system. This grouping system was initially based on a study published by Pierorazio et al. [10] and later validated in a multi-institutional study that included 20,845 radical prostatectomies, 16,176 preoperative needle biopsies, and 5501 biopsy specimens followed by radiation therapy [12]. The new grade groups are as follows:

- Grade Group 1: Gleason score ≤6—only individual discrete well-formed glands
- Grade Group 2: Gleason score 3 + 4 = 7—predominantly well-formed glands with lesser component of poorly formed, fused, or cribriform glands
- Grade Group 3: Gleason score 4 + 3 = 7—predominantly poorly formed, fused, or cribriform glands with a lesser component of well-formed glands*
- Grade Group 4: Gleason score 8 (4 + 4, 3 + 5, 5 + 3):
  - Only poorly formed, fused, or cribriform glands
  - Predominantly well-formed glands with a lesser component lacking glands**
  - Predominantly lacking glands with a lesser component of well-formed glands**
- Grade Group 5: Gleason score 9–10—lacks gland formation (or with necrosis) with or without poorly formed, fused, or cribriform glands*

*For cases with >95% poorly formed/fused/cribriform glands or lack of glands, the component of <5% well-formed glands is not factored into the grade.

**Poorly formed/fused/cribriform glands can also be a more minor component.

Five-year biochemical recurrence-free progression probabilities for radical prostatectomies were 96, 88, 63, 48, and 26% for Grade Groups 1–5, respectively. Patients assigned to Grade Group 1 have the best prognosis, and indeed many patients are candidates for active surveillance when other clinical parameters such as serum PSA, tumor stage, and volume of cancer in biopsies are taken into account [12]. This grade grouping will hopefully reduce overtreatment of indolent cases, although follow-up is still needed for Grade Group 1 cases diagnosed on biopsy as approximately 20% of patients will have un-sampled higher-grade cancer [9].

Other major modifications in the modern Gleason grading system include:

- Recommendation to report the percentage Gleason pattern 4 in cases reported as Gleason score 7. This may have implications in the clinical management as patients with low amount of Gleason 4 tumor may be considered for active surveillance rather than definitive treatment [13].
- All cribriform patterns are redefined as Gleason pattern 4. This is an important update as cribriform pattern of growth has been found to be a strong prognostic marker for

distant metastasis and disease-specific death in patients who undergo radical prostatectomy [14], and its presence also predicts biochemical recurrence [15].
- Glomeruloid structures should be graded as Gleason pattern 4.
- Mucinous carcinomas should be graded based on the underlying architectural pattern and ignoring the mucin component. Previously, all mucinous carcinomas were graded as pattern 4.

Variants of Acinar Adenocarcinoma

The majority of prostatic adenocarcinomas are usual acinar subtype, and only a small minority of them have been classified as variants. These variants have clinical significance because some are challenging to diagnose morphologically and in some variants there exist prognostic and therapeutic differences compared with usual acinar adenocarcinoma [16]. A recent WHO classification (2016) of usual acinar adenocarcinomas is given in Table 4.1 [3]. Variants that are deceptively benign looking and pose diagnostic challenge for pathologists include the atrophic, pseudohyperplastic, microcystic, and foamy gland subtypes of acinar adenocarcinoma. Variants which show poor prognostic outcome compared with usual acinar subtype include signet ring-like, pleomorphic giant cell, and sarcomatoid variants of prostatic adenocarcinoma. Other patterns are not known to differ in terms of prognosis from usual acinar adenocarcinoma, and their recognition is only of academic interest.

Atrophic variant of prostatic adenocarcinoma is seen in sporadic or post-radiation/hormonal therapy settings and is characterized by small malignant glands with scant cytoplasm, mimicking benign atrophy (Fig. 4.3a). Diagnosis is made by recognizing the infiltrating nature of the glands, the nuclear atypia, and the presence of non-atrophic usual acinar adenocarcinoma in adjacent areas. Positive AMACR expression is seen in 70% of cases, and basal markers are negative [17]. The majority of these tumors are Gleason 6 (3 + 3) with no prognostic difference from adenocarcinomas of the same grade and stage [18].

Pseudohyperplastic carcinomas are usual acinar adenocarcinoma showing cystic dilatation, epithelial hyperplasia with branching and papillary folding, and luminal undulations. Cells contain abundant, pale cytoplasm with basally located nuclei showing prominent nucleoli, a feature that differentiates them from benign hyper-

Table 4.1 Variant of acinar adenocarcinoma (WHO classification 2016)

Atrophic
Pseudohyperplastic
Microcystic
Foamy gland
Mucinous (colloid)
Signet ring cell-like
Pleomorphic giant cell
Sarcomatoid

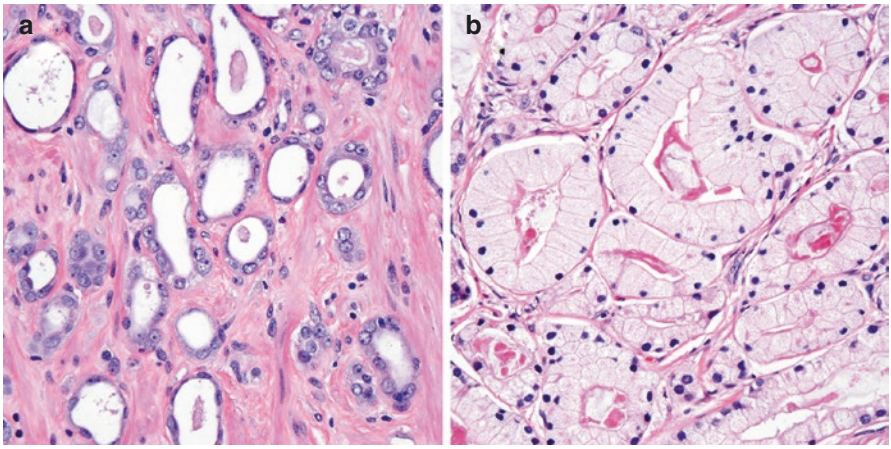

Fig. 4.3 Morphologic variants of acinar adenocarcinoma. (**a**) The atrophic variant of prostate cancer consists of small-caliber glands lined by cells with scant cytoplasm, mimicking benign atrophy. However, the cells in atrophic cancer still contain prominent macronucleoli, while benign atrophic cells do not. Also, the atrophic cancer glands do not maintain a lobular architecture (i.e., display an infiltrative growth pattern). (**b**) The foamy gland variant of prostate cancer is characterized by cells with abundant, xanthomatous (foamy) cytoplasm, as seen in this image. This variant typically has small, pyknotic-appearing nuclei located at the base of the cells, which lack prominent nucleoli. While nucleoli are often absent, recognition of this variant of prostatic adenocarcinoma is usually possible by recognizing the abundant foamy cytoplasm, sharp luminal borders, and crowded growth pattern (**a**–**b**, hematoxylin and eosin stain; original magnification for both—400×)

plastic glands. AMACR expression is seen in 77% of cases [19]. The Gleason score is usually 3 + 3 = 6, and there is no prognostic difference from similar grade and stage adenocarcinomas without pseudohyperplastic features. Of note, HOXB13 G84E-related familial prostatic adenocarcinomas have been shown to frequently have pseudohyperplastic features [20].

Microcystic adenocarcinoma is a novel variant that is characterized by cystic dilatation of malignant glands which is tenfold greater than the size of usual small acinar adenocarcinoma and typically shows rounded expansion of glands with flat luminal lining [21]. The cells lining the malignant glands show cytoplasmic volume loss giving an overall picture of deceptively looking benign cystic atrophy. Intraluminal crystalloids and intraluminal blue mucin are often present helping to identify the glands as atypical. Approximately 11% of usual acinar adenocarcinoma on radical prostatectomy show some microcystic pattern. Almost all cases (96%) demonstrate overexpression of alpha-methyacyl-CoA racemase (AMACR), and all show complete absence of basal cell markers on immunohistochemistry. These are considered Gleason pattern 3 tumors.

Foamy gland carcinoma cells show characteristic presence of abundant foamy/xanthomatous cytoplasm and small, round, pyknotic-looking nuclei without prominent nucleoli (Fig. 4.3b). Absence of nuclear atypia can make the diagnosis on limited needle core biopsy challenging. Foamy gland carcinoma is rare in its pure form

but commonly seen admixed with usual acinar adenocarcinoma. Most foamy gland carcinomas are Gleason score 6 or 7 [22]. Prognosis is the same as that of non-foamy gland carcinoma of comparable stage and grade [22].

Mucinous (colloid) carcinoma is defined as prostatic adenocarcinoma with at least 25% of the tumor comprised of extracellular mucin lakes. Using this criterion, pure mucinous carcinoma comprises only 0.2% of prostate cancers. Microscopically, it is identified as usual acinar adenocarcinoma cells floating in large pools and lakes of mucin. Intracytoplasmic mucin is generally absent. Gleason grading is done on the actual carcinoma cells/glands and ignores the mucin component. Most cases are Gleason score 7 or 8 [23].

Signet ring cell-like carcinoma is very rare and defined by carcinoma cells showing peripheral nuclear displacement and indentation by a cytoplasmic vacuole lacking intracellular mucin. At least 25% of signet ring-like cells are needed in tumors to make the diagnosis of this tumor subtype. Positivity for PSA, PSAP, and AMACR and absence of mucin staining in vacuole differentiate it from signet ring carcinoma of other non-prostatic sites. Prognosis is poor with mean survival of 28 months [24].

Pleomorphic giant cell adenocarcinoma is an exceptionally rare variant of adenocarcinoma with fewer than ten cases reported in the literature, including the single largest series of six cases [25, 26]. Tumors show giant, bizarre, anaplastic cells with pleomorphic nuclei. Atypical mitotic figures can be present with others showing concomitant small-cell neuroendocrine carcinoma, squamous cell carcinoma, and/or ductal adenocarcinoma. Staining for PSA is variable. Some cases have prior history of hormonal and/or radiation therapy before being diagnosed with this variant. All reported cases have shown admixed Gleason 9 usual adenocarcinoma. The disease course is very aggressive.

Sarcomatoid carcinoma (carcinosarcoma) is a rare biphasic tumor composed of both epithelial and mesenchymal differentiation. Approximately half of the patients diagnosed with this subtype had prior diagnosis of usual adenocarcinoma followed by treatment with hormone therapy and/or radiation therapy prior to subsequent diagnosis of sarcomatoid carcinoma. Thus, the mesenchymal component is hypothesized to evolve from the epithelial component. Molecular studies have also documented common clonal origin [27]. Mesenchymal components may include osteosarcoma, chondrosarcoma, rhabdomyosarcoma, leiomyosarcoma, liposarcoma, angiosarcoma, or other heterologous differentiation. Prognosis is poor but localized cancer can be effectively treated with local and/or systemic approaches [28].

Variants of Non-acinar Carcinoma

Non-acinar variants of prostatic adenocarcinoma are listed in Table 4.2.

Intraductal carcinoma of the prostate (IDC-P) is a new recognized entity in the 2016 WHO classification. Although primary carcinoma of prostatic ducts has been

Table 4.2 Variants of non-acinar adenocarcinoma (WHO classification 2016)

Ductal adenocarcinoma
Urothelial carcinoma
Adenosquamous carcinoma
Squamous cell carcinoma
Basal cell carcinoma
Adenocarcinoma with neuroendocrine differentiation
Adenocarcinoma with Paneth cell-like neuroendocrine differentiation
Well-differentiated neuroendocrine tumor
Small-cell neuroendocrine carcinoma
Large-cell neuroendocrine carcinoma

described in the literature as early as 1972 [29], the first comprehensive report of ductal spread of prostatic carcinoma was published by Kovi J a decade later in a series of 139 cases of prostatic adenocarcinomas [30]. The WHO describes this entity as "intra-acinar and/or intraductal neoplastic epithelial proliferation that has some features of high-grade prostatic intraepithelial neoplasia (HGPIN) but exhibits much greater architectural and/or cytological atypia, typically associated with high-grade, high-stage prostate carcinoma" [3].

The incidence of intraductal carcinoma varies among different studies depending upon on the criteria used to identify this entity, the type of specimen, and the characteristics of the cohort. In prospectively collected biopsy specimens, IDC-P was identified in 2.8% of cases [31]. IDC-P without invasive carcinoma is extremely rare and present in only 0.06–0.26% of biopsy cases [31–33]. However, the incidence of IDC-P (with or without invasive carcinoma) at radical prostatectomy goes up to 17% in one series of 901 radical prostatectomies [34].

IDC-P is thought to represent an advanced stage of this disease with intraductal spread of the prostatic carcinoma by retrograde involvement of ducts by high-grade adenocarcinoma. Histologically, IDC-P is defined as malignant cells filling large acini and prostatic ducts with preservation of basal cells and showing either solid/dense cribriform pattern or loose cribriform/micropapillary pattern with marked nuclear atypia/comedonecrosis (Fig. 4.4a). Recent recommendations are that IDC-P should not be assigned any Gleason grade. If pure IDC-P is diagnosed on prostatic biopsy, then the reporting pathologist should include a note that IDC-P is associated with high-grade and high-volume prostatic carcinoma and definitive therapy may be indicated [32].

Ductal adenocarcinoma of the prostate is a subtype of adenocarcinoma defined as large glands lined by a tall, pseudostratified columnar epithelium. It is rare in its pure form comprising only 0.2–0.4% of prostate cancer, but the incidence of ductal subtype combined with usual acinar subtype has been reported to be around 3.2% [16, 35]. Ductal adenocarcinoma is commonly located in the periurethral region, usually grows along the prostatic ducts, and also invades the prostatic stroma. Microscopically, these tumors are seen as glands with papillary or cribriform archi-

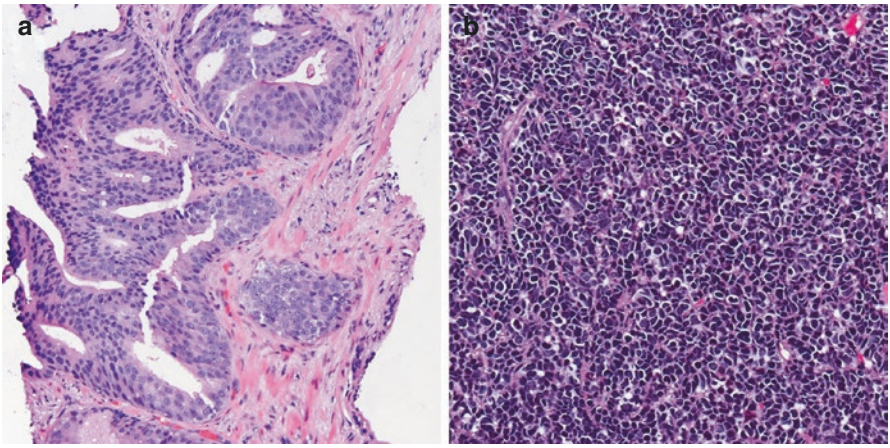

Fig. 4.4 Other variants of prostate cancer. (**a**) Intraductal carcinoma indicates the presence of malignant epithelial cells filling and expanding the native ducts and acini. Dense cribriform growth, where the malignant cells fill more than 50% of the luminal space, is one diagnostic growth pattern. (**b**) Small-cell (neuroendocrine) carcinoma of the prostate represents an aggressive variant of prostate cancer that may represent de novo disease or progression of a primary adenocarcinoma. The morphology is similar to that of small-cell carcinomas elsewhere in the body and is characterized by small *blue* cells with scant cytoplasm, salt and pepper chromatin, and nuclear molding (**a**–**b**, hematoxylin and eosin stain; original magnification for both—400×)

tecture lined by tall columnar pseudostratified epithelium. Basal cells are absent in the invasive components. The malignant cells have amphophilic cytoplasm, elongated nuclei, and often severe atypia. Prominent nucleoli, coarse chromatin, and abnormal mitotic figures are frequent. These tumors are considered Gleason pattern 4 (cribriform and papillary) or 5 (solid and comedonecrosis). No marker can differentiate ductal from acinar subtype. Prognosis is poor with pure ductal or tumors comprising predominantly ductal component [35].

Urothelial carcinoma of the urethra or bladder may involve the prostate and cause some diagnostic dilemma. Primary prostatic urothelial carcinoma, though rare, can arise from urothelial lining of primary and proximal prostatic ducts [36–38]. Morphology is similar to urothelial carcinoma of other anatomical sites. The tumor has a propensity for solid growth within ducts and acini and may show extensive involvement of the prostate without any stromal invasion. When stromal invasion is present, there is frequently associated desmoplasia and/or inflammation, both of which are uncommon in prostatic adenocarcinomas. Immunostains to help diagnose urothelial carcinoma include thrombomodulin, GATA3, p63, and high-molecular-weight cytokeratin; prostate-specific markers such as PSA, prostein, and NKX3.1 should be negative [39]. Prognosis of patients depends upon on the stage of cancer.

Squamous and adenosquamous carcinomas of the prostate are rare tumors and are thought to arise from divergent differentiation of basal cells or transdifferentia-

tion of usual acinar epithelium after radiation or hormonal therapy. Primary squamous neoplasms must be differentiated from direct or metastatic involvement of the prostate by bladder and urethral squamous carcinomas. Adenosquamous carcinomas have a glandular element along with the squamous component. Prognosis is poor with a median survival of 1 year [40].

Basal cell carcinoma of the prostate is another rare malignant tumor arising from prostatic basal cells. Microscopically, they show adenoid cystic and/or cribriform growth patterns as well as small solid nests of basal cells with peripheral palisading. Cords of cells or small tubules with hyaline rim and large nests with/without necrosis may also be seen. Cells lining the outermost layer are positive for basal markers, whereas luminal cells show immunoreactivity for CK7. Bcl-2 positivity and high Ki-67 index differentiate between basal cell carcinoma and basal cell hyperplasia [41]. A subset of basal cell carcinomas with adenoid cystic-like morphology show MYB rearrangement representing a distinct subtype of tumor [42]. It is considered to be a potentially aggressive tumor, especially those tumors with solid/nested pattern and/or necrosis.

Neuroendocrine tumors of the prostate comprise five distinct subtypes, namely, adenocarcinoma with neuroendocrine differentiation, adenocarcinoma with Paneth cell-like neuroendocrine differentiation, well-differentiated neuroendocrine tumor (carcinoid tumor), small-cell neuroendocrine carcinoma, and large-cell neuroendocrine carcinoma. Adenocarcinoma with neuroendocrine differentiation is usual acinar adenocarcinoma with scattered neuroendocrine cells frequently identified only by immunostains such as chromogranin, synaptophysin, NSE, and CD56. The routine use of markers for neuroendocrine differentiation is not recommended in routine surgical pathology reporting. Adenocarcinoma with Paneth cell-like neuroendocrine differentiation contains cells with brightly eosinophilic cytoplasmic granules, “salt and pepper” chromatin, and absence of nucleoli. Well-differentiated neuroendocrine tumors (carcinoid tumors) are tumors similar to carcinoid tumors in other sites such as lung or gastrointestinal tract. They should be diagnosed only when they are not close to usual adenocarcinoma and show PSA negativity; otherwise, they should be considered prostate adenocarcinomas with neuroendocrine differentiation. Small-cell neuroendocrine carcinomas of the prostate are aggressive tumors with morphology the same as small-cell carcinomas of the lung. Histologic features include high nuclear to cytoplasmic ratio, nuclear molding, lack of prominent nucleoli, crush artifact, geographic necrosis, apoptosis, and high mitotic rate (Fig. 4.4b). More than half of the patients have distant metastasis at diagnosis. Large-cell neuroendocrine carcinoma is an extremely rare variant of prostatic carcinoma with histologic features identical to those of large-cell carcinoma in the lung. So far, only seven cases have been reported in the single largest series by Evans et al. [43] with a few separate single case reports [44, 45]. Almost all cases arise after hormonal therapy. Histologic features show sheets and ribbons of malignant cells with peripheral palisading, large nuclei, high mitotic activity, and prominent tumor necrosis. Prognosis is dismal with rapid dissemination and death.

Immunohistochemistry

Immunohistochemistry (IHC) is a valuable diagnostic tool in difficult situations where the diagnosis of prostate cancer is not straightforward. Confirmation of the diagnosis of small foci of adenocarcinoma in needle biopsy by IHC is primarily performed using antibodies against the basal cell layer. Invasive adenocarcinomas lack basal cells and hence basal immunostains will be negative. 34β[beta]e12 and p63 are the most commonly used basal immunostains. CK5/6 is also sometimes used. A cocktail of 34β[beta]e12 and p63 increases the sensitivity of basal cell detection [46]. Interpretation of IHC must be done in the context of the H&E stain as complete or partial loss of basal cells may be seen in some benign entities such as atrophy.

AMACR (P504S or alpha-methylacyl-CoA racemase) is a cytoplasmic enzyme and selectively expressed in 80–100% of acinar adenocarcinomas with a characteristic granular staining pattern. It is a marker that is supportive of a diagnosis of adenocarcinoma but by itself is not diagnostic of carcinoma as many noncancerous lesions may also show overexpression (e.g., adenosis, high-grade prostatic intraepithelial neoplasia, nephrogenic adenomas). AMACR staining can be performed either alone or as cocktail with basal cell markers (Fig. 4.1b).

ERG immunoexpression is highly specific for neoplastic prostatic epithelium but has a sensitivity of only around 50%. It generally does not provide any added diagnostic value beyond AMACR and basal cell markers.

To differentiate high-grade prostate adenocarcinoma and urothelial carcinoma, a panel of antibodies for prostate epithelium, such as PSA, PSMA, NKX3.1, and/or P501S (prostein), and urothelium, such as GATA3, p63, and 34β[beta]e12, are used.

For differentiating prostatic adenocarcinoma and bladder adenocarcinoma, antibodies for the prostate such as PSA, PSMA, and/or prostein are used along with markers for bladder adenocarcinoma such as villin, thrombomodulin, CDX2, and CEA.

Similarly, colonic adenocarcinoma can be differentiated from prostatic adenocarcinoma with the use of PSA, PSMA, prostein, and/or NKX3.1 antibodies for prostate and villin and CDX2 for colon adenocarcinoma.

Treatment Effect

Hormonal or radiation therapy causes marked changes in histomorphology of both normal and malignant prostatic tissue; therefore, treatment history of the patient is of utmost importance to pathologist prior to reporting.

Androgen deprivation therapy (ADT) is given in various clinical settings including advanced-stage prostate cancers. Benign glands of ADT-treated patients show diffuse atrophy often with basal cell hyperplasia, immature squamous metaplasia, and urothelial metaplasia. Malignant glands after ADT show loss of luminal space and appear as clusters, rows, or single cells mimicking Gleason pattern 5 cancers. Cells become small and show nuclear pyknosis and cytoplasmic vacuolization,

often mimicking lymphocytes or histiocytes. Gleason scoring is not recommended on ADT-treated prostates that show the above treatment effects. Immunostaining with epithelial cell markers (PSA and low-molecular-weight cytokeratins) may be helpful for identification of histologically inconspicuous cancer cells showing treatment effect. AMACR expression is downregulated by ADT in some cases, such that only 45–75% of cases show AMACR expression [47, 48].

5-α[alpha] reductase inhibitors have been shown to have minimum to nil effect on morphology of prostate adenocarcinoma, and Gleason grading can be still used after treatment with these drugs [49].

Radiation therapy induces benign glands to acquire cytologic atypia and nuclear pleomorphism mimicking carcinoma. The presence of such effects throughout the entire prostate with preservation of a benign architecture can be a clue of radiation changes. Malignant cells showing radiation effect are small and often inconspicuous, show vacuolated cytoplasm, and have inconspicuous nuclei and nucleoli. Gleason grading may be applied after radiation therapy only if the effect is not marked. Post-radiation therapy biopsies of the prostate with cancer cells showing radiation changes should report this finding as these patients have similar prognosis as patients who have biopsies that are negative for carcinoma [50]. AMACR expression is typically retained and can be used along with basal cell markers to differentiate between radiation-induced atypia in benign glands and cancer cells with radiation-induced changes.

Chemotherapy-induced changes include inconspicuous collapsed glands, small tumor cells, cytoplasmic vacuolization, and intraductal and cribriform growth patterns [51, 52]. Presence of intraductal and cribriform pattern in patients treated with neoadjuvant chemotherapy shows poorer prognosis [52, 53].

Cryosurgery and thermotherapy induce nonspecific changes such as hyalinization, fibrosis, and necrosis. No therapy-specific histological changes are observed in carcinoma cells, so Gleason grading can be applied to residual carcinoma after this treatment.

Special Issues Related to Biopsy, TURP, and Prostatectomy Specimens

Needle Core Biopsy

Approximately 25–30% of patients are diagnosed with carcinoma who undergo prostatic biopsy in the current era of PSA screening and extended core biopsy [54]. Diagnosis of small foci of adenocarcinoma, especially deceptively benign-looking variants or those at biopsy edges or in crushed tissue, can sometimes be challenging for pathologists. Immunohistochemistry using a triple immunostain (p63/34β[beta] e12/AMACR) is extremely helpful in such difficult situations.

Perineural invasion identified on biopsy should always be reported as it is not only diagnostic of prostatic adenocarcinoma but also is associated with increased risk of aggressiveness and lethal prostate cancer in such patients [55]. Perineural

invasion is seen in about one-quarter of prostate cancer patients on needle biopsy and in 11% of lower-stage tumors [56]. The number of positive cores and length of core involved by tumor are also included in pathology reports as they are known to be predictors of extraprostatic extension of carcinoma [57]. Reporting the percentage of pattern 4 in Gleason score 7 tumors is recommended as it is useful in the selection of patients for active surveillance or more aggressive therapies [13, 58, 59].

Transurethral Resection of Prostate (TURP)

Detection of incidental adenocarcinoma on TURP has currently decreased due to the use of different medical therapies directed at benign prostatic hyperplasia as well as increased screening of patients prior to TURP. The use of alternative modalities of treatment such as cryosurgery, microwave therapy, and lasers had also reduced the availability of TURP specimens. However, TURP is still a viable therapeutic option for patients who failed such medical therapies or alternative forms of treatment. All TURP specimens should be examined carefully. Submission of representative tissue fragments in eight cassettes allows detection of almost all stage T1b tumors and 90% of stage T1a tumors [60–62]. If any incidental carcinoma is detected involving <5% of tissue specimen, then the remaining entire tissue should be submitted.

Radical Prostatectomy

Radical prostatectomy, either open, laparoscopic, or robotic, is a definitive treatment for clinically localized prostate cancer. In the current era of tissue-sparing surgeries, cytoreductive prostatectomy has been advocated for locally advanced and metastatic disease citing many advantages to patients [63, 64]. Eighty-five percent of prostatic adenocarcinomas are multifocal, and 70% are bilateral. Therefore, appropriate processing of RP specimen is essential for correctly diagnosing tumor grade, volume, stage, and margin positivity, all of which are parameters affecting further management and prognosis.

When received, the specimen should be weighed, measured in all three dimensions, and inked with at least two different colors to designate tumor laterality, right and left. Transverse sections from base, para-base, apex, and para-apex should be entirely submitted. A section of the seminal vesicles should also be submitted, and this section should be from the portion of seminal vesicles that is immediately adjacent to the prostate. The rest of the prostate is either entirely submitted by serial sectioning at 3–4 mm or submitting every alternate section. Each submitted section should be labeled for its accurate anatomical location.

Positive surgical margins are identified most commonly at the apex and posterolaterally and denote a significant adverse prognostic factor. A margin is reported as "positive" only when tumor is touching the inked margin. Highest Gleason score

present at positive margins is recommended to be included in the report, as is a length of the total margin positivity.

Extraprostatic extension (EPE) of tumor refers to extension of tumor into loose periprostatic fibroadipose tissue, which may occur through perineural invasion rather than direct invasion of fat. EPE is reported as focal when only a few malignant glands are beyond the prostate or when tumor involves less than a single high-power (400× total magnification) field.

Seminal vesicle involvement (stage pT3b) is diagnosed when neoplastic glands involve the muscular wall of the seminal vesicle. Involvement of intraprostatic seminal vesicle and ejaculatory duct does not constitute stage pT3b [65].

For RP specimens having two or more separate tumor nodules, this should be documented in the report and a Gleason score assigned to each nodule. Calculating the average of both scores is not recommended. Where possible, the greatest dimension of the dominant nodule or number of blocks involved by tumor can also be documented in the report. A separate stage should also be given for each individual tumor nodule.

Conclusion

Prostate cancer is the most common non-cutaneous malignancy among males and a major cause of morbidity and mortality. Establishing the pathological diagnosis of this disease is of utmost importance for accurate management and prognostication of patients. Despite recent advancements in molecular diagnostics, histopathological grading still remains one of the most significant criteria for predicting prognosis and directing treatment decisions.

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Chapter 5
Genetic Susceptibility

John P. Greene and Stephen P. Finn

Contents

Introduction

Prostate cancer is a leading cause of morbidity and mortality among men in the United States and Western Europe [1]. Widespread screening with prostate-specific antigen (PSA) and early treatment of localized prostate cancer have contributed to a decrease in age-adjusted rates of death due to prostate cancer [2]. Advancing age, diet, lifestyle-related factors, family history and ethnicity have long been recognized as contributors to the risk of prostate cancer [3]. Recent discoveries in the genetics of prostate cancer and in the acquired mutations that accumulate in prostate cancer cells have improved our understanding of the development of prostate cancer [1]. Evidence supporting the role of genetic factors comes from studies of relatives

J.P. Greene, M.B., B.Ch., B.A.O. (✉) • S.P. Finn, M.B., B.Ch., B.A.O., Ph.D.
Department of Histopathology, St. James's Hospital and Trinity College Dublin, Dublin, Ireland
e-mail: john.greene@outlook.ie

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*, Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_5

of patients with prostate cancer, founder populations, genome-wide association studies (GWAS), case-control studies and linkage analyses and from studies in patients with abnormalities in known cancer syndrome-associated genes such as BRCA1 and BRCA2 [4].

Hereditary Prostate Cancer

The risk of prostate cancer is increased approximately twofold in men with affected first-degree relatives [5]. This risk is increased further if more than one family member is affected or if there is early age of onset in a family member [6, 7]. In a cohort study of 44,000 pairs of Scandinavian twins, concordance for cancer in identical twins was higher for prostate cancer than either breast or colorectal cancer [8]. This study estimated that as much as 42% of the risk of prostate cancer could be explained by heritable factors. Interestingly, in addition to increasing the risk of developing prostate cancer, genetic factors may also influence the prognosis in these men. In a Swedish study in men with prostate cancer whose fathers also had prostate cancer, the survival of sons was similar to that of their fathers [9].

As with other cancers, familial clustering of prostate cancer has been reported. The Massachusetts Male Aging Study of 1149 Boston-area men found a much higher risk for prostate cancer among men with a family history of the disease which appeared to be independent of environmental factors [10]. Further associations between family history and risk of prostate cancer were characterized in a population-based case-control study of 1557 men aged 40–86 years [11]. At baseline, 4.6% of the cohort reported a family history of prostate cancer in a brother or father, and this was positively associated with prostate cancer risk after adjustment for age, alcohol and dietary factors. However, at least some of this familial clustering is due to increased prostate cancer screening in families thought to be at high risk [12].

This recognition that prostate cancer clusters within families has led investigators to collect multiple-case families in order to localize prostate cancer susceptibility genes through linkage analysis. It is now estimated that 5–10% of prostate cancer cases are primarily caused by high-risk inherited genetic factors or prostate cancer susceptibility genes [13–15]. Linkage analysis studies have mapped several susceptibility loci, and a number of genes have been cloned at these loci [5]. These include 1q24–25 (HPC1/RNASEL), 1q42.2–43 (PCAP), Xq27-q28 (HPCX), 1p36 (CAPB), 20q13 (HPC20), 8p22–23 (MSR1), 8q24 and 17p11 (HPC2/ELAC2) [16–26] (see Table 5.1). However, the replication of these findings remains inconsistent, with numerous genes likely to be involved in prostate cancer.

Ethnic groups and founder populations are of particular interest for genetic mapping of complex traits due to a lack of genetic heterogeneity. African-American men have the world's highest incidence of prostate cancer and a twofold higher mortality rate compared to Caucasians [27]. Admixture mapping has identified a number of chromosomal regions associated with prostate cancer in African Americans

Table 5.1 Susceptibility genes and their loci identified in linkage analyses

Gene	Location
HPC1/RNASEL	1q25
PCAP	1q42.2–43
HPCX	Xq27–28
CAPB	1p36
HPC20	20q13
8p/MSR1	8p21–23
8q	8q24

Table 5.2 Susceptibility loci identified with genome-wide association studies

Gene	Location
ZNF652	17q21.3
PRAC	17q21.3
EMSY	11q13.5
KLF6	10p15
AMACR	5p13.2-q11.1
NBS1	8q21
SRD5A2	2p23
ER beta	14q22-q24
E-cadherin gene (CDH1)	16q22.1
BRCA1	17q21
BRCA2	13q12–13
CHEK2	22q12

including the inherited variation at the 8q24 risk locus which appears to contribute to differences in African-American and European-American incidence of the disease [28–30]. Ashkenazi Jewish men have a higher incidence of mutations in BRCA1 and BRCA2 genes than the general population, and studies have reported a significant increased risk of prostate cancer in these men [31].

Although linkage studies have provided evidence that prostate cancer has a strong genetic component, identifying specific genes that contribute to the development of the disease has proven more difficult. Using gene sequencing technology, it has been possible to identify rare genes associated with an increased cancer risk [32]. Susceptibility genes with an associated increased risk for prostate cancer that have been identified include ZNF652, PRAC, EMSY, KLF6, AMACR, NBS1, SRD5A2, ER-beta, E-cadherin (CDH1), CHEK2, BRCA1 and BRCA2 [33–46] (see Table 5.2).

Single nucleotide polymorphisms (SNPs) can be used to identify candidate genes by identifying alleles that are associated with an increased susceptibility to prostate cancer [47]. Using GWAS that include SNPs, more than 100 prostate cancer susceptibility loci have been identified, explaining an estimated 30% of the familial risk for this disease [48]. Based on combined risks conferred by known risk loci, the top 1% of the risk distribution has a 4.7-fold higher risk than the average of the population [49]. Among the genes that have been identified in this way are HOXB13, MSMB, LMTK2, KLK3, CPNE3, IL16, CDH13, and HNF1B [49–53].

BRCA1 and BRCA2 Genes

The BRCA1 and BRCA2 genes are tumour suppressor genes inherited in an autosomal dominant pattern with reduced penetrance [54, 55]. The development of cancer in individuals with germline mutations in the BRCA genes requires somatic mutation of the remaining wild-type allele [56]. The BRCA1 and BRCA2 genes encode proteins that maintain genomic stability by promoting repair of DNA double-strand breaks [57]. The main functions of BRCA1 are DNA damage response and repair, transcriptional regulation and chromatin modelling [58, 59]. The role of BRCA2 is more limited to DNA repair by homologous recombination including regulating RAD51 activity, an important component of the DNA repair process [60]. Therefore, functional loss of BRCA1 or BRCA2 leads to a deficiency in repairing DNA double-strand breaks by conservative mechanisms, allowing cells to repair these lesions through other methods which are potentially mutagenic. This genomic instability may explain the increased risk of cancer caused by deleted mutations in the BRCA genes, although it is unclear why these mutations are particularly associated with certain cancers such as breast and ovarian cancer and, less commonly, prostate cancer [61].

BRCA Genes and Cancer Risk

Germline mutations in the BRCA genes have been shown to be associated with an increased risk of breast and ovarian cancer [33, 62]. The extent to which BRCA mutation carriers are at an increased risk of other cancers has been less clear; however, the presence of BRCA1 or BRCA2 mutations in men has been shown to be associated with an increased risk of developing prostate cancer [31, 43, 63–70]. As an example, in a cohort study that involved 3728 men from 173 breast–ovarian cancer families with BRCA2 mutations, the estimated relative risk (RR) of prostate cancer among BRCA2 carriers was 4.7-fold greater than controls [71] (see Table 5.3). The risk of developing prostate cancer in BRCA1 mutation carriers appears to be lower (see Table 5.4). In a multinational cohort study of 11,847 individuals with BRCA1 mutations, the risk of prostate cancer was elevated 1.8-fold in men under the age of 65, but this increase was not observed in older men [73].

Prostate cancer in men with BRCA2 mutations also appears to be associated with more aggressive histology and a substantially worse prognosis [63, 74–76]. In a study from Iceland that included 30 men with a mutation in BRCA2, prostate cancer was diagnosed at an earlier age (69 versus 74 years) and was associated with a significantly shorter survival (2.1 versus 12.4 years) [77]. Similarly, in a multinational cohort study of men with prostate cancer that included 183 men from known BRCA2 families and 119 from BRCA1 families, those from BRCA2 families had a significantly shorter survival (4.0 versus 8.0 years) [78]. A Spanish study of 2,000 men with prostate cancer confirmed the worse prognosis in mutated BRCA2 patients

Table 5.3 Case-control studies and case series in men with BRCA2 mutation

Study	Year	Prostate cancer risk (BRCA2)
BCLC [71]	(1999)	Overall: RR, 4.65 (95% CI, 3.48–6.22) Men <65 y: RR, 7.33 (95% CI, 4.66–11.52)
Thompson et al. [65]	(2001)	OCCR: RR, 0.52 (95% CI, 0.24–1.00)
Giusti et al. [66]	(2003)	OR, 2.02 (95% CI, 0.16–5.72)
Kirchhoff et al. [31]	(2004)	OR, 4.78 (95% CI, 1.87–12.25)
Agalliu et al. [63]	(2009)	OR, 1.92 (95% CI, 0.91–4.07)
Gallagher et al. [64]	(2010)	OR, 3.18 (95% CI, 1.52–6.66)
Johannesdottir et al. [69]	(1996)	999de15: RR, 2.5 (95% CI, 0.49–18.4)
Eerola et al. [67]	(2001)	SIR, 4.9 (95% CI, 1.8–11.0)
Agalliu et al. [72]	(2007)	RR, 7.8 (95% CI, 1.8–9.4)
Kote-Jarai et al. [70]	(2011)	RR, 8.6 (95% CI, 5.1–12.6)

CI confidence interval, *OR* odds ratio, *RR* relative risk, *BCLC* Breast Cancer Linkage Consortium, *OCCR* ovarian cancer cluster region, *SIR* standardized incidence ratio

Table 5.4 Case-control studies and case series in men with BRCA1 mutation

Study	Year	Prostate cancer risk (BRCA1)
Thompson et al. [73]	(2002)	Overall: RR, 1.07 (95% CI, 0.75–1.54 Men <65 y: RR, 1.82 (95% CI, 1.01–3.29)
Giusti et al. [66]	(2003)	185delAG: OR, 2.52 (95% CI, 1.05–6.04)
Kirchhoff et al. [31]	(2004)	OR, 2.20 (95% CI, 0.72–6.70)
Agalliu et al. [63]	(2009)	OR, 1.39 (95% CI, 0.60–3.22)
Gallagher et al. [64]	(2010)	OR, 0.38 (95% CI, 0.05–2.75)
Eerola et al. [67]	(2001)	SIR, 1.0 (95% CI, 0.0–3.9)
Cybulski et al. [43]	(2013)	OR, 0.9 (95% CI, 0.4–1.8)
Leongamornlert et al. [68]	(2012)	RR, 3.75(95% CI, 1.02–9.6)

CI confidence interval, *OR* odds ratio, *RR* relative risk, *SIR* standardized incidence ratio

with a significant survival advantage if patients were noncarriers (15.7 versus 8.6 years) [76].

The IMPACT trial (Identification of Men with a genetic predisposition to ProstAte Cancer: Targeted screening) is looking at the feasibility and role of PSA screening in men who are carriers for BRCA1 or BRCA2 mutations [79]. Results from the initial screening round in this study showed a detection rate for prostate cancer of 2.4%. There was an evidence of a more aggressive phenotype in these patients with more than two-thirds of the prostate cancer detected in the BRCA2 carriers being classified as intermediate or high risk. Furthermore, the only cancers detected in men younger than 50 years of age were in BRCA1 and BRCA2 carriers. A study by Castro et al. showed that BRCA carriers treated for localized prostate cancer have worse outcomes than noncarriers because they relapse and progress earlier to lethal metastatic disease [75]. This data adds to the increasing evidence that BRCA1 and BRCA2 mutation carriers develop more aggressive disease at a younger age suggesting that screening may be beneficial in this subgroup [79].

BRCA and Tumourigenesis

It has been proposed that the BRCA genes may act as tumour suppressors in prostate cells and that their functional loss predisposes to the development of premalignant prostatic lesions [80, 81]. It has been shown in animal studies that the simultaneous deletion of BRCA2 and the tumour suppressor p53 give rise to focal hyperplasia and high-grade PIN [80]. Furthermore, evidence has shown that functional BRCA1 and BRCA2 proteins may limit the metastatic potential of neoplastic cells [81]. This is achieved by downregulating MMP-9 production through inhibition of PI3-kinase/AKT and activation of MAPK/ERK pathways, which prevents cancer cell migration and invasion [82, 83].

Poly(ADP-ribose) Polymerase 1 (PARP1)

The DNA repair defect associated with mutations in BRCA1 or BRCA2 is being used to develop new targeted therapeutic approaches for prostate cancer [84, 85]. Poly(ADP-ribose) polymerase 1 (PARP1) is a nuclear enzyme which assists in the maintenance of genomic stability by identifying sites of DNA damage and recruiting repair mechanisms [86]. A number of studies indicate that tumour cells with a defect in homologous recombination, such as tumours bearing BRCA1 or BRCA2 mutations, depend on compensatory DNA repair of double-strand breaks, for which the enzyme PARP1 is essential [87, 88]. Consequently, tumours with defects in homologous recombination are hypersensitive to drugs that inhibit PARP [89]. PARP has also been implicated in the transcription regulation of the androgen receptor (AR) and has also shown antitumor activity in preclinical models of TMPRSS2-ERG-rearranged prostate cancer [90]. Additionally, PARP inhibitors suppress AR-target gene expression and tumour proliferation [91]. This had led to several studies examining the role of PARP inhibitors in prostate cancer [92–94]. Olaparib is a PARP inhibitor which has shown antitumor activity in both germline and sporadic cases of metastatic, castration-resistant prostate cancer with DNA-repair defects [93].

Susceptibility Genes

Androgen Receptor (AR) Gene

The androgen pathway and its function in the development and progression of prostate cancer has been well established, and overexpression of the AR gene has been associated with poor prognosis [95]. Altered activity of the androgen receptor caused by inherited variants of the AR gene, located on the X chromosome, may increase the risk of prostate cancer [96]. The length of the polymorphic trinucleotide CAG and GGN microsatellite repeats in exon 1 of the AR gene has been

associated with an increased risk of prostate cancer; however, data from other studies has been conflicting [97, 98]. Germline mutations in the AR gene associated with an increased risk of prostate cancer have been identified. In a Finnish study, the R726L substitution in the AR gene may confer up to sixfold increased risk of prostate cancer and may contribute to cancer development in up to 2% of prostate cancer patients [99]; however, a subsequent Finnish study did not replicate these results [100]. Therefore, germline AR mutations may only contribute to a small fraction of familial and early-onset cases of prostate cancer.

RNASEL Gene

The RNASEL gene (encodes for RNase L enzyme) has been mapped to the HPC1 (hereditary prostate cancer 1) region at 1q24–25 and regulates cell proliferation and apoptosis through the interferon-regulated 2–5A pathway [101, 102]. Interestingly, much of the evidence for a role for RNASEL in prostate cancer seems to be in cases with a positive family history, supporting the initial discovery in hereditary patients [103]. Multiple variants of the RNASEL gene have been described including Arg462G, 471delAAAG, R462Q, E265X and D541E and may be involved in up to 13% of prostate cancer cases, though the true role of RNASEL genetic variation and its influence on prostate cancer risk have been controversial [104, 105]. The R462Q variant was originally associated with an increasing risk of prostate cancer due to a significant decrease in RNASE L enzymatic activity; however, this finding has not been universally replicable [104, 106, 107]. Furthermore, results of a meta-analysis of ten independent RNASEL genotyping studies for the variants E265X, R462Q and D541E suggested that although there was no overall effect on prostate cancer risk, there was a less than twofold increase in the risk of developing prostate cancer in Caucasians with the D541E variant [108]. Missense mutations in R462Q and D541E have been shown to be associated with an increased risk of advanced-stage disease only in the pre-PSA era with no effect on survival [109].

TMPRSS2-ERG Gene Fusion

Fusions of the androgen-regulated gene TMPRSS2 to the oncogenic ETS transcription factor ERG occur in over 50% of prostate cancers [110]. It has been found to vary according to ethnic groups, to be associated with p53 mutation expression and to have a more aggressive phenotype [111, 112]. Significant association of TMPRSS2-ERG fusion-positive prostate cancer with rare variants in the DNA repair genes POLI (variant F532S) and ESC01 (variant N191S) has also been found [113]. Furthermore, linkage analysis has found the presence of an inherited susceptibility to develop the TMPRSS2-ERG fusion with several loci located on chromosomes #9, #18 and X [114, 115]. Therefore, familial aggregation of TMPRSS2-ERG could be due to an inherited chromosomal instability caused by variations in the DNA repair pathway leading to genomic instability. ERG has been also shown to

interact with the PARP1 enzymes in the DNA repair pathway, and interestingly PARP1 inhibitors have been shown to inhibit ERG-positive prostate cancer xenograft growth in a manner similar to that of BRCA1/2 deficiency [90].

HOXB13

The homeobox B13 (HOXB13) gene codes for a transcription factor that is important in prostate development [116–118]. Linkage to 17q21–22 was initially reported by the Prostate Cancer Genetics Project at the University of Michigan from pedigrees of families with hereditary prostate cancer [119, 120]. Next-generation sequencing of the 17q21–22 region identified the G84E variant of the HOXB13 gene in families with hereditary prostate cancer [116, 121]. Researchers have demonstrated that the HOXB13 G84E mutation is present in about 5% of prostate cancer families, predominantly of European descent, and have shown it to be associated with an increased prostate cancer risk [122] (see Table 5.5). In Europe, the prevalence of the HOXB13 G84E is highest in the Nordic countries, especially Finland and Sweden with a prevalence among men diagnosed with familial prostate cancer of 8.4% [125]. In the Reduction by Dutasteride of Prostate Cancer Events (REDUCE) study, an international multicentre chemoprevention trial of 3508 subjects, the HOXB13 G84E mutation was only present in Caucasians, with the highest prevalence in Northern Europeans, followed by Western Europeans and North Americans with no carriers identified in Africa, Australia, Latin America and the rest of the European population [124]. A number of studies have

Table 5.5 HOXB13 and risk of prostate cancer

Study	Year	Prostate cancer risk (HOXB13)	Men with family history of prostate cancer
Akbari et al. [123]	(2012)	OR, 5.8 (95% CI 1.3 to 26.5)	Not assessed
Breyer et al. [122]	(2012)	OR, 7.9 (95% CI 1.8–34.5)	OR, 11.8
Chen et al. [124]	(2013)	OR, 4.14 (95% CI: 1.38–12.28)	Not assessed
Laitinen et al. [125]	(2013)	OR, 7.1 (5.5–9.3)	OR, 8.8 (95% CI 4.9–15.7)
Kote-Jarai et al. [126]	(2015)	OR, 2.93 (95% CI 1.94–4.59)	OR, 4.53 (95% CI 2.86–7.34)
Karlsson et al. [127]	(2014)	OR, 3.5 (95% CI, 2.4–5.2)	OR, 6.6 (95% CI, 3.3–12.0)
MacInnis et al. [128]	(2013)	ASI, 16.4 (95% CI 2.5–107.2)	Not assessed
Hoffman et al. [129]	(2015)	OR, 3.63 (95% CI 2.48–5.85)	Not assessed
Huang et al. [130]	(2014)	RR, 4.51 (95 % CI 3.28–6.20)	OR, 7.27 (95 % CI 4.02–13.15)

CI confidence interval, *OR* odds ratio, *RR* relative risk, *ASI* age-standardized incidence

confirmed an increased risk of prostate cancer in patients with the HOXB13 G84E variant [116, 122, 124, 126]. In a study of 5083 unrelated subjects with prostate cancer and 1401 controls, there was a 20-fold increase in the frequency of the HOXB13 G84E mutation in men with prostate cancer compared with those without it (1.4 versus 0.1 percent) [116]. Similarly, in the 4-year follow-up of the REDUCE study, the prostate cancer detection rate was 53.8% among mutation carriers and 22.0% among noncarriers, with a relative risk of 2.45 [124]. In a second case-control study of familial prostate cancer, investigators genotyped 928 familial prostate cancer probands and 930 control probands without a personal or family history of prostate cancer and found the point estimate of the odds ratio, adjusted for age, was 7.9 among carriers of the mutation [122]. The estimate was greater among cases with a family history of three or more relatives affected (OR = 11.8), compared to a family history of only two affected (OR = 5.8). In a British case-control study assessing the prevalence of HOXB13 G84E, investigators identified the variant in 0.5% of healthy controls and 1.5% of prostate cancer cases and found the presence of HOXB13 G84E to be associated with a 2.93-fold increased risk of prostate cancer [126]. The risk was even higher among men with family history of prostate cancer supporting the hereditary link.

The penetrance estimates for prostate cancer development in HOXB13 G84E mutation carriers have also been reported. A study from Sweden found HOXB13 G84E to be prevalent in more than 1% of the population and to be associated with a 3.5-fold increased risk of prostate cancer with an estimated 33% lifetime risk of prostate cancer [127]. Furthermore, an Australian study reported age-specific cumulative risk of prostate cancer of up to 60% by the age 80 years [128].

HOXB13 expression has been linked to advanced pT stage, high Gleason grade, positive lymph node status, high preoperative PSA levels, TMPRSS2:ERG fusion, PTEN deletions, AR expression, cell proliferation, reduced PSA expression and early PSA recurrence; however, it has not been found to have an effect on prognostic outcomes and overall or cancer-specific survival [126, 131, 132]. It has also been demonstrated that the prostate cancer risk-associated T allele of rs339331 enhances HOXB13 chromatin binding and drives allele-specific upregulation of the rs339331-associated gene RFX6 which might have a role in prostate cancer cellular transformation [130]. It appears that HOXB13 has an important role in prostate cancer development; however, the mechanism by which it contributes to the pathogenesis of prostate cancer remains unknown.

Mismatch Repair (MMR) Genes and Prostate Cancer

Lynch Syndrome

Lynch syndrome is an autosomal dominant disorder caused by a germline mutation in one of the mismatch repair (MMR) genes, MLH1, MSH2, MSH6 or PMS2 [133]. Chromosomal deletion, point mutation or epigenetic inactivation by hypermethylation in a second allele predisposes to a lack of MMR protein function, leading to

an accumulation of mutations [134]. This can lead to malignant transformation of cells and tumour formation with a mutated phenotype, demonstrated by the presence of microsatellite instability (MSI) and lack of one or more of the four MMR proteins on staining by immunohistochemistry (IHC) [135]. There is an increased risk of several cancers in patients with Lynch syndrome including colorectal, endometrial, ovarian, gastric, small intestinal, pancreatic, ureteral, brain and sebaceous gland adenocarcinomas [136]. Screening for colorectal cancer and prophylactic surgery for gynaecological cancers have been shown to improve outcomes in these patients [137, 138]. Prostate cancer is currently not considered part of the Lynch syndrome spectrum, and data for the association has been inconclusive [139–141]. However, a number of studies have shown the cumulative lifetime risk of prostate cancer to be increased in individuals with Lynch syndrome, ranging from twofold to fivefold higher than in the general population [142–145].

Loss of MMR protein expression has been shown in prostate cancer tumours in patients with Lynch syndrome [146, 147]; however, this has been rarely detected in patients with hereditary prostate cancer [148], suggesting that Lynch syndrome is unlikely to be implicated in the majority of cases of familial prostate cancer [139]. Furthermore, patients with Lynch syndrome do not appear to have an earlier onset of prostate cancer or a more aggressive phenotype [143].

MSH2

There is some evidence that prostate cancer is more commonly diagnosed in men with an MSH2 mutation compared to men with a mutation in one of the other MMR genes [142, 143, 149–152]. A German study identified cases of prostate cancer among men who were positive or obligate carriers of MSH2 mutations; however, they found no increased incidence of prostate cancer [153]. The investigators noted a median age of 59 years at diagnosis, younger than the average age at diagnosis, suggesting a marginal association between MSH2 mutation and risk of prostate cancer. Rosty et al. have shown that MMR gene mutation carriers have at least a twofold or greater increased risk of developing MMR-deficient prostate cancer, with the risk being highest for MSH2 mutation carriers [154]. Except for Rosty et al. most studies have been underpowered to observe any differences in prostate cancer risk by specific MMR gene mutations [145]. Large cohorts will be required to measure separate prostate cancer risks for specific MMR gene mutation carriers.

Fanconi Anaemia

Fanconi anaemia (FA) is a rare disorder of chromosomal instability characterized by bone marrow failure, developmental anomalies and an increased incidence of myelodysplasia, leukaemia and solid tumours [155, 156]. The prevalence of FA is

1–5 cases per 1 million persons, and the heterozygous carrier frequency is about 1 case per 300 persons [157]. Germline mutations, somatic mutations and epigenetic silencing have all been shown to occur in FA genes [158]. FA is caused by biallelic mutation of any 1 of the 16 known genes and can be either autosomal or X-linked recessive, depending on the inherited gene. Of the 16 genes (FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCL, FANCM, FANCN/PALB2, FANCJ/BRIP1, FANCO/RAD51C, FANCP/SLX4 and FANCQ/ERCC4), three of them, FANCD1, FANCN and FANCJ, are identical to the DNA repair genes BRCA2, PALB2 and BRIP1 [159–161]. The protein products of these genes function cooperatively in the FA-BRCA pathway which plays a central role in DNA repair and the maintenance of genomic integrity [159].

The FA Pathway

After DNA damage, FA proteins form a nuclear complex that mediates the monoubiquitylation of the FA protein FANCD2 [159–161]. This monoubiquitylated FANCD2 colocalizes in nuclear foci with proteins involved in DNA repair, including BRCA1, FANCD1/BRCA2, FANCN/PALB2 and RAD51 [161–168]. FANCJ interacts directly with BRCA1 and is a member of the DNA helicase family [169]. FANCN interacts with FANCD1/BRCA2 and is required for its homologous recombination and checkpoint functions [170]. In the absence of an intact FA pathway, cells are sensitive to spontaneous and DNA damage-induced chromosomal breaks leading to tumourigenesis [171]. Clinical trials are now testing the use of PARP inhibitors in patients with FA pathway defects [172].

FA Genes and Prostate Cancer

Initial studies of cancer risk in FA heterozygotes found a higher rate of cancers; however, subsequent studies have not confirmed this risk [173–176]. Due to the conflicting data from other studies and the relative rarity of FA, it is difficult to confirm these findings. In a British study of FA families, there was no higher incidence of cancer detected; however, 2 prostate cancer cases were observed in 33 obligate carriers, with an overall relative risk of prostate cancer in carriers which was calculated to be 3.089, an incidence which was higher than expected [177]. In a founder population cohort study of Finnish FA patients, the prevalence of 6 FA-causing mutations in over 1800 breast cancer and 565 prostate cancer cases was analysed [178]. All mutations were recurrent, but no significant association with cancer susceptibility was observed for any. Further analysis from the prostate cancer cohort revealed several carriers both among affected and unaffected males, but the frequencies were roughly the same and without any statistical significance. Although clearly deleterious, the tested heterozygous mutations in the FA pathway do not act as

high- or moderate-risk alleles for prostate cancer in the general population; however, there could be a modest increased risk in prostate cancer in some FA heterozygotes which merits further investigation in larger cohort studies [179].

DNA Adducts

Polycyclic aromatic hydrocarbons (PAH) and heterocyclic amines (HCA) are environmental contaminants and known carcinogens (1). PAHs and HCAs are thought to derive their carcinogenic properties through their ability to form DNA adducts. 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is the major HCA generated from cooking meats at high temperatures, and exposure has been shown to induce prostate cancer in animal studies. PhIP induces cancer by the formation of PhIP-DNA adducts [180]. This formation of DNA adducts can lead to DNA replication errors and increase the potential for carcinogenesis. DNA adducts have been detected in prostate cells, but the exact nature of adducts with respect to prostate cancer risk factors and histology is unclear [181]. African ancestry is strongly associated with PhIP-DNA adduct levels in non-tumour prostate cells [182]. Furthermore, the SULT1A1 genotype and enzyme activity has been suggested to be associated with DNA adduct levels and ethnicity [183]. However, further studies indicate the SULT1A1 genotype does not appear to be associated with increased genetic susceptibility to prostate cancer [182, 184], and overall, elevated levels of PhIP-DNA adducts do not appear to significantly increase prostate cancer risk, independent of ethnicity [185].

Conclusion

Case-control studies, linkage analyses, admixture mapping and GWAS have identified a number of candidate genes associated with prostate cancer susceptibility. Similarly, studies of ethnic and founder populations have identified inherited genetic factors associated with a higher risk of prostate cancer. However, the replication of these findings remains inconsistent, with numerous genes likely to be involved. GWAS have provided evidence supporting the genetic complexity of prostate cancer. It is also likely that there could be significant variation in the contribution of various genes and SNPs to prostate cancer risk in various ethnic groups. Additional studies will be required to determine whether genes or SNPs can be combined with PSA levels and other clinical factors to identify men who are at particularly high risk of being diagnosed with prostate cancer. The finding that the FA–BRCA pathway is intimately involved in the response to DNA damage and repair and may confer potential susceptibility to prostate cancer has spurred further research in this area. Furthermore, tumour cells with a disrupted DNA repair pathway are hypersensitive to PARP inhibitors, and these agents have been shown

to be efficacious in prostate cancer. Moving forward, genes identified through GWAS may eventually have a role in prostate cancer screening and as targets for therapeutic targets.

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Chapter 6
Racial Differences

Francesca Khani and Brian D. Robinson

Contents

Differences in the Epidemiology of Prostate Cancer Among Ethnicities

Incidence and Mortality Across Ethnic Populations Within the United States

Within the United States, the prostate cancer incidence rate is the highest in African American men (AAM), followed by Caucasian American men (CAM) and Hispanic men, and the lowest incidence rate in American Indians/Alaska Natives and Asian/Pacific Islanders (Fig. 6.1) [1]. Although over the past decade, the incidence of prostate cancer has been declining overall, having decreased significantly from 2002 to 2011 by 3.4% among all men, it has decreased the least by only 2.9%

F. Khani, M.D. (✉) • B.D. Robinson, M.D.
Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, New York Presbyterian Hospital, New York, NY, USA
e-mail: frk9007@med.cornell.edu

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*, Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_6

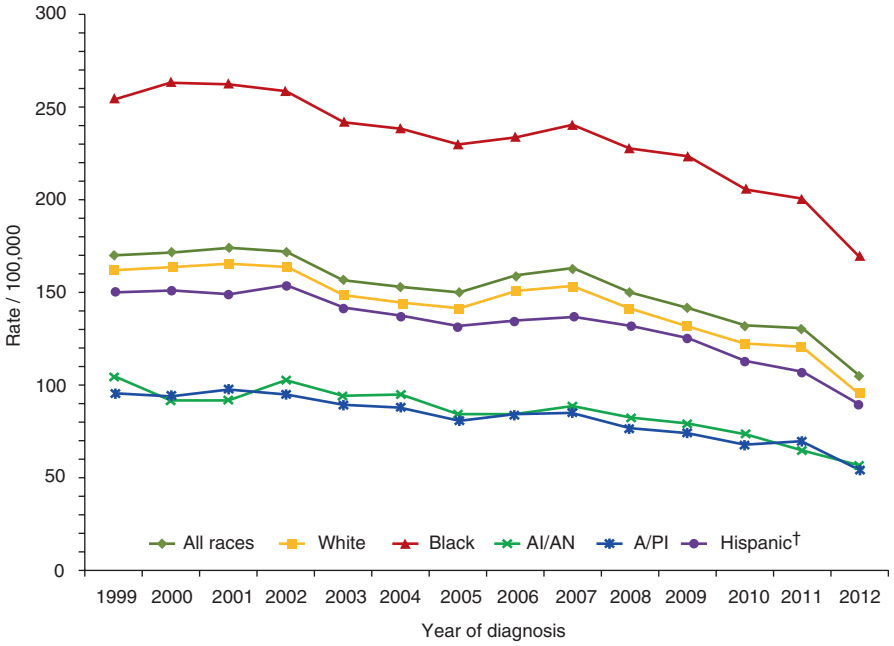

Fig. 6.1 Prostate cancer incidence rates by race and ethnicity in the United States from 1999 to 2013. Reproduced from the Center for Disease Control (CDC) (Accessible at www.cdc.gov)

among AAM compared to the 3.8% decrease among CAM, 4.2% decrease in Hispanic men and in American Indian/Alaskan Native men, and 3.7% decrease among Asian/Pacific Islander men [2], although the reasons for the observed decreased incidences remain largely unknown.

With respect to prostate cancer mortality in the United States, AAM also have the highest rate of death from prostate cancer than any other ethnic group, followed by CAM, Hispanic, American Indian/Alaska Native, and Asian/Pacific Islander men (Fig. 6.2) [1]. Like the incidence trends, mortality trends have improved over the past decade, having decreased significantly by 3.3% among all men. In contrast, the trend in mortality rate has improved the most for AAM with a 3.8% decrease compared to a lower rate of decrease in all other ethnic groups [2], suggesting that improvements indeed have been made in earlier detection and treatment of prostate cancer, particularly in AAM.

Overview of Potential Etiologies of Observed Racial Differences

Given that AAM still have the highest incidence and mortality from prostate cancer than all other ethnic groups, most studies in the current literature have focused on identifying the reasons for the disparity observed particularly between AAM and CAM. Of course, a variety of sociological factors have been postulated to contribute to incidence and/or mortality differences, including access to care, attitudes toward

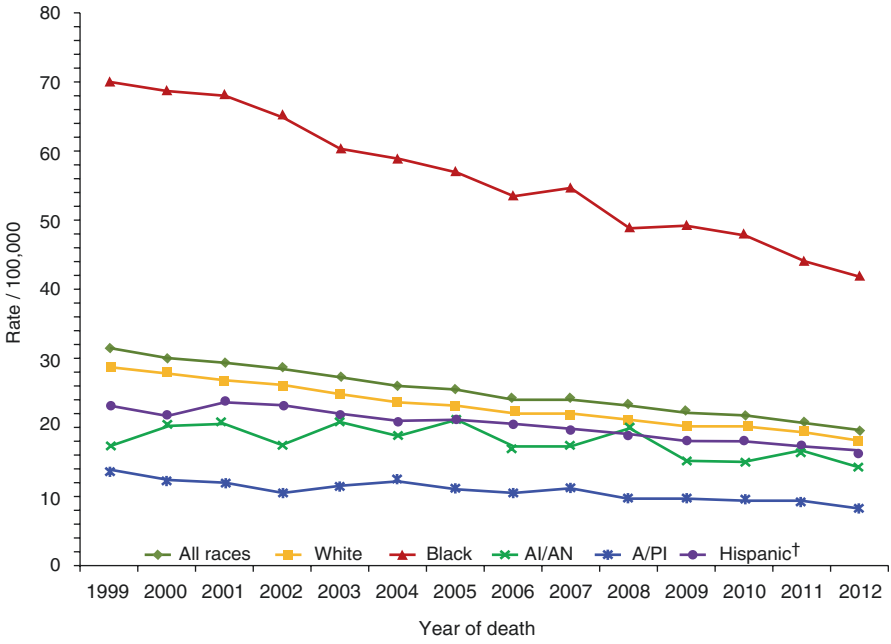

Fig. 6.2 Prostate cancer death rates by race and ethnicity in the United States from 1999 to 2013. Reproduced from the Center for Disease Control (CDC) (Accessible at www.cdc.gov)

care, socioeconomic factors and educational disparities, differences in type and aggressiveness of treatment, and dietary fat intake [3]. While there is literature to support that the racial disparities observed may be attributable to such sociological factors [4–6], these factors do not completely account for the incidence and prognostic differences observed [7–12]. Furthermore, in one study which used a large international cohort of patients, AAM and Afro-Caribbean men presented with more aggressive disease features than CAM and had lower rates of biochemical recurrence, even when other clinicopathologic variables were controlled [13]. In combination with studies that have demonstrated that prostate cancer in AAM may exhibit a faster growth rate and/or earlier transformation from latent to aggressive disease when compared to CAM [14–16], molecular and genetic differences between prostate cancers in AAM and CAM likely play a prominent role in these disparities. The current literature on the specific molecular and genetic differences observed will be discussed further in section "Molecular and Genetic Differences" of this chapter.

Incidence and Mortality in Populations Globally

It is perhaps more difficult to accurately compare the true global incidence and mortality of prostate cancer among different ethnic groups due to the absence of centralized data collection stratified by race as well as differences in disease detection/screening. However, nationality may serve as a proxy for race in more

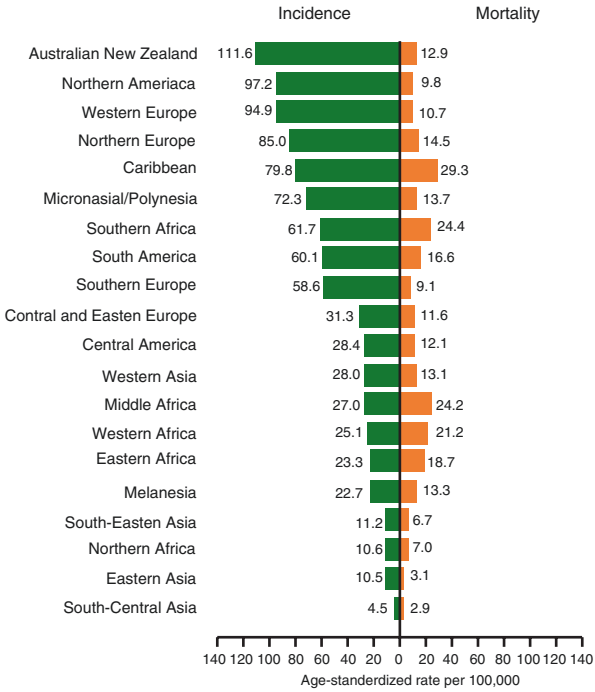

Fig. 6.3 Global cancer statistics prostate cancer incidence and mortality rates by world area. (Reproduced from Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin. 2015 Feb 4;65(2):87–108)

homogenous regions of the world. Global cancer statistics on prostate cancer show that it is more commonly diagnosed in developed countries with the highest incidence in Australia/New Zealand, Northern America, Northern and Western Europe, and some Caribbean nations, and the lowest in Asian nations (Fig. 6.3) [17], although much of this variation reflects differences in the use of prostatic-specific antigen (PSA) screening [18]. Global incidence data also helps in identifying genetic susceptibility to prostate cancer that exist within certain populations and suggest that a heritable susceptibility loci exist within the Northern European genome [19] and within people of West African and Afro-Caribbean descent [18, 20–22]. Of note, men of West African ancestry from the Caribbean and South America have a similar incidence and mortality from prostate cancer to African Americans [23]. Specific susceptibility loci have been identified within these populations and others which will be further discussed in section "Molecular and Genetic Differences."

It is noteworthy that East Asian populations overall have the lowest incidence of prostate cancer on the global spectrum, and the lack of widespread PSA screening in these countries does not fully account for the low incidence [24]. Although the incidence of prostate cancer is increased in Asian immigrants in the United States compared to those in their native countries [25–28], Asian immigrants in the United States still have a lower incidence of the disease compared to AAM and CAM. Risk factors associated with economic development have been postulated to explain the increase in incidence among Asian immigrants, including increased

consumption of animal fat, obesity, and physical inactivity [18]. In addition, soy foods, which are more frequently consumed in Asian cultures, have been reported in several studies to be associated with a 25–30% reduced risk of prostate cancer [29–31], an interesting observation considering that soy products contain phytoestrogens that may mimic estrogen in the body and have been found to inhibit numerous prostate cancer cell lines' growth [32] and prostate carcinogenesis in rats [33]. The etiology of the decreased incidence of prostate cancer among Asian populations is likely multifactorial, but biological differences in heritable susceptibility loci as well as acquired genetic and epigenetic changes have also been observed and are believed to contribute [24], further discussed in the section, "Molecular and Genetic Differences."

Molecular and Genetic Differences

The molecular and genetic differences observed in prostate cancer between different races may be broadly classified into subcategories including inherited genomic alterations, acquired molecular alterations, DNA methylation, microRNA expression, carcinogen-DNA adduct levels, and protein/biomarker expression.

Inherited Genomic Alterations

Mutations in Androgen Receptor Pathway Genes

Heritable mutations and variations in genes involved in androgen receptor pathways are believed to contribute to prostate cancer pathogenesis (see Part IV, Chap. 20). Differences have been observed mostly between CAM and AAM, specifically in androgen receptor (AR), CYP3A4, SRD5A2, and CYP17.

Androgen Receptor (AR)

In the AR gene, differences have been observed among different ethnic populations in the lengths of two polymorphic trinucleotide repeats (CAG and GGC), which encode polyglutamine and polyglycine tracts, respectively, in the N-terminal domain of the androgen receptor protein [34]. Shorter repeats lead to increased transcriptional activity of AR [35], and several studies have shown that AAM have significantly shorter repeat lengths than that observed in CAM [34, 36, 37]. While some earlier studies had shown shorter repeat lengths to be associated with a higher risk for developing prostate cancer, distant metastases, and fatal disease [38–40] and were particularly associated with higher stage disease in AAM in one study [41], while other more recent studies have demonstrated an absence of correlation

between repeat length and prostate cancer risk in AAM [42–44]. In a relatively large study by Powell et al. involving AAM and CAM with prostate cancer, repeat lengths did not correlate with disease extent or other clinicopathologic factors, and longer repeat lengths were found to be associated with a 52% increased risk of recurrence in CAM and AAM combined [45]. The conflicting data on the significance of the number of CAG/GGC repeat lengths in AR and prostate cancer risk and prognosis suggest that it likely is not a major contributor to the increased incidence and worse prognosis of prostate cancer in AAM compared to CAM.

CYP3A4

CYP3A4 encodes a protein in the cytochrome P-450 family and is associated with oxidative deactivation of testosterone. A particular germline genetic variant involving a single nucleotide polymorphism (SNP) in the 5′ flanking region of CYP3A4 (A to G transition) was found to be associated with higher clinical stage and grade of prostate cancer in CAM [46]. This SNP confers the variant G allele (referred to as the CYP3A4 G variant or CYP3A4*1B), which is most frequently present in AAM when compared to CAM, Hispanic, or Asian American men [47–49]. The variant G allele has also been associated with higher clinical grade and stage in older men (>65 years) and was predictive of disease progression [47]. While the variant G allele was strongly associated with prostate cancer exhibiting aggressive characteristics at diagnosis in AAM and little association with prostate cancer risk in CAM in one study [49], another study by the same group showed that increasing copies of the G allele was associated with poorer progression-free survival among CAM with prostate cancer but not among AAM [48]. Although the variant G allele in CYP3A4 is more commonly present in AAM and has been associated with prostate cancer risk and aggressive disease in both CAM and AAM, it is uncertain as to whether it exerts any causative functional effect on the biology of the disease versus exhibiting a mere association with African American race.

SRD5A2

SRD5A2 encodes an isozyme of 5 alpha-reductase which is expressed in the prostate and involved in androgen conversion of testosterone to its more active metabolite, dihydrotestosterone (DHT). Intraprostatic levels of DHT have been suggested to have a role in racial variations of prostatic cancer risk [50]. While particular SNPs and TA repeat alleles in this gene have been identified, with differing frequencies observed among AAM, CAM, Hispanic, and Asian populations [51–54], a meta-analysis revealed little to no effect on prostate cancer susceptibility [55]. Interestingly, however, a more recent large study examined SNPs in both SRD5A2 and a related gene, SRD5A1, and identified several SNPs which were independently associated with biochemical recurrence in white and Asian men [56], suggesting that there may be prognostic significance to certain SNPs in these genes.

CYP17

CYP17 encodes the cytochrome P450c17a enzyme which enables testosterone biosynthesis in the gonads and adrenal glands. Polymorphisms in this gene have been associated with prostate cancer risk in multiple studies [57–60] and have been found to increase prostate cancer susceptibility particularly among AAM in other studies and in a meta-analysis [61–63].

CYP17 variants have also been identified in Asian populations. One recent study found a particular CYP17 variant, (along with variants in HSD17B2 and ESR1, two other steroidogenic pathway genes), to be associated with disease progression in CAM and decreased survival in Taiwanese men; this polymorphism (CYP17A1) was also linked to DHEA-S circulating hormonal levels in these populations as well [64], suggesting that there may be some clinical significance to this variant, warranting future studies. Furthermore, the CYP17A2 variant was shown to be associated with prostate cancer risk in a case-control study within the Japanese population [65].

Chromosome 8q24 Variants

A variant on chromosome 8q24 families (allele −8 of the microsatellite DG8S737) was initially identified in a study of Icelandic families and was found to confer a heritable risk of prostate cancer, based on three case-control series in different populations. In this study, Amundadottir et al. discovered that 13% of general population and 19% with prostate cancer carry one copy of this variant compared to 30% of AAMs and 41% of affected AAMs carrying one copy, resulting in a 16% population attributable risk which likely contributes to higher incidence of PCa in AAM [66]. This study was further supported by an admixture mapping study by Freedman et al. which indicated a major unidentified risk gene for cancer at this locus in AAM [67]. A follow-up study in this cohort demonstrated SNPs within the 8q24 region which portended a higher risk of prostate cancer among AAM than in CAM [68], and several additional independent studies and further validation studies have confirmed the association between African American ancestry and prostate cancer susceptibility loci in the 8q24 region [68–72]. 8q24, as well as 17q susceptibility loci, have additionally been found to contribute to prostate cancer risk in multiethnic Asian cohort of Singaporean Asian men [73], indicating that the risk is not unique to AAM.

Although the significant 8q24 variants are not in regions that align to a known gene and do not alter the coding sequence of a protein [68], some prognostic significance to these variants have been identified. In a study by Helfand et al. in which tumor characteristics were examined in carriers of 8q24 susceptibility alleles, they found these tumors to be significantly more likely to have a Gleason score of 7 or higher and lymph node metastasis [74]. In another study by Whitman et al., a particular polymorphism was identified only in people of African ancestry, where it was associated with an increase in non-organ-confined prostate cancer at prostatectomy and a trend toward early biochemical recurrence [75]. The mechanism for how these variants influence the risk and aggressiveness of prostate cancer is uncertain, and it may be a futile pursuit unless it would somehow improve disease management.

EphB2

EphB2 maps to chromosome 1p36 and encodes the tyrosine kinase receptor EPHB2. EPHB2 is believed to have an essential role in cell migration and maintenance of normal tissue architecture and is believed to function as a tumor suppressor gene involved in prostate carcinogenesis and progression [76]. A germline nonsense mutation (3055A.T; K1019X) was found to be associated with a threefold increased risk of familial prostate cancer in AAM from high-risk families [77]. In a later follow-up study by the same group, common variation within the EphB2 locus was found to be associated with the risk of sporadic prostate cancer in AAM where certain SNPs had significant protective effects, while others increased the risk of developing the disease [78]. A genome-wide association study (GWAS) in a Japanese population also identified a linkage susceptibility locus on 1p36 [79].

Glutathione S-Transferase (GST) Genes

GST genes encode enzymes associated with detoxification activity, affecting the level of carcinogenic metabolites created by endogenous steroid hormones and exogenous chemical substances. In a study with long-term clinical follow-up by Agalliu et al. the null phenotype of GSTM1 was associated with increased prostate cancer mortality in a Caucasian population, while GSTP1 and GSTT1 genotypes were not associated with any significant outcomes [80]. Similarly, a meta-analysis on polymorphisms in the GST genes found that the GSTM1 null genotype conferred an increasing risk of prostate cancer in CAM and Asians, and no relationship was found between GSTT1 and GSTP1 status and risk of prostate cancer [81]. Another meta-analysis involving both East Asians and CAM living in Asian countries also found that the GSTM1 null genotype conferred an increased risk of disease [82]. The GSTM1 null genotype is rare in AAM and likely does not contribute to disease risk in this population [81]; however, one study found that the GSTT1 null genotype in was associated with increased biochemical recurrence in AAM with prostate cancer compared to those having GSTT1 present, and the authors concluded that GSTs may hold promise as therapeutic targets in more advanced prostate cancers particularly in AAM [83], although it appears that more substantial work may be needed in this area.

Acquired Molecular Alterations

There are several relatively recently discovered acquired molecular alterations observed in human prostate cancer that have been shown to occur with greater or lesser frequency in certain ethnic groups. The significance of each of these recurrent molecular alterations is discussed in greater detail in other chapters, while this section will emphasize the racial variations that have been observed in these alterations.

Chromosome 8 Abnormalities

Frequent chromosomal gains and losses have been observed with equal frequency by comparative genomic hybridization in tumors from both AAM and CAM, suggesting that sporadic prostate cancers from both groups develop by similar chromosomal mechanisms [84]. In particular, chromosomal loss of 8p and gain of 8q frequently have been observed. While an early study by Washburn et al. observed a racial difference in the frequency of 8p loss [85], a more recent larger study found no racial disparity in 8p allelic loss at tumor initiation or progression between AAM and CAM [86]. However, in a study which compared clinically localized tumors in radical prostatectomy specimens to tumors which exhibited biochemical recurrence and progression in AAM only, a gain of MIR151 at 8q24.3 and/or loss of NKX3.1 at 8p21.2 was more prevalent in the tumors with poorer outcome. These gains/losses even indicated the presence of pre-existing metastatic disease at the time of radical prostatectomy in a subset of patients [87]. In another study which examined prostate cancers in AAM and compared them to those in a historically available CAM cohort, loss of 8p21 and gain of 8q24 (as well as other chromosomal gains and losses) were observed with higher frequency in clinically localized tumors in AAM as they were in metastatic lesions from CAM [88]. Taken together, these studies suggest that chromosomal gains and losses in 8q24 and 8q21, respectively, may be indicative and/or contribute to the more aggressive disease observed in AAM.

ERG Rearrangements

Since the discovery of ERG rearrangements in prostate cancer, multiple recent studies using a variety of methods consistently have shown ERG rearrangements to occur in approximately 50% of tumors from CAM, but in only about half (24–31%) of tumors from AAM [89–92] and occur in even fewer (8–21%) tumors from various Asian populations [90, 93–96]. Although the prognostic significance of ERG rearrangements has been heavily debated, ethnic differences in the prevalence of ERG rearrangements may have diagnostic implications, especially with investigations currently underway on urinary screening tests involving its detection [97–100].

PTEN Loss

Genomic deletions in tumor suppressor PTEN has recently emerged as a biomarker for aggressive prostate cancer throughout the literature [101]. Although PTEN deletions are generally associated with ERG rearrangements which are known to be less frequent in AAM, only one study to date has compared the frequency of PTEN deletions between prostate cancers in AAM and CAM. In this particular study involving approximately 200 patient tumors, PTEN deletions were found in only 6.9% of tumors from AAM compared to 19.8% of tumors from CAM, and this difference

approached statistical significance after adjusting for clinicopathologic parameters. Although this suggests that PTEN loss may not be a major contributor to the more aggressive disease observed in AAM, larger, more robust studies are needed in order to confirm or refute this preliminary finding. In a similarly sized study comparing prostate cancers in Chinese men to a predominantly Caucasian cohort from the United Kingdom (UK), PTEN genomic deletions were found in only 14.3% of cases in the Chinese cohort compared to 42.3% in the UK cohort. The etiology of the racial differences observed in the frequency of PTEN mutations, as well as ERG rearrangements, is yet unknown.

SPOP Mutation

More recently discovered through whole-genome and–exome sequencing of prostate cancers, nonsynonymous somatic mutations in SPOP have been identified as recurrent molecular alterations in a subset of prostate cancers which lack ERG rearrangements [102, 103]. In two studies utilizing the same AAM cohort, no significant differences were observed in the frequency of SPOP mutations among prostate cancers in men of different ethnic and demographic backgrounds [89, 104], and thus these alterations likely do not contribute to the racial differences observed.

Differential DNA Methylation

Differences in the epigenetic phenomenon of DNA methylation in prostate cancer have been observed among different races. In particular, hypermethylation of CpG islands in the promoter region of GSTP1 has been of particular interest due to the role of the protein (glutathione S-transferase) in lessening chemical carcinogens and reactive oxygen species. While two studies found similar levels of GSTP1 hypermethylation between prostate cancers from AAM and CAM [105, 106], the study by Kwabi-Addo et al. additionally reported that promoters of two other cancer-linked genes, TIMP3 and NKX2–5, were hypermethylated with repressed expression in prostate cancers from both CAM and AAM as well [105]. Remarkably, however, these two promoters were hypermethylated in normal prostate tissue from AAM only [105], suggesting that AAM may have an increased susceptibility to environmental variables which increase the risk of prostate cancer. Furthermore, a more recent nested-case control study found that benign prostate biopsy samples with methylation of RARB, another gene whose methylation confers and increased risk of prostate cancer [107], was associated with a statistically significant increased risk of subsequent prostate cancer in AAM over CAM [108]. In addition, APC methylation was found in this study to be associated with a higher risk of developing high-grade tumors in AAM than in CAM [108]. Although there are few studies to date on differential methylation stratified by race, the current studies suggest that

methylation of particular genes associated with prostate cancer carcinogenesis may be an important contributor to the racial disparities observed. Furthermore, elucidation of methylation data on different genes has the potential to improve risk stratification and algorithms for screening and diagnosis among men of different ethnicities, emphasizing the importance of future studies in this area.

MicroRNA Expression

MicroRNA (miRNA) expression is believed to be involved in prostate cancer carcinogenesis and may have important prognostic implications [109]. The expression of certain miRNAs have been found to be differentially expressed in benign prostate tissues between AAM and CAM, particularly miRNA30c, miR-301, miR-219, miR-261, and miR-1b1 [110]. In prostate cancer cell lines obtained from CAM and AAM from tumors of similar stage and grade, prostate cancer cells in AAM showed the greatest expression of miRNA-26a among all cell lines tested [111]. This study also found that a general increase in miR-26a expression toward more aggressive cell lines in both AAM and CAM [111]. In a different study, miR-151 was found to be increased (which coincided with a decrease in the expression of NKX3–1, the gene it regulates) in AAM who had progressive disease after radiation/hormone therapy compared to its expression in AAM with no progression after radical prostatectomy [87]. Taken together, these recent studies suggest that the differential expression of certain miRNAs between AAM and CAM may confer prognostic significance; thus, further work should be continued.

DNA Damage Markers

Carcinogen-DNA adducts, also known as polycyclic aromatic hydrocarbon (PAH)-DNA adducts, induce mutations that contribute to carcinogenesis and their expression has been postulated to vary between prostate cancers from AAM and CAM. Preliminary evidence in a study involving about 900 patients by Nock et al. demonstrated an association between smoking and PAH-DNA adduct levels which differed by race in AAM and CAM and was modified by common genetic polymorphisms in PAH metabolism conjugate genes [112]. In a larger study involving a historical cohort of more than 6500 men by Tang et al. elevated PAH-DNA adducts in benign prostate were found to be significantly associated with increased risk of prostate cancer in AAM but not CAM, with an overall 60% increased risk and greatest risk within 4 years of follow up; the authors concluded that this may reflect a carcinogenic process in AAM that was not yet histologically detectable [113]. PAH-DNA adduct levels seem to be another potentially promising marker involved in racial disparities observed in prostate cancer.

Protein/Biomarker Expression

Expression of certain proteins and serum biomarkers has been found to be related to prostate carcinogenesis and prognosis. Other chapters discuss some of these processes in more detail, but this section will highlight the racial differences observed in this area.

Vitamin D and Vitamin D Receptor Expression

High serum levels of vitamin D have been associated with a significantly decreased risk of lethal prostate cancer in CAM [114], and it is also known that darker skin reduces body's ability to generate vitamin D from sunshine [115]. It therefore has been hypothesized that low vitamin D levels may contribute to aggressive prostate cancer development in AAM. In an early study by Williams et al., vitamin D receptor polymorphisms did not predict pathologic features of prostate cancer, although a particular allele was found to be protective against recurrence in CAM with locally advanced disease treated by radical prostatectomy [116]. In the largest and perhaps highest impact study to date in this area, involving over 900,000 men in a SEER database, Taksler et al. found that counties with the lowest ultraviolet (UV) index had higher prostate cancer incidence rates for both CAM and AAM, and although the incidence of prostate cancer in AAM was higher overall, the difference in incidence between the races was less in countries with higher UV indices compared to the lowest [117]. Furthermore, they found that the mortality rates from prostate cancer decreased with increasing UV index for CAM, but surprisingly, mortality rates actually increased for AAM with increasing UV index, for reasons not yet elucidated [117]. It is plausible that differential Vitamin D levels contribute to prostate cancer disparities among races, but further studies are needed.

SPINK1

Overexpression of the protein SPINK1, a low molecular weight trypsin inhibitor, in prostate cancer cells has been associated with worse prognosis in multiple studies [118–120], although the largest recent study showed no difference [121]. In the first study to examine racial differences in SPINK1 overexpression in prostate cancers from AAM and CAM, we found its overexpression to be more frequent in AAM than CAM (23.8% versus 8.2%), a difference which remained statistically significant after adjusting for clinicopathologic factors [89]. As a serum biomarker, another study found SPINK1 to be more frequently increased in AAM compared to EAM and predicted advanced Gleason scores and biochemical recurrence in a race-dependent manner [122]. Similarly, using gene expression profiling, a recent study from the same group found SPINK1 overexpression to be more frequent in tumors

from AAM and overall correlated with prostate cancer-specific mortality in men who had experienced biochemical and clinical recurrence after prostatectomy [123]. Together, these studies strongly suggest a role of SPINK1 as a potential biomarker for aggressive disease and may be more useful in AAM, in whom it is overexpressed more frequently.

Biomarker Signatures

Identifying prognostically and/or diagnostically relevant biomarker signatures for prostate cancer has become an area of increased interest. In a study by Kim et al. using a six biometric feature combinations involving alpha-methylacyl-CoA racemase (AMACR), androgen receptor (AR), and Ki67 by immunofluorescent assays, all six biomarker features were found to be significantly expressed at higher levels in AAM than in CAM on multivariate analysis, a profile which was significantly associated with progression in a prior study [124]. Similarly, in a multi-institutional study, mRNA expression levels of 20 validated biomarkers reported to be associated with prostate cancer initiation and progression were compared between tumors of CAM and AAM, and 6 (ERG, AMACR, SPINK1, NKX3–1, GOLM1, and AR) were found to show statistically significant differential expression in AAM [122]. Furthermore, this study found that dysregulation of AMACR, ERG, FOXP1, and GSTP1 as well as loss-of-function mutations for tumor suppressors NKX3.1 and RB1 predicted risk of higher stage disease in an ethnicity-dependent manner [122]. Biomarker signatures such as these may contribute to the racial disparities observed in outcomes between CAM and AAM and have potential for risk-stratifying patients of different ethnicities, an important step in overcoming the disparities observed.

Conclusion: Importance of Racial Differences in Influencing Diagnosis and Management

Identifying biological differences in prostate cancer among men of different races has the potential to influence diagnosis and management. Differences in disease screening among at-risk populations such as AAM may be warranted [125], and there may be a necessity for implementing different guidelines for active surveillance, a topic that has been recently heavily debated throughout the oncology literature [15, 126–131]. Given what is known about disparities in clinical outcomes among men of different ethnicities, the question arises of whether to treat cancers more aggressively in certain populations such as AAM. Further understanding of the underlying biological differences in tumors from men of different ethnicities underlies this question, and thus, more research in the area of racial differences in prostate cancer is warranted.

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Chapter 7
Cancer Stem Cells

Yanjing Li and Jiaoti Huang

Contents

Cancer Stem Cell Theory

Defining Tumor Heterogeneity

Tumors were once believed to be monoclonal and arise from a single cell. Subsequent observations challenged this notion. Starting from the 1960s, a series of tumor transplantation studies unveiled tumor heterogeneity [1, 2]. The concept that tumors are heterogeneous not only refers to the variability among tumors of the same type in different patients but more commonly emphasize the cellular differences within one single tumor. Specifically, the cancer cells within an individual tumor display both morphological heterogeneity and functional heterogeneity. Histological examination of tumor samples reveals remarkable differences in cellular morphology and

Y. Li, Ph.D. • J. Huang, M.D., Ph.D. (✉)
Department of Pathology, Duke University School of Medicine, Durham, NC, USA
e-mail: Jiaoti.Huang@Duke.edu

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*,
Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_7

antigen expressions within the same tumor. In 1978, Fidler [3] demonstrated that only a subpopulation of cells within the neoplasms show fatal metastatic potential. By comparing the numbers of lung metastasis in 17 mice receiving B16 melanoma clones derived from same parental cell line, he observed that the clone sublines gave rise to widely different numbers of lung colonies, indicating that the parental tumor was heterogeneous, containing cells of both high and low metastatic potentials.

Recently, an intratumoral multiregion genome mapping carried out by Gerlinger and colleagues [4] indicated that the tumor heterogeneity has been underestimated. By exome sequencing, chromosome aberration analysis, and ploidy profiling on multiple spatially separated samples obtained from primary renal carcinomas and associated metastatic sites, the authors observed significant molecular heterogeneity within the same tumor. They conclude that intratumor heterogeneity can lead to underestimation of the tumor genomics landscape portrayed from single tumor biopsy samples and may present major challenges to personalized medicine and biomarker development.

Cancer Stem Cell Hypothesis

As stated above, tumors contain heterogeneous cellular subpopulations, which contribute differently in the progression of malignancy. Based on these early observations, investigators proposed two theoretical models, hierarchical model and stochastic model, to explain this intratumor variability (Fig. 7.1). According to stochastic model, every cancer cell has the potential to form tumor, but the probability of them entering the cell cycle is low and is controlled stochastically. In contrast, hierarchical model predicts that only a particular subpopulation of cancer cells are tumor-initiating cells, giving rise to the bulk tumor cells which are themselves not tumor initiating. Gradually, increasing evidences favors the hierarchical model. One of the earliest studies conducted by Bonnet and Dick [5] demonstrated that the human acute myeloid leukemia is originated from CD34+ subpopulation of tumor cells and is organized as a hierarchy. These CD34+ cells, also termed leukemia-initiating cells, were able to differentiate in vivo into leukemia blasts and possess proliferative and self-renewing capabilities.

The cellular hierarchical model laid the foundation of cancer stem cell hypothesis. The cancer stem cells, or tumor-initiating cells, stay at the apex of the tumor hierarchy, have the exclusive ability to give rise to tumor progenitor cells and mature cells that are more differentiated and have a limited life span, and drive the growth and spread of cancer. The establishment of cancer stem cell hypothesis has important therapeutic implications. Because cancer stem cells are less proliferative, they tend to be resistance to conventional therapies such as chemotherapy and radiation therapy. As a result, although these treatments can kill bulk tumor cells and cause initial tumor remission, the tumor often relapses since the engine of tumor growth, CSCs, survives such treatments and can reinitiate tumors. It is hoped that by developing drugs specifically targeting cancer stem cell population to cause their apoptosis or differentiation, we may achieve definitive cancer cure.

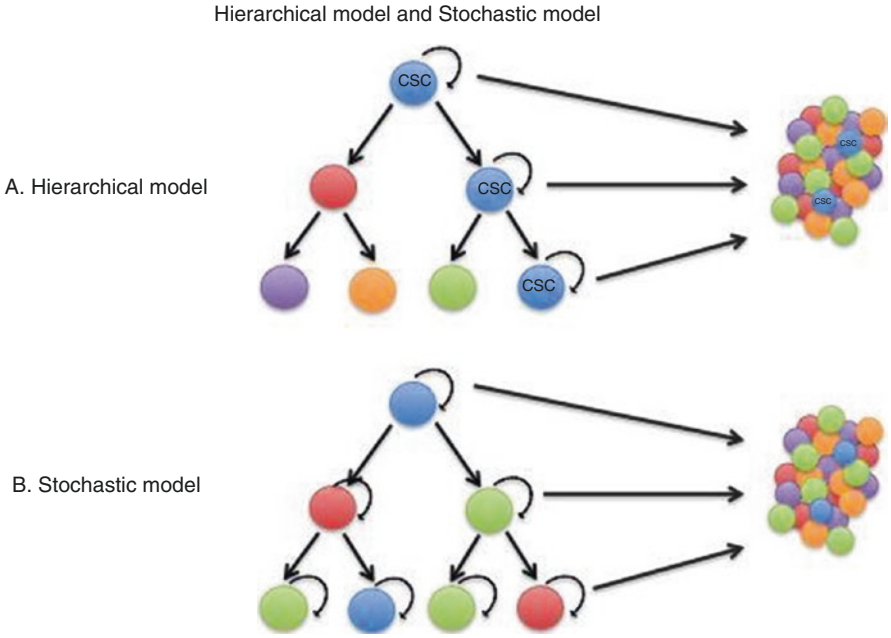

Fig. 7.1 Hierarchical model and stochastic model. (**a**) Shows the hierarchical model. Only cancer stem cells (CSCs) can give rise to bulk of tumor cells, whereas the rest of the tumor cells are not tumor initiative. (**b**) Shows the stochastic model. Every tumor cell has the ability to form a tumor, but their probability of entering the cell cycle is controlled stochastically

Evidence of Cancer Stem Cell

Tissue-specific cancer stem cells were first discovered in leukemia and myeloma, where studies showed that only a small subset of cancer cells is capable of giving rise to tumor. In 1937, Furth and Morton [6] performed single malignant leukocyte inoculation in 97 mice, of which only 5 developed leukemia, indicating that the frequency of tumor-propagating cells is around 1–2%. Later on, both in vitro colony formation assay of myeloma cells and in vivo transplantation assay of leukemic cells supported that only 1–4% of the cell population were clonogenic [2, 7]. This minor population of cells were therefore termed leukemia stem cells, as they are capable of initiating tumor and as clonogenic as normal hematopoietic stem cells.

The first report of isolating cancer stem cells from solid tumor was breast cancer. By performing in vivo tumor formation assay in NOD/SCID mice, Al-Hajj et al. [8] distinguished a tumorigenic population in breast tumor from non-tumorigenic population by cell surface markers CD44 and CD24 and revealed that the CD44+CD24− cell population is cancer stem cells in breast cancer with heterogeneous tumor cells. Subsequently, cancer stem cells have been identified in various types of tumors, including brain cancer, ovarian cancer, colon cancer, prostate cancer, melanoma, etc. Singh et al. [9] demonstrated the existence of brain tumor stem cell (BTSC) through the use of neural stem cell marker CD133 and showed that the

Table 7.1 Identified CSC populations in various cancers

Type of cancer	CSC selection phenotype	Reference
Breast cancer	CD44+ CD24−	Al-Hajj et al. (2003) [8]
Brain cancer	CD133+	Singh et al. (2004) [9]
Colon cancer	CD133+	Ricci-Vitiani et al. (2007) [10]
Ovarian cancer	CD44+ CD133+ ALDH+	Bapat et al. (2005) [11] Ferrandina et al. (2009) [14] Silva et al. (2011) [15] Kryczek et al. (2012) [16]
Pancreatic cancer	CD133+ (CXCR4+)	Hermann et al. (2007) [12]
Melanoma	CD20+	Fang et al. (2005) [13]
Prostate cancer	CD44^{+}/α[alpha]2β[beta]1$^{+/hi}$(CD133+)	Collins et al. (2005) [17] Patrawala et al. (2007) [18]

CD133+ cell population possess the ability of self-renewal and proliferation as well as differentiation into tumor cells that phenotypically recapitulate the tumor they were derived from. Based on the same surface marker, Ricci-Vitiani [10] identified human colon cancer stem cells and showed that the CD133+ cell population, which is around 2.5% of the total colon cancer cells, can form tumor when subcutaneously injected into immunodeficient mice. For human ovarian cancer, Bapat and colleagues [11] showed the existence of a single tumorigenic clone from the ascites sample of a patient with malignant grade IV serous ovarian adenocarcinoma. Hermann [12] identified human pancreatic cancer stem cell by cell surface marker CD133 and further demonstrated that the CD133+CXCR4+ cancer stem cells are responsible for tumor metastasis. In the study of melanoma, Fang et al. [13] found that a fraction of CD20+ melanoma cells possess tumor-initiating capability and that CD20 can be a potential therapeutic target for melanoma (Table 7.1).

Prostate Cancer Hierarchy and Discovery of Prostate Cancer Stem Cell

Several studies have addressed the issue of prostate cancer intratumoral cellular heterogeneity and hierarchy. Based on the biopsies of patients with metastatic castration-resistant prostate cancer, Roudier [19] and colleague reported the remarkable degree of tumor cellular heterogeneity among different patients as well as at multiple sites within the same patient. This cellular variability includes different degrees of glandular differentiation and PSA expression, different proportions of neuroendocrine (NE) cells, and a variety of histological patterns. Patrawala et al. [18] sorted different cell populations by cell surface markers from human xenograft tumor and revealed the hierarchy of tumorigenicity in the order of CD44+α[alpha]2β[beta]1+/hi =CD44+α[alpha]2β[beta]1−/lo > CD44−α[alpha]2β[beta]1+/hi > CD44−α[alpha]2β[beta]1−/lo.

Given the heterogeneous tumorigenicity of cancer cells in the prostate and the essential role of cancer stem cells in therapy resistance, the identification of

prostate cancer stem cells by various cell surface markers has been an important research area in the past two decades. In 1999, Craft et al. [20] identified an androgen receptor-negative (AR-) cell subpopulation that is responsible for the expansion of prostate cancer under castration treatment and androgen-independent growth. They also reported that this AR- cell population is only present at a frequency of 1 in 105–106 androgen-dependent cells in human prostate cancer xenograft LAPC9.

An important functional study to identify cancer stem cells from primary human prostate cancer was done by Collins et al. in 2005 [17]. By purifying a cell subpopulation with the normal prostate stem cell markers—CD44/α[alpha]2β[beta]1/CD133—they discovered that the CD44+/α[alpha]2β[beta]1hi/CD133+ phenotypic cells from both primary and metastatic prostate tumors possess cancer stem cell characteristics, including extensive capability of proliferation, self-renewal, differentiation, and invasion. Subsequently, the role of CD44+ cell population in prostate cancer tumorigenesis was further studied by several groups. Patrawala et al. [21] showed that CD44+ PCa cells are more proliferative, tumorigenic, clonogenic, and metastatic than their CD44− counterparts in xenograft tumors. Also, the CD44+ PCa cells expressed "stemness genes" including Oct3/4 and others and underwent asymmetric cell division, which are the hallmarks of stem cells. Hurt et al. [22] demonstrated that the CD44+CD24− cell population in LNCaP cell line showed stem cell features by performing both in vitro and in vivo tumorigenic assays.

Apart from those typical cell surface markers, it has been shown that aldehyde dehydrogenase (ALDH) enzyme activity plays an important role in epithelial homeostasis by regulating cell proliferation, differentiation, and even drug resistance. Van den Hoogen et al. [23] reported that ALDH-high prostate cancer cells possess both tumor-initiating and metastasis-initiating potential, suggesting that ALDH-based cell sorting can be used to identify tumor-initiating cells in prostate cancer.

One important criterion in the identification of cancer stem cells is that as stem cells, they do not express or express low level of differentiation markers. Prostate-specific antigen (PSA) is a well-known luminal differentiation marker of prostate cancer cells. By comparing the stem cell properties between PSA-/low and PSA+ cells, Qin et al. [24] showed that PSA-/low cells have robust tumor-initiating capability and may represent cells of origin of castration-resistant PCa. In another study, Rajasekhar et al. [25] identified that a subpopulation of AR-/PSA- cells possess stem cell-like qualities by showing their ability to form spheroids and recapitulate the tumor heterogeneity in serial xenotransplantation. This sphere-forming population expresses stemlike basal cell markers SOX9, MET, and CK5 and shows little expression of differentiation markers Nkx3.1, ZO-1, CK18, and p63.

Metastatic castration-resistant prostate cancer (CRPC) was treated with chemotherapy, including docetaxel and cabazitaxel. However, patients with this type of disease will ultimately become resistant to these drugs. Domingo-Domenech et al. [26] identified a subpopulation of CRPC cells that survived long-term docetaxel exposure and revealed that this chemoresistant and castration-resistant cell population show potent tumor-initiating capability and overexpress NOTCH and Hedgehog signaling pathway molecules, which regulate canonical survival molecules including Akt and Bcl-2.

Cellular Origin of Prostate Cancer

The normal prostate epithelium consists of basal, secretory (luminal), and neuroendocrine (NE) cells. The luminal cells are the predominant cell type in the epithelium and are terminally differentiated. They are located toward the glandular lumen, express high level of androgen receptor (AR) and prostate-specific antigen (PSA), and depend on androgen for growth. Basal cells comprise the outer layer of the glands and acini and separate luminal cells from the stroma. They are relatively undifferentiated, maintain proliferative potential, and express low or nearly undetectable AR, are negative for PSA, and are androgen independent. It is well accepted that prostate cancer generally follows a sequence of benign glands to high-grade prostatic intraepithelial neoplasia (HGPIN) to invasive cancer. HGPIN is a precursor lesion of prostate cancer in which the luminal cells show malignant features but are not invading the stroma as the basal cells are still present albeit in reduced numbers, similar to in situ carcinomas of various organs. Invasive prostate cancer, the vast majority of which is histologically classified as adenocarcinoma, is composed of malignant luminal-type tumor cells and devoid of basal cells. Since the malignant cells in HGPIN and cancer are morphologically similar to the benign luminal cells (with the exception of malignant features) and express AR and PSA by immunohistochemistry, it has traditionally been assumed that all prostate cancers have their origin in luminal cells. An important caveat is that luminal cells are terminally differentiated, while proliferation occurs in the basal cell compartment.

Approaches to Investigate Cellular Origin of Cancer

In vivo tumor regeneration assay of putative tumor-initiating cells after genetic manipulation is a common approach for studying the cellular origin of cancer. Both genetically engineered mouse models and in vivo tissue recombination models have been used. Transgenic or knockout mice with tissue-specific gene manipulation technology are used to explore the roles of oncogenes or tumor suppressors in different cellular contexts. In this approach, tissue- or cell-type-specific promoter is required to drive the expression of an oncogene or Cre-mediated knockout of a tumor suppressor gene. Ideally, only one cell population is expected to generate tumor which recapitulates the human cancer being modeled. For instance, tissue-specific knockout of Pten biallele in different prostatic cell subpopulations has been used to study the cellular origin of prostatic adenocarcinoma. This method is also termed as “lineage tracing”—tracking individual cells as they undergo transformation. With the tissue recombination model, purified cells (human or rodents) are genetically modified by lentiviral vector-mediated expression of oncogenes or knockdown of tumor suppressors, which are then mixed with supportive stroma and transplanted under the renal capsule or subcutaneously for tumor induction. This approach often relies on sorting of defined subpopulations of cells using cell surface markers. The advantage of this approach is that human cells can be studied directly.

Cells of Origin in Prostate Cancer

More than two decades ago, Okada and colleagues [27] revealed that the major parts of cells of prostatic adenocarcinoma are luminal cells but not basal cells, based on keratin profiles of 25 patients with prostatic adenocarcinoma and 10 patients with normal/hyperplastic prostate. Follow-up studies have also confirmed the cancerous expansion of luminal cells and absence of basal cells in prostate cancer [28], leading to the general belief that luminal cells are the cellular origin of prostate cancer.

Several studies have investigated the role of luminal cells during prostatic tumorigenesis in mouse model. Ma et al. [29] generated a novel prostatic tissue-specific Pten knockout mouse model using luminal cell-specific promoters and observed epithelial hyperplasia, neoplasia, and invasive prostate carcinoma, accompanied with luminal cell expansion and loss of basal cell population. Subsequently, a groundbreaking study by Wang et al. [30] identified a novel luminal stem cell, termed CARNs (castration-resistant Nkx3–1-expressing cells), as a target of prostate carcinogenesis. By performing genetic lineage tracing of Nkx3–1-expressing cells, they uncovered a rare luminal cell population that expresses Nkx3–1 homeobox gene and is castration resistant with stem/progenitor properties during prostate regeneration. In addition, CARN-specific deletion of Pten could initiate high-grade prostatic intraepithelial neoplasia (PIN) and carcinoma, suggesting that they can be the cells of origin of prostate cancer. A human counterpart of CARNS has yet to be discovered.

Though basal cells are not present in prostate cancer, several studies revealed that histological characterization of cancer does not necessarily correlate with the cells of origin. Mulholland et al. [31] showed that specific deletion of Pten gene in prostatic basal cells can initiate primary prostate cancer. In another study, Lawson et al. [32] compared the tumorigenic ability of murine prostatic luminal and basal/stem cells after ex vivo genetic manipulation to overexpress Erg, AR, and/or Akt. By performing in vivo prostate regeneration assay, they found that the basal/stem cells regenerated epithelia hyperplasia, PIN, and carcinoma, whereas luminal cell grafts failed to grow. The first study that investigated the cellular origin of human prostate cancer was done by Goldstein et al. [33] by introducing similar genetic changes into human prostatic basal and luminal cells. Similarly, they observed that only basal cells developed prostate cancer reminiscent of luminal-like cancer in human, a finding that has been confirmed by additional studies [34, 35]. Interestingly, a study by Choi et al. [36] showed that in mice, both basal and luminal may serve as targets for prostate cancer initiation.

Benign prostate and adenocarcinoma contain rare NE cells that are quiescent. Small cell neuroendocrine carcinoma (SCNC) is composed of highly aggressive NE tumor cells which is often seen in patients with a history of adenocarcinoma who have failed hormonal therapy. There is in vitro and in vivo evidence supporting a transdifferentiation model whereby luminal-type cancer cells transdifferentiate into NE tumor cells [37] which are consistent with an adaptive mechanism. However, recent studies have shown that the p53, Rb, and Pten are often mutated or deleted in SCNC in comparison to adenocarcinoma, and MYCN and Aurora Kinase

A genes are often amplified and overexpressed, findings that may be consistent with a clonal selection process [38–40]. In other words, it is also possible that certain genetic alterations occur in the preexisting NE cells of adenocarcinoma during hormonal therapy, leading to their rapid proliferation and aggressive behavior, resulting in SCNC.

Cancer Cells of Origin vs Cancer Stem Cells

It is worth noting that cancer stem cells (CSCs) and cancer cells of origin (CCO) are two distinct concepts. Cancer stem cells are cancer cells, which stay at the apex of tumor hierarchy, capable of generating tumor progenitor cells and mature tumor cells and sustaining the tumor growth, whereas cancer cells of origin are normal cells, which have the potential to initiate tumor after acquiring genetic mutation. By this definition, CSCs and CCOs refer to tumor-propagating cells and tumor-initiating cells, respectively. So far, majority of data points to tissue-specific adult stem cells as CCOs. Adult stem cells are quiescent, self-renewing, long-living, and multipotent, which are the properties required of CCOs. In addition, lineage tracing study comparing the tumor-initiating property of adult stem cells, trans-amplifying cells, and differentiated cells after introducing genetic mutation indicates that adult stem cells may be CCOs in multiple tumors. However, CSCs may arise from either adult stem cells, trans-amplifying cells, or differentiated cells (Fig. 7.2).

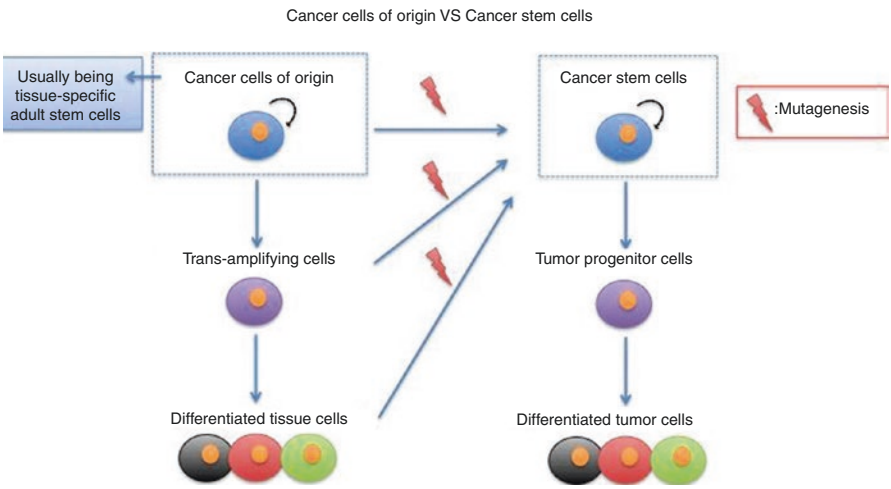

Fig. 7.2 Comparison of cancer cells of origin and cancer stem cells. Cancer cells of origin usually refer to tissue-specific normal adult stem cells, which can differentiate into trans-amplifying cells and then differentiated tissue cells. Cancer stem cells are tumor cells, which can give rise to tumor progenitor cells and the bulk of tumor cells. Cancer stem cells can be originated from adult stem cells or trans-amplifying cells or differentiated tissue cells after receiving certain mutagenesis

Characterization of Cancer Stem Cells

The Properties of Normal Stem Cells

Given that cancer stem cells share many phenotypic and functional properties with normal stem cells, it is useful to discuss the properties of normal stem cells in order to better understand the biology of cancer stem cells. Normal stem cells, from pluripotent embryonic stem cells to multipotent tissue-specific adult stem cells, possess two major properties: self-renewal and differentiation. Self-renewal is the ability to produce more stem cells with the same development and proliferation potential during asymmetrical or symmetrical cell division. It is the most important and distinguishing quality of stem cells since it enables the expansion and maintenance of this undifferentiated cell pool and prevents stem cells from exhaustion. During self-renewal, stem cells maintain their undifferentiated states by repressing the expression of genes involved in differentiation. The intracellular signaling of self-renewal is regulated by extracellular signals from the niche, the microenvironment that maintains stem cells and regulates their function in tissue. Another important feature of stem cells is their ability to differentiate into tissue-specific specialized cells. Unlike self-renewal, differentiation is the process where stem cells change into another cell type, along with changes in cell size, morphology, metabolic activity, and function. Differentiation occurs throughout the development of multicellular organisms. In adults, the adult stem cells play an important role in maintaining and replenishing the tissue in which they reside. They are normally quiescent for a long period of time and remain undifferentiated until they receive the signal from the niche, which initiates the migration and differentiation of adult stem cells into trans-amplifying progenitor cells which will further differentiate into mature terminally differentiated cells to fulfill the function of the tissue/organ. Abnormality in stem cell function can affect tissue regeneration and results in cancer.

Characterization of Cancer Stem Cells

Cancer stem cells and normal stem cells share several similarities, such as the capability of self-renewal and differentiation into multicellular lineages. These properties enable cancer stem cells to maintain and expand the tumor. Multiple in vitro and in vivo experiments have proven that cancer stem cells, which represent a rare subpopulation in tumor, showed stem cell properties and extensive proliferative potential that drive the formation and growth of tumor. In in vitro cell culture, putative cancer stem cells show higher proliferative activities than the rest of tumor cell population. Tracing cancer stem cells during several rounds of cell division showed that cancer stem cells can undergo both symmetric cell division for self-renewal and asymmetric cell division (ACD) to regenerate an identical daughter cell and a more differentiated tumor cell. Moreover, molecular analysis revealed that cancer stem cells of a specific tumor express higher level of stemness genes but lower level of

tissue-specific genes compared with the rest of the tumor cell population. In addition, in vivo tumor initiation assay also confirmed that cancer stem cells are capable of initiating tumor formation in immunodeficient mice, whereas noncancer stem cells of the same tumor failed to do so or generate a much smaller tumor. Serial transplantation of CSC and non-CSC showed that CSCs are more clonogenic and tumorigenic compared with non-CSC counterpart isolated from the same tumor.

Characterization of Prostate Cancer Stem Cells

In addition to the general properties of cancer stem cells, prostate cancer stem cells also show unique properties. Androgen and androgen receptor signaling is essential for prostate cancer. The differentiated tumor cells express androgen receptor and rely on androgen for growth. However, prostate cancer stem cells do not express androgen receptor and they are castration resistant. Therefore, prostate CSCs will survive hormonal therapy, are able to repopulate tumor, and lead to CRPC (castration-resistant prostate cancer) following androgen ablation therapy.

Identification of Cancer Stem Cells

Cancer stem cell hypothesis posits that only a small subpopulation of tumor cells has the potential to initiate the tumor and promote the expansion and metastasis of tumor. In order to identify this particular subpopulation within the tumor, multiple tests are required to demonstrate that the putative CSCs have certain biological properties associated with stem cells. The gold standard is in vivo transplantation assay, where a single tumor cell transplanted into an immunodeficient mouse can generate a tumor similar to the original human tumor. In addition, various in vitro assays need to be performed to further support that they possess those CSC characteristics as discussed above.

Identification of Putative Prostate Cancer Stem Cells Within the Tumor

Hoechst side population assay is a technique to identify and purify cancer stem cells in various types of tumor. This method is based on the differential uptake of Hoechst dye by CSCs and differentiated tumor cells. Hoechst 33342 DNA dye is usually actively taken up by live cells, whereas cancer stem cells and early cancer progenitor cells are able to pump out Hoechst dye via ATP-binding cassette (ABC) transporters, resulting in low Hoechst dye concentration within those cells. By performing flow cytometry after Hoechst dye staining of whole tumor cell population, distinct

cancer stem cell population can be obtained. Multiple studies have successfully isolated SP from a variety tumor types and verified that this population possess cancer stem cell properties. However, Hoechst staining condition needs to be optimized according to cell type of interest, which is critical for this technique.

Another method to identify CSCs is by cell surface markers of the corresponding normal tissue stem/progenitor cells. It is assumed that the CSCs may share many similarities with normal stem cells of the same tissue, including cell surface markers. Based on the fact that normal prostate epithelial stem cells express CD44/integrin α[alpha]2β[beta]1, Patrawala et al. [18] isolated putative prostate cancer stem cells which are double positive for these markers and verified their tumorigenic potential. So far, CD44+/integrin α[alpha]2β[beta]1+ has become established biomarkers for prostate cancer stem cell purification.

The BrdU pulse-labeling assay can be used to identify slow cycling cells, which is also termed as label-retaining cells (LRCs). CSCs are considered more quiescent than progenitor cells and differentiated tumor cells. After being exposed to BrdU "pulse," tumor cells with proliferative ability will be labeled as BrdU is incorporated into DNA during DNA synthesis. Faster cycling cells, including progenitor cells and differentiated tumor cells, will gradually dilute inner nuclear BrdU concentration or become BrdU negative after culture in BrdU-negative media, whereas putative CSCs, which have longer cell cycle time, will remain BrdU positive and can be purified and isolated.

Another intriguing method to enrich CSCs is based on their property of chemo- and radiation resistance. Chemotherapeutic drugs, such as docetaxel, target actively proliferative cells including tumor progenitor cells and terminally differentiated tumor cells. As a result, CSCs will be enriched after long-term exposure to these drugs due to their quiescent nature. Domingo-Domenech et al. [26] identified a docetaxel-resistant subpopulation in prostate cancer and demonstrated that this subpopulation possesses potent tumor-initiating capacity, lacks differentiation markers, and also overexpresses the Notch and Hedgehog signaling pathways.

Assaying Function of Putative Prostate Cancer Stem Cells

A series of in vivo and in vitro assays have been developed to confirm the biological properties of the purified putative CSCs. They include in vivo serial transplantation assay, in vitro clonogenic and sphere formation assays, assessment of proliferative capacity, etc.

The gold standard of measuring CSC properties is in vivo serial transplantation assay, which assess the tumor initiation capacity of purified subpopulation. Putative CSCs should be able to initiate tumors that recapitulate patient tumor histology and can be serially transplanted. Putative CSC and non-CSC counterparts can be purified from cultured cells or xenograft tumors or from patient. Purification method can be chosen from above (sect. "Identification of Putative Prostate Cancer Stem Cells Within Tumor"). In order to compare the tumorigenic potential, a limiting

dilution assay will be performed by transplanting the same number of CSCs and non-CSCs from 1, 10, 100, 1000, to millions in mice. Cell number should be examined before these cells are resuspended in 20–30 μl culture medium and mixed with concentrated Matrigel. Then cell/Matrigel mixture will be injected into the flanks of mice. Tumor development should be monitored from the second week after injection by measuring tumor incidence (i.e., number of tumors/number of injections), latency (time from injection to detection of palpable tumors), and tumor weight. After 4–6 months, animals should be terminated and tumors will be harvested for further examinations.

In vitro clonogenic assay (or colony formation assay) and sphere formation assay are established to assess the reproductive viability of single tumor cells. 100–1000 dissociated CSCs and non-CSCs per well are plated in a 6-well plate and cultured for 10–14 days. The resulting colonies, composed of 50 or more cells, can be counted under a microscope. For sphere-forming assays, cells are plated in 6-well ultralow attachment plate to allow colonies to grow into 3D structure. CSCs, which possess higher tumorigenic potential, generate more colonies than their non-CSC counterpart. In addition, colonies generated from sphere formation assay can be collected and passaged into a new well to form secondary spheres. During serial sphere passaging, CSCs would exhibit robust clonogenic capacity, whereas non-CSCs would gradually lose the ability to form colonies as they have limited tumorigenic potential.

To assess the proliferative capacity of purified subpopulations of cells, multiple experiments can be performed, including growth curve, BrdU incorporation assay, etc. Growth curve is the simplest way to compare the doubling time of both CSCs and non-CSCs at defined time points. To directly determine the proliferative capacity, BrdU incorporation assay can be performed to assess the ratio of S-phase cells, which are undergoing proliferation. CSCs, which are more quiescent than their non-CSC counterpart, would show longer doubling time and fewer cells in S-phase but more G0/G1 cells from BrdU assay. Asymmetric cell division (ACD) is another property of CSCs during cell proliferation which can be studied by labeling specific CSC markers and tracing the marker expression in daughter cells. During ACD, CSC would self-renew by generating an exact copy of itself and at the same time differentiate into a mature tumor cell which lacks the expression of the specific CSC marker.

Signaling Pathways in Cancer Stem Cells

Cancer stem cells share several signaling pathways with normal stem cells, including Wnt-β[beta]-catenin, JAK-STAT, Hedgehog, BMI1, Pten-PI3K/Akt, Sox2, and NF-kB pathways, which regulate self-renewal and proliferation of cancer stem cells. In prostate cancer, the deregulation of these pathways has been associated with the tumorigenic property of prostate cancer stem cells. Bisson and Prowse [41] showed that the activation of Wnt-β[beta]-catenin pathway contributes to the formation of prostaspheres generated from cancer stem cells during in vitro culture. Blocking Wnt signaling inhibited self-renewal of prostate cancer stem cells resulting in

reduced size of prostaspheres. Similar result has been obtained by other groups. Hsieh and colleagues [42] revealed that the microRNA320 inhibits tumorigenic properties of prostate CSCs including tumorsphere formation and chemoresistance through the downregulation of Wnt-β[beta]-catenin pathway. However, the full activation of Wnt-β[beta]-catenin pathway to maintain CSC self-renewal requires the upregulation of Bmi-1, a polycomb group transcriptional repressor, which has been shown to be associated with normal prostate tissue regeneration and initiation of prostate cancer [43]. The activation of IL-6-JAK-STAT3 signaling pathway has been found in several types of cancer. Kroon et al. [44] reported that the putative prostate CSCs secrete higher level of IL-6 than the non-CSCs and inhibition of JAK-STAT3 pathway significantly suppressed the clonogenic capacity of CSCs by in vitro colony formation assay and in vivo xenograft model. The upregulation of IL-6-JAK-STAT3 may be due to the loss of AR expression in prostate CSC [45]. Similar to the Wnt-β[beta]-catenin pathway, Hedgehog (Hh) signaling also affects the normal development and regeneration of prostate as well as tumorigenesis. Studies have shown that the activation of Hh plays an essential role in transformation of normal prostate progenitor cells into tumor stem cells therefore initiating cancer [46, 47]. Recent study conducted by Domingo-Domenech et al. [26] showed that inhibition of NOTCH and Hedgehog pathway depleted the prostate tumor-initiating cell population identified by docetaxel resistance. Pten/PI3K/Akt pathway is known as key regulator for cancer progression. Pten knockout mice has been used as a common model for prostate cancer. Dubrovska and colleague [48] showed that knockdown of Pten in putative prostate CSCs identified by surface marker CD44+/CD133+ increases the clonogenic and tumorigenic potential as demonstrated by in vitro sphere formation assay. Moreover, in vivo deletion of Pten can induce the expansion of Sca1+/BCL2+ prostate basal stem/progenitor cells, resulting in malignant transformation and tumor initiation [49]. Sox2 is a key transcriptional factor that maintains the pluripotency of embryonic stem cells. However, it also plays a critical role in self-renewal and anti-apoptosis of prostate cancer stem cells [50–53]. In another study, Rajasekhar and colleagues [54] identified a prostate tumor-initiating cell (TIC) population by human pluripotent stem cell markers TRA-1–60, CD151, and CD166 and showed that these TICs exhibited enhanced levels of NF-kb, which promotes in vitro and in vivo tumorigenesis of prostate CSCs.

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Part II
Molecular Signatures of Primary Prostate Cancer

Chapter 8
Prostate Cancer Risk: Single Nucleotide Polymorphisms (SNPs)

Robert J. Klein

Contents

Introduction

While the exact cause of prostate cancer remains unknown, numerous lines of evidence support the contention that there is a strong hereditary component to prostate cancer. For instance, a study comparing monozygotic and dizygotic twins in Scandinavia found that monozygotic twins were more likely to both be diagnosed with prostate cancer compared to dizygotic male twins [1]. Similarly, numerous studies have observed that relatives of men with prostate cancer have a higher risk of developing prostate cancer [2]. These data suggest that inherited genetic factors play a key role in the development of prostate cancer, but they do not reveal the underlying genetic architecture.

When considering the genetic architecture of disease, one needs to consider both the frequency with which an allele is observed in the population and the effect it has on the risk of developing disease [3]. For instance, mutations in the breast and ovar-

R.J. Klein, Ph.D.
Department of Genetics and Genomic Sciences,
Icahn School of Medicine at Mount Sinai, New York, NY, USA
e-mail: Robert.klein@mssm.edu

© Springer International Publishing AG 2018

B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*,
Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_8

ian cancer susceptibility gene BRCA2 have been implicated in risk for prostate cancer as well; one report estimated that a specific BRCA2 founder mutation increased the risk of prostate cancer threefold in men [4]. As these mutations are relatively rare in the population, they only explain a small fraction of the heritability of this disease [3]. In contrast, numerous common polymorphisms have been statistically associated with prostate cancer risk. While prevalent in the population, each variant only confers a slight increase in risk. For instance, the first such SNP described in prostate cancer, rs1447295, increases the risk of prostate cancer by about 15–60%, depending on the population [5].

This chapter will primarily focus on the latter type of variants—common polymorphisms that modestly alter disease risk. We will begin by providing an overview of genome-wide association studies (GWASs) and how they are used to identify such common polymorphisms. Next, we will discuss GWAS of prostate cancer and the risk loci they identified. Then, the utility of these findings in understanding disease biology and as predictive markers will be elucidated. Finally, less common polymorphisms of more substantial effect on risk will be briefly described.

Genome-Wide Association Studies (GWASs)

While family-based linkage studies historically were used to identify rare diseases that cause disease in a Mendelian fashion, this approach does not work if the causal mutation is both common (found in many individuals in the population) and low penetrance (many people with the causal mutation do not have the disease) [6]. Identification of common disease predisposition alleles with low penetrance therefore requires a new approach. GWASs make use of high-density genotyping microarrays to genotype from 100,000 to several million SNPs at once. As the genetic history of the human population has resulted in correlation, or linkage disequilibrium (LD), between nearby variants [7], such arrays comprehensively assess all common variants in the genome. Because of LD, however, one initially finds a marker SNP associated with the disease that is in LD with the functional mutation [8]. Therefore, while facilitating the initial discovery of a locus of interest, as will be discussed below, LD makes it more difficult to pinpoint the causal, functional mutation(s) at any such locus.

To understand GWAS, it is necessary to understand the methods used to select the SNPs to be included on the genotyping microarrays. These arrays are primarily manufactured by two companies—Affymetrix and Illumina—and the specific choice of SNPs that can be included depends in large part on the restrictions of the chemistry each company uses in their assay [9, 10]. Given these restrictions, the goal of the genotype design is to maximize the number of SNPs "captured" by the design, either directly or through LD. Typically, a hard filter of a correlation (r2) between SNPs of 0.8 was used for a given population, and the array was designed to maximize the number of SNPs captured at that threshold [11]. While early designs focused on populations of European ancestry, later designs with larger numbers of

genotyped SNPs expanded to include LD patterns from populations of Asian and African ancestries as well.

Though genotyping microarrays have been able to include denser sets of SNPs, there is also a need to determine the best guess of a genotype that isn't genotyped on the microarray. To that end, statistical methods for imputation have been developed. All of these methods depend on a reference panel, typically data from the 1000 Genomes Project. Given the input of the genotyped SNPs from the microarray platform, these algorithms use the reference panel to generate the best guess for which haplotypes are found in the genotyped individuals and fill in the missing data from the haplotype panel. Common programs to conduct these analyses include IMPUTE [12, 13], BEAGLE [14, 15], and MACH [16]. Thus, with current genotyping technology, it is possible to essentially test every common variant in the genome for association with a given phenotype.

GWAS of Prostate Cancer

Given this ability to assess the genotype of all of the common polymorphisms in a set of individuals, combined with the previously reported heritability of prostate cancer, it was natural to ask whether any common polymorphisms contribute to risk of this disease. Numerous approaches to this question have been undertaken over the last decade, with the end result that over 100 variants associated with prostate cancer have been identified. Interestingly, the first common polymorphism associated with prostate cancer risk was not identified by a GWAS, but was identified independently by two groups taking two distinct approaches. One group started by doing family-based linkage analysis using the extensive genealogy available for families in Iceland. Using this approach, they identified a region on chromosome 8q24 linked to risk of prostate cancer [5]. In a separate study, another group took advantage of the fact that prostate cancer is more common in African-American men than European-American men. They hypothesized that one reason for this disparity could be that there are one or more prostate cancer risk alleles that entered the African-American population from African rather than European ancestors. Using an approach called admixture mapping, they identified the same region of chromosome 8q24 as having African ancestry compared to European ancestry more often than expected by chance in African-American individuals with prostate cancer [17].

Following these studies, numerous genome-wide association studies of prostate cancer risk were conducted by several groups. In almost all these cases, a nested design was used in which the full set of genotypes was generated for a subset of samples, and only SNPs for which evidence of association was observed were brought forward and genotyped in a larger number of individuals. This study design was chosen because it is more cost-efficient with almost no loss of power [18]. It is worth briefly mentioning each of these sets of studies, as they highlight different study design decisions that influence the results that were found. One set of studies was conducted on the founder population found in Iceland. Using the

extensive genealogical records linking the population along with a large population-based set of genome-wide genotypes, this group identified several polymorphisms associated with prostate cancer risk [19–22]. A US-based study used a nested case-control design, in which cases and matched controls were selected from larger cohort studies for genotyping. This set of studies identified additional SNPs associated with prostate cancer risk [23, 24]. Among the first prostate cancer GWAS, the largest number of associated loci came from a study of individuals from the United Kingdom and Australia. This study design selected cases that had a family history of disease or early age of onset and controls that had extremely low PSA levels [25]. With this study design, any observed effect of a SNP on disease risk is amplified relative to a standard case-control design as the effect of a genetic factor is likely to be more important in these selected cases and the controls are unlikely to have undiagnosed prostate cancer. Using this initial GWAS data and increasing numbers of samples for replication, numerous prostate cancer risk loci were identified [25, 26].

From these studies, it became clear that new strategies would be needed to identify additional prostate cancer risk loci. One strategy that has been employed successfully is to study prostate cancer in non-European populations. All of the above studies—both from Europe and the United States—focused on individuals of European ancestry. However, if there are variants associated with prostate cancer risk that are of low frequency in European but no other populations, or if there are variants whose effect is restricted to certain populations, European-focused studies would not identify them. To address this, studies in non-Europeans, both in the United States and Asia, have been performed. GWAS conducted using a standard design in individuals from Japan [27] and China [28] both identified additional risk loci, as did a study in African-American men [29].

However, the real limiting step in the power of GWAS is recruiting a large number of individuals and genotyping them on SNPs across the genome. To help solve this problem, current GWAS in both prostate cancer and other diseases are conducted as multinational consortia incorporating data from numerous individual studies. As different studies are often genotyped on different platforms, the first step in such meta-analyses is to impute the genotype of all of the variants from a common reference panel, such as the 1000 Genomes Project [30], in each of the underlying studies. Then, association tests are conducted on a SNP-by-SNP basis, either by testing each underlying study separately and combining the results through meta-analysis or testing all of the studies together. Using this approach, increasingly large prostate cancer GWASs were conducted enabling increasing numbers of variants to be identified that are associated with prostate cancer risk [31–33].

While the typical GWAS looks for single nucleotide changes associated with disease risk, it has been found that changes in the number of copies of a genomic segment present in the genome be observed between people [34] and are associated with disease risk [35]. To investigate this in prostate cancer, DeMichelis et al. conducted a case-control study using a SNP genotyping array that was also designed to capture such copy number variation (CNV) [36, 37].

However, instead of focusing on the SNPs on the array, they asked if the copy number differed between cases and controls. They identified two loci, not previously associated with prostate cancer, at which copy number associates with prostate cancer risk [36]. Given that many common, polymorphic CNVs are well tagged by SNPs, the potential role of functional CNVs at SNP-identified prostate cancer risk loci cannot be discounted [38].

In summary, to date GWASs have identified approximately 100 variants associated with the risk of developing prostate cancer. Taken together, these SNPs explain about 33% of the familial risk of prostate cancer (at least in men of European ancestry) [33]. However, while the association between these SNPs and prostate cancer is clearly statistically significant, as will be discussed below, their implication for understanding disease biology and in predicting who will be at risk for prostate cancer remains less clear.

Insights into Disease Biology

One reason why one may wish to conduct a GWAS is to gain new insight into the etiology of a disease. To translate results from GWAS into such an understanding requires moving from associated genetic variants to their mechanism of action. This task is made more complicated by the phenomenon of LD, through which common genetic variants are correlated with their neighbors in the genome [7]. When the idea of GWAS was first proposed, it was assumed that the causal variant would affect the protein-coding sequence of a gene [6]. However, with time it became clear that the causal variant in most cases is noncoding [39]. Therefore, new approaches would be needed to understand the biology underlying GWAS hits.

One promising hypothesis is that many GWAS results, in prostate cancer and elsewhere, alter transcriptional regulatory elements and therefore the transcriptional program in the cell. Motivated by the search for causal variants in GWAS, it has been observed that SNPs in regulatory regions and evolutionarily conserved regions, even if far from known genes, are under selective constraint in the human lineage [40]. More recently, with the completion of the first production phase of the Encyclopedia of DNA Elements (ENCODE) [41], it has been observed that many GWAS signals are enriched in putative transcriptional regulatory regions [42]. These disease-associated variants also tend to overlap expression quantitative trait loci (eQTLs), genetic loci associated with changes in nearby gene expression [43].

Several lines of evidence, both measured across prostate cancer risk loci and targeted at particular loci, support this regulatory hypothesis. Several studies have found prostate cancer risk SNPs to be eQTLs for nearby genes; in many cases, these associations are tissue specific [44–49]. More recently, it was observed that at many prostate cancer risk loci, risk-associated SNPs disrupt transcriptional regulation [50]. These principles are best illustrated by two examples.

From the initial GWAS of prostate cancer, the most promising variant for functional understanding was rs10993994, a SNP in the promoter of the MSMB gene [24, 25]. MSMB codes for β[beta]-microseminoprotein (β[beta]-MSP), a major secretory product of the prostate [51] that has been suggested to have tumor-suppressive properties [52]. This SNP is located 57 nucleotides upstream of the transcription start site for the gene MSMB; reporter assays suggest that rs10993994 may be functionally responsible for altered promoter activity at this locus [53–55]. Intriguingly, MSMB codes for β[beta]-MSP, a small secreted protein initially purified from human seminal fluid [56–58]. This protein is one of the three primary secretory products of the prostate [51]. While the natural physiological role of this protein is unclear, it does display characteristics of a tumor suppressor. Decreased MSMB expression is observed in malignant prostate tissue, especially more advanced disease [59–62]. Several functional studies support the hypothesis that MSMB has tumor-suppressive properties. Exogenous addition of purified β[beta]-MSP in cell culture induces apoptosis of cancer cells [52]. Tumor size is reduced when exogenous β[beta]-MSP is injected with prostate cancer cells in a rat xenograft model [52, 63]. Overexpression of MSMB has been shown to reduce survival in a clonogenic survival assay [64]. Clinically, the risk allele of rs10993994 associates with decreased levels of β[beta]-MSP in blood and semen [65, 66]; similar results are seen both at the mRNA and protein level [48, 67, 68] in prostate tissue. eQTLs at the mRNA and protein level with rs10993994 have been described for both adjacent genes [48] and genes on other chromosomes.

The first locus to be functionally dissected in prostate cancer was on chromosome 8q24. The initial SNP identified at this locus was found through both linkage and admixture analysis [5, 17]. Surprisingly at the time, this SNP is located in a gene desert far from any annotated gene; the closest gene, MYC, is several hundred kilobases away, though it is an important oncogene. Further studies of prostate and other cancers revealed a complex interplay of SNPs, where distinct SNPs at this locus are associated with risk for different types of cancer including bladder cancer [69], breast cancer [70], chronic lymphocytic leukemia [71], colorectal cancer [72–74], glioma [75], lymphoma [76], ovarian cancer [77], and renal cell carcinoma [78]. Though eQTL analysis has not identified association between the prostate cancer risk alleles and MYC expression [79], several indirect lines of evidence support the hypothesis that the variants at 8q24 influence MYC expression. First, several of the cancer risk SNPs at 8q24 appear to be in tissue-specific enhancers that are in proximal to the MYC promoter in the native three-dimensional chromosomal conformation [80, 81]. One such SNP, associated with risk of both prostate and colorectal cancer, has been found to lie in a regulatory element whose knockout leads to altered expression of several genes, including MYC, in colorectal cancer cells [82]. Knocking out the orthologous enhancer in mice results in decreased Myc expression in the colon and decreased numbers of polyps in a mouse model of colorectal cancer [83]. Finally, a SNP at this locus associated with breast cancer was found to correlate with expression levels of targets of MYC, further supporting the hypothesis that the cancer risk SNPs at 8q24 function by altering regulatory elements that influence MYC.

SNPs as Predictive Biomarkers

GWASs are also potentially useful in that the disease-associated variants potentially can be used as predictive markers, even in the absence of a biological understanding of function. The first application of this approach to prostate cancer was in 2008, when it was suggested that five SNPs could be used as a predictive marker of prostate cancer risk [84]. More recently, extending this approach to include 66 prostate cancer risk SNPs and weighting SNPs by the magnitude of their effect on risk, Pashayan and colleagues suggested that this SNP-based risk prediction could be used to guide screening decisions and reduce overdiagnosis [85]. However, unlike in the case of diseases where no good biomarkers currently exist, PSA screening does predict who has a cancerous prostate, albeit with controversy regarding overtreatment. Therefore, in determining the clinical significance of SNPs, it is useful to consider how SNPs compare to PSA in risk prediction. By this standard, a recent study found that SNPs perform worse than PSA in predicting either any disease or aggressive disease and that simply adding SNPs to PSA does not markedly improve the prediction [86].

Recent studies in several diseases have suggested a polygenic model of disease, in which numerous SNPs, each with a small effect, contribute to disease risk. For instance, a study of schizophrenia found that including SNPs for which the evidence of association was only nominal improved the predictive accuracy of the genetic model [87]. In a different modeling approach, it has been shown that a substantial fraction of the heritability of human height can be explained by the set of common SNPs genotyped on a common microarray platform [88]. Similar approaches have been used to demonstrate the polygenic nature of other common diseases, including cardiovascular disease and rheumatoid arthritis [89, 90]. In the case of prostate cancer, however, the picture is more mixed. On the one hand, early studies on building predictive models with a large number of nominally associated SNPs did not improve the accuracy of the models [91]. On the other end, modeling the fraction of the variance explained by the complete set of common SNPs suggests that the heritability of prostate cancer due to these common variants alone ranges from 0.3 to 0.8 [92, 93]. Further studies in larger cohorts will be needed to determine the extent to which SNPs can be useful predictive markers in prostate cancer.

Summary

In sum, genome-wide association studies have identified numerous SNPs associated with the risk of developing prostate cancer. While each of these SNPs alone only modestly alters a man's risk of prostate cancer, together common genetic variants may explain a large proportion of the risk of prostate cancer in the population. The use of these SNPs as predictive markers, however, will need to be carefully defined. Identification of SNPs associated with prostate cancer can also give insight into disease biology. As developing a functional explanation for a single associated SNP

is an extensive undertaking, only several prostate cancer risk loci have been well characterized. Looking forward, integrative approaches will be more likely to give insight into disease biology from GWASs.

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Chapter 9
Copy Number Variation/Chromosomal Aberration

Olivier Elemento

Contents

Introduction

Prostate cancer remains a major cause of cancer-related mortality. Recent studies employing next-generation sequencing have revealed that multiple genomic alterations underlie the formation and the evolution of prostate tumors [1–4]. These include point mutations and indels in genes such as FOXA1, SPOP, and MED12 as well as structural variations, which frequently involve multi-megabase genomic regions that contain several to many genes. Structural variations include genomic losses, i.e., the deletion of large genomic fragments, genomic gains, i.e., duplication of large genomic fragments, and also translocations and inversions. Just like other mutations, structural variations can be clonal (a majority of tumor cells have it) or

O. Elemento, Ph.D.
Caryl and Israel Englander Institute for Precision Medicine, Institute for Computational Biomedicine, Department of Physiology and Biophysics, Weill Cornell Medicine, New York, NY, USA
e-mail: Ole2001@med.cornell.edu

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*, Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_9

subclonal (only a fraction of cells have it) [3, 5]. Structural variations typically involve DNA breaks and religation. The genomic breakpoints that ensue can be complex and include additional nucleotides added during religation [6]. Recent preclinical and clinical data shows that a significant number of CNAs and CAs are potentially targetable. In this chapter, we review recent knowledge about copy number alterations and chromosomal aberrations. We also review knowledge about the nascent but increasingly important field of germ line copy number variations as source of prostate cancer risk.

Somatic Copy Number Alterations in Prostate Cancer

Recent interrogation of the TCGA prostate cancer genome results reveals 58 copy number alterations by GISTIC2, including deletion of CHD1 (5q21.1), gain of MYC (8q24.21), and deletion of CDKN1B (12p13.1) (http://www.cbioportal.org/study.do?cancer_study_id=prad_tcga). Well-known intrachromosomal gene fusions such TMPRSS2-ERG are also linked to copy number alterations, specifically deletions as deletion of intervening DNA gives rise to gene fusion [7]. PTEN is also frequently deleted in prostate cancer, although the deletion involves complex intragenic events [1]. These CNAs correspond to recurrently altered, minimal genomic regions. In individual patients, altered regions can be much larger and involve entire chromosomal arms [8].

The role and function of these somatic CNAs (SCNAs) in prostate tumor formation and maintenance is only partially known, and few have been directly linked to tumorigenesis. CHD1 was recently linked to cell invasion [9] and to an increased rate of other chromosomal alterations [10]. A variety of mouse models of PTEN loss have shown a role for PTEN in tumor progression and metastasis [11].

Recent computational algorithms allow exploration of clonality of SCNAs and can inform on the timing and order of mutations [5]. These analyses, when applied to prostate cancer genomic data, have revealed that certain SCNAs are frequently clonal, e.g., NKX3–1 and TMPRSS2-ERG. Such clonality is consistent with these alterations arising early in the evolutionary history of these tumors [3]. Other events such as PTEN and CDKN1B loss are frequently subclonal, consistent with later occurring events [3]. Indeed, these SCNAs are associated with higher-stage and advanced disease [2, 12]. The discovery of recurrent mutations and intrachromosomal gene fusions supports the molecular subclasses of prostate cancer, a topic that is addressed in Chaps. 10 and 30.

A recent multicenter study showed that many CNAs are also uniquely found in advanced tumors (CRPC), as a result of mechanisms to overcome treatment especially androgen deprivation. This study showed, for example, that AR is frequently amplified in CRPC [4]. The genetic landscape of castrate-resistant prostate cancer is discussed in Chap. 18.

Somatic Chromosomal Aberration

It was noted in Berger et al. (2011) that complex "chains" of rearrangements could be observed in many prostate cancer cases. Such chains occurred as if broken DNA ends had been shuffled and religated to one another in a novel configuration, thus creating novel compound chromosomes. A later study employing whole-genome sequencing (WGS) further characterized this phenomenon, which was termed "chromoplexy," and showed that it can be found in nearly 90% of prostate tumors [3] (see also Chap. 10). Analysis of chromoplexy events suggested a model of "punctuated" tumor evolution where considerable genomic shuffling and rearrangements occur over just a few events in prostate cancer. Chromoplexy has also been observed in other tumor types including hematological malignancies [13]. It is distinct from the related event of chromothripsis in that it involves two or more chromosomes. Analysis of chromatin interaction data from Hi-C experiments suggested that rearranged breakpoints are closer to each other in the nucleus than other regions, perhaps favoring religation events [3, 14].

Correlation with prostate cancer clinical grade suggested that structural alterations observed in prostate tumors (many of which resulting from chromoplexy) may be linked to the aggressive clinical status of high-grade prostate tumors [3].

Targetable Copy Number Alterations in Prostate Cancer

MYC amplifications may eventually be targetable using BRD4 inhibitors [15]. PTEN losses may be targetable using PI3K, AKT, or mTOR inhibitors, either alone or in combination with other inhibitors [16]. The long tail of tumor-specific SCNAs may also give rise to targetable weaknesses. For example, we recently showed that FANCA somatic hemizygous deletions were associated with sensitivity to cisplatin in a metastatic prostate cancer case if the wild-type (WT) allele was inactivated due to a germ line event [8]. In metastatic prostate cancer with neuroendocrine differentiation (NEPC), gene amplification of AURKA and MYCN were found in 40% of cases. These tumors were shown to be highly sensitive to aurora kinase inhibitor therapy in vitro [17], prompting a clinical trial (https://clinicaltrials.gov/ct2/show/NCT01799278).

Germ Line Copy Number Variants That Predispose for Prostate Cancer

In the past years, extensive germ line copy number variants have been uncovered. A recent paper showed that several of these variants are correlated with increased or decreased prostate cancer disease risk [18]. This includes non-genic copy number

variants overlapping with enhancer epigenetic marks and involved in chromatin interactions with other genes [18]. Genetic susceptibility and single nucleotide polymorphisms (SNPs) associated with the risk of developing prostate cancer are discussed in Chaps. 5 and 8, respectively.

Techniques for CNA/CA Detection

Traditionally the detection of CNA has been performed using DNA fluorescence in situ hybridization (FISH) and related techniques such as TaqMan. The advent of whole-genome analysis and first microarray then next-generation sequencing has revolutionized our capacity to discover and detect SCNAs. One of the first whole-genome approaches for SCNA detection was array-based comparative genomic hybridization (CGH). In aCGH, DNA probes are immobilized on glass slides in thousands of discrete locations collectively called a microarray. DNA from a tumor sample is labeled with a fluorescent dye, and DNA from a control sample (a patient's own germ line DNA from blood or cheek swab) is labeled with a distinct dye. Both DNA samples are mixed and applied to the microarray, leading to hybridization. The comparative strength of hybridization reflects the comparative abundance of each DNA region in the tumor sample. In the related technique known as SNP arrays, only tumor samples are analyzed using the same hybridization principle. Miniaturization of the microarray platforms means that millions of probes can be assayed in parallel, thus allowing high-resolution SCNA analysis.

Array-based platforms are being replaced by next-generation sequencing approaches where millions of DNA fragments are directly sequenced using techniques such as Illumina sequencing. In these assays, detection of copy number losses and gain is correlated to number of short reads mapping to each genomic segments. A larger than expected number of short reads mapping to a genomic segment indicate a gain, while lower than expected numbers indicate a loss. The short-read nature of all current next-generation sequencing technologies makes it difficult to reconstruct structural variation breakpoints; nonetheless, this is being offset by longer-read sequencing, e.g., Moleculo and Oxford Nanopore. Optical mapping strategies such as the Bionano Genomics Irys may soon allow single-molecule analysis of structural variants, allowing resolution of complex breakpoints. In many cases, SCNA needs to be validated using an orthogonal technology. Technologies for validation include DNA FISH and digital polymerase chain reaction (PCR) but also expression analysis using immunohistochemistry (IHC).

Whole-genome approaches for SCNA/CA detection need sophisticated computational analysis software. This is because individual probe measurements for microarray are noisy, in part due to unequal GC content, spatial biases, and other sources of bias. Moreover, individual probe signals must be converted into genomic segments. This is typically done using a process called segmentation, performed using tools such as DNA copy [19]. Approaches such as Excavator [20] and CONTRA [21] allow segmentation of tumor/normal read count log ratios for

markers such as genes or exons. More recent approaches combine tumor/normal read count log ratios with minor allele frequencies. Once detected, clonality of SCNA can be analyzed using tools such as CLONET [5].

Once segments are called, recurrently altered segments must be identified, and the critical genes within each segment must be identified. The latter involves identifying the minimum recurrent region, that is, the minimal region that is commonly altered across several tumors. This is commonly done using the GISTIC approach [22]. P-values for each segment can be calculated by comparing the score at each locus to a background score distribution generated by random permutation of the segment locations in each sample.

Visualization of copy number alteration data, which collectively involve hundreds if not thousands of patients, can be performed using interfaces such as the cBioPortal [23].

Other non-SCNA chromosomal aberrations can be detected from WGS using a variety of programs. Translocations can be detected using BreakDancer [24] and Delly [25] among other programs (see also Chap. 10). The ChainFinder program can, for example, be used to identify chained rearrangements observed in chromoplexy [3]. The detection of structural variants is a dynamic area with significant room for improvement, as indicated by the limited overlap between outputs of existing programs.

Discussion

One of the main challenges in the detection and treatment of prostate cancer is the significant molecular heterogeneity of tumors in general [26]. Approaches to decipher this heterogeneity are becoming available. For example, single-cell WGS and detection of single-cell-specific SCNA are now possible as recently established for breast cancer [27]. Further analyses using either patient-derived xenografts or tumor organoids, which preserve genomic features of original tumors including structural variants, will be required to expand actionability of frequent copy number alterations and gene fusions [28]. Extensive DNA damage linked to chromoplexy may also be targetable using synthetic lethality approaches. In fact, synthetic lethality may be a remarkably efficient approach for exploiting genomic structural losses or gains frequently observed in tumors [29].

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Chapter 10
Gene Fusions

Andrea Sboner

Contents

History of Gene Fusions

Gene fusions, or chimeric transcripts, can be defined as transcripts that are not colinear in the genome. Two main mechanisms can generate gene fusions: (1) genomic rearrangements or structural variations, where areas that are normally distant on the genome are "relocated," and (2) cis/trans-splicing, a posttranscriptional event, where two genes are transcribed into a single mRNA and then spliced, or when two mature transcripts are "reassembled" to generate a new fusion transcript—both cases without genomic alterations [1]. In cancer, the majority of chimeric transcripts arise from genomic rearrangements, although important cis/trans-splicing events have been reported, including in prostate cancer [2–4].

A. Sboner, Ph.D.
Department of Pathology and Laboratory Medicine, Institute for Computational Biomedicine, Englander Institute for Precision Medicine, Weill Cornell Medicine, New York Presbyterian Hospital, New York, NY, USA
e-mail: ans2077@med.cornell.edu

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*,
Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_10

Chromosomal aberrations were hypothesized to be linked to human cancer since the beginning of the twentieth century, when theories about the genetic origin or cancer were first proposed [5, 6]. However, it was only about 50 years later that these aberrations were recognized as hallmarks of cancer development with the discovery of the "Philadelphia chromosome" in chronic myelogenous leukemia (CML) by the seminal work of Peter Nowell and David Hungerford [7, 8]. In the following decades, thanks to improved cytogenetics techniques, it was shown that the "Philadelphia chromosome" is caused by a translocation between chromosome 9 and chromosome 22 resulting in a novel fusion protein (BCR-ABL) [9, 10]. During the 1970s and 1980s, the scientific investigation in this field led to the discovery of other translocations, such as IGH-MYC in Burkitt's lymphoma, and since then the number of rearrangements associated with cancer kept growing [11–15].

In the late 1990s, the recognition that BCR-ABL-positive CML patients could be treated with tyrosine kinase inhibitors and increase their overall survival to 30 years instead of 2–3 years is likely the most successful story in cancer research and ushered us in the era of targeted therapy or precision medicine [16–21].

Historically, gene fusions were deemed more prevalent in hematological malignancies and mesenchymal neoplasia (soft tissue tumors) than in epithelial cancers, and only a few isolated examples from breast and prostate cancer cell lines were known [22–24]. This erroneous notion was likely due to methodological and technical bias: chromosome quality for cytogenetic studies was much higher in hematologic malignancies, and heterogeneity in the epithelial neoplasm acted as an additional confounding factor [25]. The work by Mitelman and colleagues to catalog gene fusions demonstrated that no biological reason could explain a bias of gene fusions toward hematological malignancies [13]. However, it was not until the discovery of a highly recurrent gene fusion in a common epithelial cancer that a broader role of chimeric transcripts was fully recognized.

Discovery of Recurrent Gene Fusions in Prostate Cancer

In 2005, Tomlins et al. reported the first case of highly recurrent fusions in a common solid tumor—prostate cancer—when they discovered that the transmembrane protease, serine 2 (TMRPSS2—21q22.2) gene is fused with v-ets avian erythroblastosis virus E26 oncogene homolog (ERG—21q22.3) or the ets variant 1 (ETV1—7p21.2), by using a computational approach called cancer outlier profile analysis (COPA) [26]. Intriguingly, fusion transcripts involving these two genes were well known in Ewing's sarcoma. Tomlins et al. performed 5′ rapid amplification of cDNA ends (RACE) and identified TMPRSS2, an androgen-regulated gene, as common partner [26]. They also observed mutual exclusivity of TMPRSS2-ERG and TMPRSS2-ETV1 fusions. Several subsequent studies confirmed their high prevalence in prostate cancer where 30–50% of the cases harbor these fusions [27–33].

Tomlins et al.'s seminal work was the first study demonstrating that recurrent gene fusions can play a role in common carcinomas as well. Soon after, the search for fusions in common solid tumors was revitalized, and several others have been reported in lung, breast, colorectal, ovarian, brain, bladder, gastric, thyroid, and renal cancer, in melanoma, and in other rare cancer types [24, 34–45].

ETS Fusions in Prostate Cancer

Of note, the novel fusions detected in prostate cancer included members of the ETS family of transcription factors as in Ewing's sarcoma, where as many as 90% of these sarcomas harbor a fusion between EWS (Ewing's sarcoma gene) to FLI1 [46]. This family includes about 27 genes divided in 11 subfamilies all sharing the DNA-binding domain (ETS domain) [47]. They play a role in many biological processes including development, proliferation, differentiation, apoptosis, cell migration, and angiogenesis [48–50]. The fusion transcript commonly generates a chimeric protein by linking the N-terminal region of EWS to the ETS domain of FLI1. ERG is also implicated in about 5% of Ewing's sarcomas, and in rare cases, other ETS genes are fused with EWS. In leukemia, different fusion genes involving ETV6 have been detected [51]. Typically, the first exons of EWS, which encode a transactivating domain, generate chimeric proteins that regulate the expression of ETS target genes in addition to the ETS genes themselves.

In prostate cancer, TMPRSS2-ERG is by far the most common fusion (~50% of cases) resulting from an interstitial deletion between the two genes which are ~3 MB apart on chromosome 21 or from a more complex translocation without loss of genetic material [26]. The role of TMPRSS2 is mostly to provide a strong activating promoter, but there can be several different chimeric transcripts, the vast majority including the first exon of TMPRSS2 and exons 4 and 5 of ERG [27, 52]. Importantly, the ETS DNA binding domain is preserved in most of the chimeric transcripts, although a few variants may lead to a truncated ERG protein. Interestingly, these isoforms can be concomitantly expressed in a single sample, suggesting that alternative splicing can further increase the diversity of fusion isoforms [52, 53].

Other members of the ETS family (ETV4, ETV5, ELK4, and FLI1) have been involved in gene fusions in prostate cancer albeit at lower frequencies (~1–10%) [3, 54–57]. While the most recurrent partner of ERG is TMPRSS2, several 5′ partners have been identified for the other ETS genes, many being under androgen regulation (see Table 10.1).

ETS fusions result from underlying genetic rearrangements with an exception: SLC45A3-ELK4, caused by a cis-splicing, or read-through, event. In this case, a pre-mRNA is transcribed across the gene boundaries, and then splicing generates a chimeric transcript by joining exon 1 of SLC45A3 and exon2 of ELK4 [3, 4, 56]. In contrast to other gene fusions, SLC45A3 is not exclusive to prostate cancer cells, but it can be detected in benign prostate tissues, although at much lower levels.

Table 10.1 Ets prostate cancer fusions

ETS partner	5' partner	Frequency	Androgen regulation
ERG	TMPRSS2	~50%	Upregulation
	SLC45A3	~3%	Upregulation
	NDRG1	~2%	Upregulation
	HERPUD1	~1%	Upregulation
ETV1	TMPRSS2	~5–10%	Upregulation
	SLC45A3		Upregulation
	HERV-K17 (FLJ35294)		None
	HERV-K		Upregulation
	EST14		Upregulation
	C15orf21		Downregulation
	FOXP1		Upregulation
	HNRPA2B1		None
	ACSL3		Upregulation
ETV4	TMPRSS2	~1–5%	Upregulation
	DDX5		None
	CANT1		Upregulation
	KLK2		Upregulation
ETV5	TMPRSS2	<1%	Upregulation
	SLC45A3		Upregulation
ELK4	SLC45A3[a]	<1%	Upregulation
FLI1	SLC45A3	<1%	Upregulation

[a]SLC45A3-ELK4 is a read-through

Non-ETS Fusions in Prostate Cancer

The discovery that roughly 50% of prostate cancer cases harbor a gene fusion naturally raised the question: are there other fusions characterizing the group of ETS-negative cases? Palanisamy et al. analyzed a set of 15 cases including prostate cancer samples and identified chimeric transcripts involving genes in the RAF kinase pathway: SLC45A3-BRAF and ESRP1-RAF1 [37]. These findings are particularly important because of the potential therapeutic options available with RAF and MEK inhibitors in advanced cancer. Pflueger et al. interrogated a set of 25 samples enriched for ETS-negative cases and did identify novel gene fusions involving non-ETS genes including CDKN1A, CD9, IKBKB, PIGU, and RSRC2 [58]. All these fusions were detected at very low frequency, but, surprisingly, they were all associated with cases already harboring the TMPRSS2-ERG fusion, suggesting that a common mechanism driving the generation of chimeric transcript is active in this subgroup. Recently, Yu et al. identified eight novel recurrent fusion transcripts with frequencies ranging from ~3% to 8% that are associated with prostate cancer recurrence by analyzing three different cohorts [59].

Mechanism of Gene Fusion

What is the mechanism generating these fusions? TMPRSS2-ERG may be explained by the genomic proximity of the two partners (~3 MB apart on chromosome 21). Proximity may also be a factor for other fusions, via the reorganization of genomic regions that brings together distant loci, for example, by genomic looping. In addition, as most 5′ partners are prostate specific and androgen regulated, transcriptional regulation seems to play a role. Evidence has shown that AR binding can favor this reorganization of the genome [60, 61]. Moreover, AR can promote double-strand genomic breaks by recruiting enzymes such as topoisomerase II beta, cytidine deaminase (CDA), or ORF2 endonuclease [61, 62].

Additional indications supporting this hypothesis came from the study by Berger et al. The researchers interrogated whole-genome sequencing (WGS) of seven prostate cancer samples, with and without ERG rearrangements [63]. Interestingly, all the samples showed evidence of interdependent complex "chains" of genomic rearrangements, where distant areas of DNA are "shuffled" and religated in a novel configuration. This observation was confirmed and extended in a subsequent study by Baca and colleagues [64]. Their analysis provided further evidence for these "chains" of translocations, sometime accompanied by loss of genomic material near the breakpoints, a phenomenon named chromoplexy [64] (see also Chap. 9). They observed that, in several samples, more than one chain is present, with variable numbers of genes per chain, ranging from 3 to 40 genes. Computational analysis suggested that these chains coordinately induce structural alterations, thus simultaneously disrupting several genes. Importantly, the ETS-positive group exhibited a different pattern of chromoplexy than the ETS-negative class. In ETS-positive cases more inter-chromosomal rearrangements were present than in ETS-negative tumors. This observation led to the speculation that distinct processing mechanisms underlie the formation of the chains in these two groups. The authors hypothesized that in ETS-positive cases DNA damage may occur at "transcriptional hubs," where genes across multiple chromosomes are co-regulated, perhaps via transcription factors. This hypothesis was supported by the fact that genes regulated by the androgen receptor are typically included in these chains. This observation provided further support of a link between active transcription and DNA damage [61, 62]. In contrast, chromoplexy events in ETS-negative tumors exhibited a high number of intrachromosomal events, thus resembling chromothripsis, a phenomenon where a single catastrophic event "shatters" a chromosome [65–68]. In the ETS-negative group, a higher number of rearrangements were present, especially in tumors with associated focal deletion of CHD1, a chromatin-modifying enzyme, which may regulate genomic stability [69]. Given the widespread nature of chromoplexy in prostate cancer, its role may be to coordinately deregulate several genes and thus confer selective advantage to cancer cells that otherwise, by a gradual accumulation of alterations, would not be viable [64]. Furthermore, by using a computational

method to assess the clonality of the alterations, this study showed that multiple chromoplexy events occur at different times during cancer progression [64].

Altogether, the results suggested a model of punctuated evolution of prostate cancer, where "simultaneous" disruption of several cancer genes by chromoplexy events drives the tumor development and progression. Although this hypothesis still awaits experimental validation, it is nevertheless reasonable to speculate that chromoplexy is one of the underlying driving processes in prostate cancer that may explain the higher number of copy number alterations and fusions in this tumor type and also offer one plausible explanation of the discovery of multiple gene fusions in a single sample.

Role of Gene Fusions in Prostate Cancer

Gene fusions can exert their oncogenic potential via two main mechanisms: (1) by generating a novel fusion protein product, such as the BCR-ABL case creating a novel tyrosine kinase, or (2) by altering the transcriptional regulation via the juxtaposition of a new promoter and 5'UTR sequence upstream of a proto-oncogene resulting in aberrant expression of the genes [23]. In the case of prostate cancer, the latter seems to be the preferred mechanism of action as the majority of ETS fusions encode a truncated or null fusion protein. The 5′ partner, almost always regulated via androgen signaling and typically prostate specific, drives the overexpression of the 3′ partner of the fusion. The exact role of these fusions in cancer development and progression is still a matter of active research, especially in the case of novel, less common chimeric transcripts that have been discovered since then. Most studies in literature thus focused on ETS fusions. Several lines of evidence suggest that they likely act as "gatekeeper": the fusion itself is not sufficient to form fully develop tumors, but in concert with other alterations, it confers cells the full neoplastic potential [70–72]. ETS fusions are early events that are also present in high-grade prostatic intraepithelial neoplasia (PIN) [33, 73, 74]. TMPRSS2-ERG has not been found in benign prostatic lesions or proliferative inflammatory atrophy [75]. Genomic studies using next-generation sequencing have also confirmed that ERG rearrangements are early events in prostate development [64, 76]. Overexpression of ERG or ETV1 in cell lines increases cell migration and invasion, but in murine models, no invasive cancer develops. ERG overexpression only in conjunction with PTEN deletion, TP53 mutations, or other tumor suppressor gene alterations is thought to lead to a more aggressive phenotype [77–79].

Clinical Implications of Fusions

The success of imatinib for the management of leukemia patients with BCR-ABL1 fusions is the most remarkable example of the clinical impact of gene fusions on cancer treatment. FDA-approved tyrosine kinase inhibitors (TKIs) for ALK fusions in non-small cell lung cancer and PDGFR fusions in dermatofibrosarcoma

protuberans, hypereosinophilic syndrome/chronic eosinophilic leukemia, and myelodysplastic syndrome/myeloproliferative neoplasm are now available (http://www.fda.gov/Drugs/ScienceResearch/ResearchAreas/Pharmacogenetics/ucm083378.htm) [15]. Off-label use of TKIs may be promising for the management of patients with these alterations in other tumor types. In prostate cancer, targeting TMPRSS2-ERG fusions has been shown to be a promising therapy [80].

Despite these exciting advances in cancer treatment, the more common clinical application of gene fusions is diagnostic or prognostic. The correlation of fusions with tumor subtypes, for example, makes them excellent candidates as diagnostic biomarkers. In some cases they are pathognomonic event, such as in epithelioid hemangioendothelioma (EHE) and in solitary fibrous tumor (SFT) [81–83]. In prostate cancer, the detection of TMPRSS2-ERG in urine has shown high specificity (93%) and positive predictive value (94%), and the combination with other genes can improve prostate cancer early detection [84, 85].

Gene fusions are also relevant to stratify risk, whenever heterogeneous outcomes are associated to fusion status in otherwise homogenous malignancies, an example being the MLL fusion status in AML [86]. In prostate cancer, however, the presence of ETS fusion is still highly debated as prognostic indicator with several studies reporting conflicting findings [29, 30, 78, 87–91]. These differences may be related to the different clinical settings and outcome measures and different genetic background of the cohorts. Indeed, lower frequencies of ERG rearrangements have been reported in Asian and in African-American cohorts [92–97]. Another explanation of the difference in clinical outcomes could be the diversity of the chimeric variants: TMPRSS2-ERG cases harboring an interstitial deletion have poorer outcome than those without loss of genomic material. Also, different N-terminal truncated ERG proteins may have different functional activity in binding to promoters of ETS target genes [90, 91, 98, 99].

Fusions in Advanced Prostate Cancer

A recent study looking at metastatic tissue of prostate cancer patients confirmed the presence of ETS rearrangements and fusions involving BRAF and RAF1 but also found novel potentially clinically relevant fusions in PIK3CA/B and RSP02, albeit at very low frequencies (~1.3%) [100]. This global analysis of genomic alterations in metastatic samples showed that new alterations, including fusions, arise in the context of therapeutic intervention, warranting more investigations to fully understand the impact of treatment on cancer biology.

Technology for Discovery and Validation

The history of the Philadelphia chromosome is deeply connected to the technological advances that allowed inspections at increasingly higher resolution into the genome. Nowadays, the tremendous technological advances in sequencing have expanded our

capabilities of detecting and investigating the role of chromosomal rearrangements in cancer development and progression. Massive parallel sequencing, or next-generation sequencing (NGS) as it is more commonly known, has indeed enabled the investigation of genomic and transcriptomic alterations that occur in cancer at an unprecedented depth. The resurgence of the hunt for novel fusions is undoubtedly linked to the advent of RNA sequencing (RNA-seq). In a typical experiment, poly-A selected transcripts are fragmented, resulting in "snippets" of about 250–400 bp, and both ends of each fragment are then sequenced (see Fig. 10.1). This process will produce millions of paired sequences (or reads), hence the name paired-end RNA-seq. Reads are then aligned to a reference sequence, e.g., the human genome, to identify their provenance, a process called mapping. As one can imagine, identifying paired reads where the two ends are mapped to two distinct genes is an indication of the presence of a fusion transcripts (Fig. 10.1). Moreover, some reads may also provide information about the actual fusion junction sequence and thus indicating the transcript breakpoints. However, this seemingly straightforward analysis is complicated by the presence of artifacts due to several sources of noise:

1. Mismapping of the reads: Determining the exact location of the read is a daunting process because of repetitive or paralogous sequences; this is also compounded by the presence of single nucleotide polymorphisms (SNPs), somatic mutations, RNA editing, or sequencing errors.
2. Library preparation artifacts due to inefficient A-tailing leading to random ligation of cDNA molecules; this is particularly relevant in the case of highly expressed genes because the high number of mRNA fragments makes more likely to result in artificial chimeric transcripts.

Several computational tools have been devised to address these challenges; however, the reduction of false positives is still one of the major hurdles for reliable automatic detection of chimeric transcripts [53, 101–113].

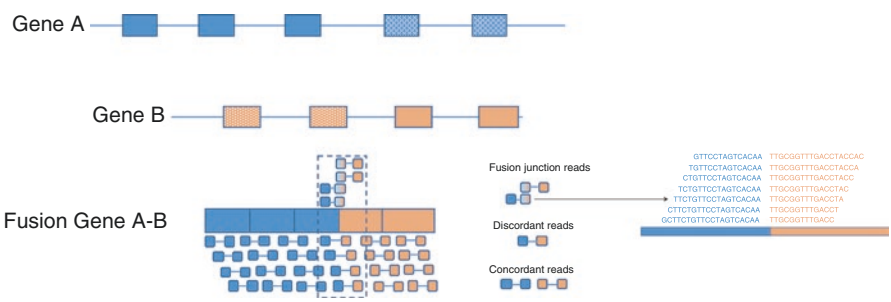

Fig. 10.1 Schematic example of the identification of fusion transcripts from RNA-seq experiments. Assuming genes A and B are transcribed left to right, the first three exons of gene A are fused with the last two exons of gene B to generate a fusion transcript with five exons (fusion genes A–B). A typical paired-end RNA-seq experiment determines the sequences of the ends of mRNA fragments that are about 250–400 nucleotide long (in the *dotted box*). Only the discordant paired-end reads (where the two ends are from a different gene) and the fusion junction reads (single ends that include the fusion junction) provide evidence for a fusion transcript

Laboratory verification of the computational predictions is critical [114, 115]. Reverse transcriptase-polymerase chain reaction (RT-PCR) and/or fluorescence in situ hybridization (FISH) are the two most common validation methods adopted, which are able to confirm the presence of the predicted chimeric transcripts (PCR) and the potential involvement of genomic rearrangements (FISH).

Conclusions

Structural rearrangements leading to gene fusions have been recognized as important driver alterations in several malignancies. The history of their discovery has been closely associated to the technological improvements that have expanded our capabilities to "view" the genome at higher resolutions. Nowadays, sequencing technology is our most advanced "microscope" into the genome, with nucleotide base resolution. The search for novel gene fusions has greatly benefitted by this technology, with more than 9000 fusions identified in the last 3 years only [15].

New exciting opportunities lie ahead: sequencing technology is now providing longer reads that will help overcome some of the limitations of short reads. Indeed, longer reads can help in reconstructing the full chimeric transcript and reduce the large number of false positives affecting current methods. Moreover, improvements in extracting and sequencing RNA from archival material can provide the possibility to exploit the vast number of specimens available in every pathology department around the world, expanding our understanding on the prevalence of gene fusions across many different tumor types and the mechanisms of action.

As cost of sequencing continues to decrease, the combined analysis of structural genomic variations and transcriptomics will better refine our computational predictions and provide more reliable methods to detect and understand the role of gene fusions in cancer biology, from development to progression of the disease.

Improved extraction and sequencing technology will also enable the detection of fusions and rearrangements from plasma and circulating tumor cells. Effective monitoring of the progression of the disease during treatment will provide timely information regarding emergence of resistance and will prompt for a change of therapeutic intervention, fulfilling one of the goals of precision medicine.

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Chapter 11
Gene Expression Analysis

Michael Ittmann

Contents

Introduction

Alterations in gene expression in cancer are fundamental to the aberrant biology of cancer cells. Traditionally, genes are thought of as heritable units that encode proteins, and indeed changes in expression of protein-coding genes are critical in the pathogenesis of cancer. A modern definition for a gene has been proposed to be "a union of genomic sequences encoding a coherent set of potentially overlapping functional products" [1]. This definition includes many types of transcripts that do not encode proteins and yet have important cellular functions that determine phenotype. These include transcripts such as miRNAs and long noncoding RNAs (lncRNAs), which are important in the biology of prostate cancer. There is growing evidence that such lncRNAs may be extremely useful for diagnosis and prediction of prognosis and as therapeutic targets in prostate cancer. These types of transcripts can be and have been studied using gene expression arrays [2], but this chapter will focus on protein-coding transcripts. See Chap. 16 for an in-depth discussion of miRNAs and lncRNAs.

M. Ittmann, M.D., Ph.D.
Department of Pathology and Immunology, Baylor College of Medicine, Houston, TX, USA
e-mail: mittmann@bcm.edu

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*,
Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_11

Recent studies have concluded that about 80% of the variation in protein levels can be linked to differences in mRNA levels after correcting for methodological bias [3]. The remainder of protein variation can be accounted for by protein degradation and translational control. Thus, much of the variation in the proteome is controlled by mRNA abundance. In addition to the multiple factors controlling gene transcription in normal cells, mRNA levels in cancer cells can be linked to stable genomic alterations such as gains or losses of genes (copy number variation), the presence of gene fusions, promoter mutations, and alterations in DNA methylation. Such changes at the genome level are fundamental to the pathogenesis of cancer and often achieve phenotypic expression primarily by changes in mRNA levels. See Chaps. 9 and 13 for an in-depth discussion of copy number and epigenetic alterations in prostate cancer.

With the advent of the molecular biology revolution in the 1970s, mRNA levels could be measured, albeit laboriously, with techniques such as Northern blotting. The discovery of polymerase chain reaction (PCR) and later quantitative reverse transcription PCR (Q-RT-PCR) made analysis of gene expression much easier and more quantitative. Indeed, using Q-RT-PCR arrays, hundreds of genes can be assayed in multiplexed formats, and such arrays are currently widely available for focused analysis of specific gene sets. However, high-throughput technologies such as expression microarray analysis have revolutionized our ability to carry out unbiased analysis of gene expression alterations in cancer. In expression microarray analysis, thousands of cDNAs or oligonucleotides corresponding to individual genes are spotted on solid surface such as a glass slide or silicon chip. Fluorescently labeled cDNAs, generated from cellular RNA, are then hybridized to the chip, and after washing to remove nonspecific signals, the chip is scanned. The fluorescent signal is proportional to the abundance of the corresponding RNA used for labeling. The use of cDNAs on arrays has been largely supplanted by the use of oligonucleotides designed to hybridize to specific gene segments. In addition, some formats use beads rather than a solid surface. In any format, expression levels of tens of thousands of genes can be assayed simultaneously, dramatically increasing the ability to interrogate gene expression in a biological sample in a comprehensive manner.

Biological Considerations

Gene expression array technology has been used to study prostate cancer in two major ways. One major type of analysis is studying changes in gene expression in vitro using prostate and prostate cancer cell lines. In this approach, large-scale changes in gene expression can be assessed in different cell lines or more commonly a single cell line under different conditions. This technique can assess the impact of gene knockdown or overexpression, drug treatments, alterations of environmental conditions, etc. on gene expression versus appropriate controls [4–6]. In recent years, such approaches have been used extensively to understand the underlying biology of prostate cancer. In general, biological duplicates or triplicates are needed

for each cell line for each condition to allow more reliable comparisons of gene expression in the two biological states.

The other major use of gene expression arrays has been the study of human and to a lesser extent mouse tumor tissues. The study of human prostate cancer tissues was certainly one of the first major applications of this technology and has yielded many important insights into prostate cancer biology and has identified novel diagnostic and prognostic biomarkers [7–10]. Most published studies have used cancer tissues (and corresponding benign tissues) from radical prostatectomy specimens. Such tissues are potentially readily available given that tens of thousands of radical prostatectomies are performed each year in the United States alone. However, specific precautions are needed to minimize "warm ischemia," i.e., the interval between devascularization and rapid freezing of tissue for later analysis. This is a particularly important consideration in robotic prostatectomies, since warm ischemia can be prolonged in such specimens since they may remain in the patient after devascularization [11]. However, with collaboration between surgeons, pathologists, and other health-care personnel, tissue can be collected in a manner which minimizes warm ischemia and maximizes RNA integrity [11–13]. However, it should be noted that it is impossible to completely eliminate warm ischemia in a surgical specimen. Generally, 15–30 min of warm ischemia is excellent, and up to 60 min is probably acceptable [14]. Of course, tissues with warm ischemia can be used for some studies, particularly DNA analysis, but mRNAs tend to be labile. Thus mRNA integrity can be a surrogate marker of overall tissue quality assuming that RNAs are not degraded during extraction.

One important consideration in the analysis of human prostate cancer specimens is that there is a significant amount of non-cancer cells in such tissues including tumor stroma, benign prostate tissue, and inflammatory cells (both in benign prostate and tumor tissue). It should also be noted that the benign tissues can undergo atrophy and a variety of epithelial metaplasia and hyperplasia. In addition, high-grade prostatic intraepithelial neoplasia (HGPIN) can be present in the non-tumor tissues. In general, it is best to use highly enriched tumor samples for microarray analysis to minimize the contribution from benign tissue components. While there is no official standard, this author believes 50% tumor is a reasonable minimum, although usable data can be obtained with less enriched tissues. However, it should be noted that prostate cancer foci can be difficult to identify by gross examination, and inevitably there will be selection bias for larger and more cellular tumors (often with higher Gleason score) if one uses only more cellular tumor samples. That said, it can be argued that smaller tumors with lower Gleason scores are not of great interest since they rarely result in patient mortality.

It is known that a significant fraction of the alterations in gene expression in prostate cancer versus benign tissue is derived from the cancer stroma [15]. One method of insuring gene expression profiles which are derived from the cancer cells, cancer stroma, benign epithelium, or benign stroma is to carry out laser-capture microdissection [16, 17]. This technique is quite labor intensive but yields more specific results. Generally, amplification of the RNA obtained is required to yield sufficient quantities for gene expression analysis, and controls should always be

carried out to insure that no bias is occurring during amplification [16, 17]. A relatively small number of studies have been carried out using this technique, but they have revealed novel paracrine interactions of tumor stroma with the cancer cells that biologically relevant to tumor progression [18].

If one is comparing cancer tissues to benign tissues from the same prostate, there are also caveats. Since most cancers arise in the peripheral zone, it is presumably better to use this tissue for comparison. Benign prostatic hyperplasia, which arises in the transition zone, is known to induce extensive changes in gene expression and is extremely common in radical prostatectomy specimens [19]. It should also be noted that the same issues of tissue heterogeneity of benign tissues apply to the control benign samples. In particular it is important to exclude high-grade prostatic intraepithelial neoplasia (HGPIN) in the benign samples. However, it would also important to exclude severe acute and/or granulomatous prostatitis, which can be seen in radical prostatectomy specimens. Unfortunately, it can be difficult at times to discern from published reports what are the criteria used, if any, to exclude benign tissues for analysis. Finally, it should be noted that benign adjacent tissues from radical prostatectomies may not be truly "normal" since there is evidence for premalignant field effects in such tissues [20–22]. In practice, age-matched normal prostate tissue is very hard to obtain. Prostate can be harvested from organ donors, but such men are almost always considerably younger than the typical prostate cancer patient. Prostate tissue can also be obtained from radical cystoprostatectomies for treatment of bladder cancer, but one must be cautious about potential gene expression changes induced by bladder cancer treatments, i.e., BCG, etc. Thus, no perfect control tissue is readily available, but carefully characterized benign peripheral zone tissue is probably the best tissue to use in practice.

Metastatic tissues have also been used for gene expression arrays [23, 24]. Such tissues are not commonly obtained in routine clinical practice except for palliative procedures (channel TURPs, relief of spinal cord compression, etc.), but several leading institutions have developed rapid autopsy programs to obtain tissue from lethal prostate cancer. Of course, it should be noted that there is some contamination of normal tissues from various sites as well as tumor stroma, so not all of the RNA is derived from cancer cells in these tissues.

Given that most gene expression arrays have been done on clinically localized disease treated by radical prostatectomy or heavily treated end-stage disease, there is little known about specific gene expression patterns in other clinical prostate cancer states such as treatment-naïve advanced local disease or treatment-naïve metastatic disease to the lymph nodes, bone, or other sites. With emphasis on targeted therapy, biopsy and molecular analysis of such disease states is likely to become more common in the research setting, allowing for further studies of such tissues.

While all of the above caveats may seem to detract from the utility of gene expression analysis of cancer tissues, in reality major advances have arisen from such studies as will be outlined below. However, it is best to be aware of these issues and try and minimize their impact on future studies. In addition, one must keep them in mind when interpreting data from various studies, particularly being aware that not all genes altered in prostate cancer tissues are actually altered in cancer cell themselves.

Expression microarrays can also be used to analyze mouse tissues or human tissues in mice. Analysis of xenografts of human tissues in immunocompromised mice is straightforward and can be used to examine treatment effects, impact of genetic alterations, etc. similar to studies in tissue culture [25–27]. Gene expression arrays can also be used to analyze gene expression in genetically engineered mouse models of prostate cancer [28, 29]. This can give unique insights into the biology of prostate cancer with genetically defined lesions. The mouse prostate consists of distinct lobes [30] (ventral, dorsal, lateral, and anterior) with their own distinct gene expression profiles [31]. Early small lesions can be examined in each lobe (with appropriate controls), while larger cancers usually invade adjacent tissues and cannot be divided into lobes. A number of laboratories have recently used a combined approach in which both human and mouse expression data are mined to define key regulatory pathways in prostate cancer [32–34].

Analytical Considerations

A key element in obtaining accurate gene expression array data is the quality of the input RNA. This is commonly expressed as an RNA integrity number (RIN) or RIN value [35]. The maximum value of RIN is ten, which is quite hard to achieve in practice. Generally, a RIN number of >7 is considered necessary for acceptable quality for RNA from fresh tissues. RNA can be partially degraded due to prolonged ischemia, slow freezing, thawing of tissue samples, and at many points during RNA extraction due to inadequate technique. The use of formalin-fixed paraffin-embedded (FFPE) samples for large-scale mRNA microarray analysis has remained challenging. RNA quality and degradation can be variable, and to date mRNA from frozen tissues remains the gold standard [36]. However, more focused mRNA analysis is certainly possible using specifically designed analytical platforms. DNA analysis in FFPE is more easily performed due to its higher stability but again is more difficult than analysis of DNAs from frozen tissues.

It goes without saying that meticulous attention to detail for reverse transcription, labeling, hybridization, washing, and scanning is critical to obtain accurate results. It is beyond the scope of this review to examine these factors in details, particularly since multiple platforms and approaches are in use, each with distinct technical requirements. That said, several studies have shown generally high concordance rates between different platforms and techniques of labeling [8].

There are two basic approaches to array hybridization. Two-color hybridization uses dyes of two different colors labeled to RNAs representing two conditions, i.e., Cy3 for cancer and Cy5 for matched benign. This gives a direct readout of the relative expression of any probe by the ratio of fluorescence at the two appropriate wavelengths. One-color arrays use only a single dye, and intensity is measured directly and compared to intensity in other arrays directly. The assumption is that labeling and hybridization are relatively similar for all arrays and that any differences between samples in different batches can be accounted for by mathemati-

cal normalization and correction of batch effects. The two methods yield similar results; one-color approach is the dominant mode of analysis and in practice gives robust data [37].

The number of oligonucleotide probes in an array for any given gene is variable. If multiple probes for a single gene show similar alterations of intensity in a given analysis, it adds confidence that the observed change reflects biology and is not an artifact. Of course differences between probes for the same gene may also reflect alternative RNA species arising by alternative splicing, alternative promoters, etc. Alternative splicing plays an important role in prostate cancer biology [38]. For example, changes in alternative splicing of FGF receptors [39] and androgen receptor [40] are well documented in prostate cancer. One can use microarrays to detect alternative splicing using either custom-built arrays or arrays with all known exons [41]. The former is used for more focused analysis since it targets known slice junctions. The latter reveals differences in exon usage but cannot really document the full repertoire of alternatively spliced transcripts. As a hypothetical example, if one detects decreased intensity of exon 2 of a given gene and exon 6 shows increased intensity, it can be hard to determine if these two alterations are occurring in the same or in independent transcripts or both. That said, exon arrays can provide important clues to potentially biologically important alternative RNA species.

Data Analysis

After hybridization, arrays are scanned, and quality control performed. These files are then used to generate gene expression values using programs such as Bioconductor. Data is normalized to adjust the overall chip brightness of the arrays to a similar level [42]. This is needed since differences in labeling efficiency, hybridization, and wash conditions result in differences in signal between arrays. Techniques such as loess normalization, total intensity normalization, quantile normalization, or invariant set normalization can be used. Batch effects also need to be assessed and corrected for in larger experiments.

Of course, data analysis performed depends on the design of the specific experiment or study. Quite often, the goal of the microarray experiment is to define genes that are differentially expressed between two biological states, i.e., cancer and normal, and treated and untreated. Statistical analysis using t-tests can be used to define statistically significant differences between the two sets of data. However, such tests have significant issues in very large datasets. Simplistically, with 60,000 features, using a cutoff of $p < 0.01$ will yield ~600 false positive signals; if 6000 genes are differentially expressed, 10% of these are likely to be false positives. This has led to the use of the false discovery rate or permutation testing to estimate rates of false positivity [43]. While in-depth discussion of statistical approaches to the analysis of microarray data is beyond the scope of this chapter, users need to be aware that such false positive is always an issue. Of course, the actual fold changes are strong indicators of biological significance, i.e., a threefold increase is more likely to be bio-

logically significant than a 10% increase. It should be noted that fold increases tend to be underestimated at the higher end in expression microarrays due to technical factors with array hybridization such as saturation of probe on the array, so that two- or threefold changes in expression are usually highly significant. Outlier analysis has also emerged as important method of identifying differentially expressed genes that are biologically significant [44, 45]. Of course, correlation of gene expression analysis data with copy number analysis, mutations, and other genomic analysis can provide strong support for the importance of a given alteration. For example, if a gene with loss of expression also undergoes frequent homozygous deletion, it is a strong indication that the gene may be a critical tumor suppressor.

In addition to identifying specific genes that are differentially expressed in two datasets, more complex patterns of linked gene expression can be sought. Cluster analysis can be carried out to identify natural groupings of genes that may reflect biological subtypes or other natural groupings. A variety of approaches can be used such as hierarchical clustering, K-means clustering, and principle components analysis. The data is commonly visualized using heat maps [46]. Another useful approach is gene set enrichment analysis to compare a given gene expression signature to a gene set indicative of a specific function, chromosomal location, or regulation [47]. It should be noted that of the thousands of genes that are differentially expressed in prostate cancer and benign tissues, we only understand the biological significance of a fraction of these genes. Thus, we are only beginning to understand the "big data" that has been unleashed over the last 15 years, and novel approaches almost certainly allow new insights into prostate cancer.

An important aspect of gene expression data is that much of it is publicly available. Most published data is deposited in publicly available websites. Of course, analysis of such data requires significant skill. Other sites, such as Oncomine [48] and cBioPortal [49], are usable by general cancer scientists and clinicians and are a very useful avenue for hypothesis testing and generation.

Microarray-Based Discoveries in Prostate Cancer

Prostate Cancer Biology and Pathogenesis

The discovery of recurrent fusion of the androgen-regulated TMPRSS2 gene to the ETS transcription factors, particularly the ERG gene, in the majority of prostate cancer lesions, has led to a paradigm shift in the study of prostate [44]. This discovery will be discussed in greater detail in the next chapter but was based on outlier analysis of gene expression data by the Chinnaiyan group [44]. The TMPRSS2/ERG (T/E) fusion gene occurs in approximately 50% of prostate cancers [50–58]. Experiments in prostate cancer cells containing the T/E fusion [44] indicate that the TMPRSS2 promoter, which contains androgen receptor (AR)-responsive promoter elements [59], increases ERG expression in response to androgens. The ubiquitous activity of AR in prostate cancer cells thus results in high expression of ERG fusion

transcripts. Immunohistochemical studies have shown that ERG overexpression is almost never seen in benign prostate epithelial cells. The high frequency of this genetic alteration argues that it plays a key role in the pathogenesis of those prostate cancers bearing the fusion gene. Indeed, it has been shown that downregulation of the T/E fusion gene by stable shRNA or liposomal siRNA targeting the fusion gene results in markedly reduced tumor growth in vivo [60, 61]. Thus, like the BCR-ABL gene in chronic myelogenous leukemia, it is an attractive therapeutic target as well as a potential diagnostic marker.

Another major finding growing out of gene expression microarray studies is the finding that serine protease inhibitor Kazal type 1 (SPINK1) is overexpressed in approximately 5–15% of prostate cancers [45, 62–64]. SPINK1 overexpression is essentially mutually exclusive with ERG overexpression and thus constitutes a distinct subtype of prostate cancer. Many but not all studies have found SPINK1 overexpression to be associated with adverse outcome [45, 62, 65]. SPINK1 has been shown potentiate EGFR signaling and as an extracellular protein is potentially therapeutically targetable [63].

Diagnostic Biomarkers

Surgical pathologists frequently face the problem of trying to determine if a small cluster of glands in a needle biopsy is malignant. A very early discovery using expression microarrays was that alpha methyl-acyl CoA racemase (AMACR) [9, 66, 67] is markedly increased in prostate cancer. This has led to the widespread use of immunohistochemistry to detect this marker as an adjunct to diagnosis of difficult lesions [64, 65]. Typically, this is combined with basal cell markers such as p63, CK5/6, and/or high molecular weight cytokeratin (34β[beta]E12), which are absent in cancer lesions. While these are caveats about this approach, using a combination of a positive marker such as AMACR along with negative basal cell-specific markers has proven to be a powerful diagnostic tool [68]. However, AMACR is not prostate cancer specific and can be expressed in benign prostatic glands and benign mimics of prostate cancer and high-grade PIN [68, 69]. Similarly, basal cells may be absent in some benign mimics of prostate cancer, particularly in a small clusters of glands. ERG is more specific to prostate cancer and thus is also useful as an immunohistochemical adjunct to diagnosis [70, 71]. Its utility is somewhat limited by the fact that it is present in only ~50% of prostate cancers in European-Americans [72] and ~20% of prostate cancers in African-Americans [73, 74]. See Chap. 27 for further discussion.

Detection Biomarkers

Given the limitations of prostate-specific antigen (PSA) testing, it is clear that better biomarkers for the early detection are needed of clinically significant prostate cancer. A number of protein-coding genes initially detected as elevated in prostate cancer by gene expression arrays have been included in potential multiplexed panels to detect

prostate cancer using urine including T/E and SPINK1 (see above) as well as GOLPH2 [75] and GOLM1 [76]. Recently increasing interest has focused on non-coding RNAs including lncRNAs and microRNAs. Novel blood-/serum-based tests are also under active development, although none are as yet in widespread clinical use. See Chap. 27 for additional discussion of this rapidly evolving area.

Prognostic Biomarkers

Given the highly heterogeneous clinical behavior of prostate cancer, it is critical to define those men requiring treatment and, hopefully, the optimal treatment needed. A number of genes identified by gene expression analysis have been proposed as potential biomarkers of disease aggressiveness such as EZH2 [23, 77] and SPINK1 [45]. One can also seek to define patterns of gene expression that are predictive of clinical outcome and response to therapy, etc. A number of such signatures have been proposed [10, 78–88] including signatures based on stromal markers [89]. One signature based on gene expression data is now commercially available.

(Oncotype Dx for prostate cancer). Whether such signatures will achieve widespread usage is not yet clear. In-depth discussion of such approaches is beyond the scope of this chapter but is discussed in detail in Chap. 29.

Prostate Cancer Classification

Expression array analysis has proven to be extremely powerful in defining biological and clinically linked subclasses of cancer in a number of malignancies, notably in lymphoma [90] and breast cancer [91]. Expression microarray analysis suggests a classification of localized prostate cancer that certainly has biological significance and perhaps clinical significance as well. As described above, approximately 50% of localized prostate cancers have ERG overexpression due to the presence of the T/E fusion gene. Another 10–15% of prostate cancers have overexpression of ERG via alternative fusions or overexpression of other ETS factors. SPINK1 is overexpressed by approximately 10% of prostate cancers. A final category is defined by mutation of the SPOP gene (see Chap. 12 for details). Of note, these categories are almost mutually exclusive and account for approximately 80% of all prostate cancers. See Chap. 30 for further discussion.

RNA-Seq and Future of Expression Microarrays

Gene expression arrays have had a tremendous impact on our understanding of the pathobiology of prostate cancer over the last 15 years. However, the use of gene expression microarrays is now being supplemented by the use of RNA-Seq, i.e., the

use of next-generation sequencing to sequence RNAs. Gene expression measured by this technology is highly concordant with measurements using one-channel microarrays [92]. RNA-Seq also has significant advantages over gene expression arrays. First, it can discover novel genes and is not limited by what is arrayed on the chip. Second, it has an almost limitless linear measurement range, while gene expression microarrays have limited sensitivity for low-expression genes and show signal saturation for highly expressed genes. Third, exon usage and alternative splicing can be more accurately evaluated. Finally, fusion genes, point mutations, and small deletions can be identified. While RNA-Seq is still more expensive than gene arrays, its cost is decreasing and is approaching the cost of gene arrays. This higher cost and more complex data analysis is still limiting penetration of RNA-Seq into prostate cancer analysis of gene expression, but it is clear that RNA-Seq is likely to substantially replace gene arrays in the future.

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Chapter 12
Next-Generation Sequencing

Anne Offermann and Sven Perner

Contents

Introduction

Until 2004, Sanger sequencing was the commonly applied DNA sequencing method and is now considered the "first-generation" technology. The main disadvantage of Sanger sequencing is the relatively low throughput, which means low amount of DNA sequence per sequencing reaction. Due to the requirement for electrophoretic separation of DNA fragments, the number of parallel sequencing reactions is limited. Thus, Sanger sequencing is an accurate, but very time-consuming and more cost-intensive method to sequence large numbers of genes and/or samples [1]. Therefore, in 2004 the goal of the National Human Genome Research Institute (NHGRI) was to develop a more cost-effective and time-efficient sequencing technology. As a result, time and cost per base pair sequenced dropped dramatically by achieving massive parallel sequencing of millions of DNA templates. These "second-generation" sequencing methods enable clonal amplification of single DNA templates, which are separated on a support matrix followed by cyclic

A. Offermann, M.D. • S. Perner, M.D., Ph.D. (✉)
Pathology of the University Hospital Schleswig-Holstein,
Campus Luebeck and Research Center Borstel,
Leibniz Center for Medicine and Biosciences,
Luebeck and Borstel, Germany
e-mail: sven.perner@uksh.de

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*,
Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_12

sequencing. Oligonucleotides bind to the terminal ends of the DNA fragment and are immobilized to a support matrix, and the template is amplified using polymerases. All platforms require preparation of a sequence library by preprocessing DNA for sequencing. Therefore, DNA is sheared into a platform-specific size as well as adapter ligation to the 3′ and 5′ ends of DNA fragments. Methodologies regarding preparation of sequence libraries, support matrices, sequencing chemistry, as well as signal detection differ depending on the sequencing platform offered by different companies [1, 2]. In the following chapter, we describe some of the "next-generation" sequencing techniques and the frequency and role of point mutations and indels in primary prostate cancer.

Next-Generation Approaches

Whole Genome Sequencing

Whole Genome Sequencing (WGS) enables the most comprehensive analysis of genomic DNA comprising protein coding regions as well as intergenic and regulatory regions such as promoters and enhancers. WGS provides complete information about genetic alterations including single-nucleotide variations (SNV), indels, amplifications or deletions, complex rearrangements, and copy number variations (CNV). While exome and targeted sequencing is limited to coding regions and known genes, respectively, WGS has the potential to detect rare and novel genetic alterations across all 3 billion base pairs in human genome. Additionally, recent studies identified breakpoints in balanced chromosomal rearrangements (named "chromoplexy") by using WGS, which would be invisible to other sequencing applications. Disadvantages are high cost per sequenced base pair as well as generation of an enormous amount of data with still unknown functional or clinical relevance [2].

Whole Exome Sequencing

Whole exome sequencing (WES) requires specific probes which have been designed complementary to all known protein coding DNA regions (exons) representing about 2% of the whole genome. To sequence selected regions of the genome, two technologies enable to prepare a DNA library following template sequencing. Capture of selected DNA fragments can be performed by using multiplex primer pairs combined with template DNA followed by PCR or by hybrid capture. Recent studies found that WES is less sensitive for detecting exome variants and shows less uniform sequencing quality compared to WGS. However, advantages of WES include cost-efficiency as well as a more selective generation of data about known and biological relevant genes [2].

Targeted Sequencing

Sequencing of selected protein coding genes requires designing a DNA library similar to WES described above. Predesigned or self-assembled gene panels can be used for sequencing studies. Focusing on a preselected gene set of interest reduces time efforts and costs. It is obvious that targeted sequencing can only be used for specific questions and not to identify novel gene regions harboring mutations [2].

Selected Sequencing Platforms

Illumina Technology

Illumina technology uses the sequencing by synthesis approach, and all enzymatic and sequencing steps take place in a flow cell. Templates for sequencing are first amplified into polymerase colonies (polonies) via bridge amplification to increase the signal-to-noise ratio. Oligonucleotides complementary to the template molecule are attached to the surface of the flow cell and hybridize to the sequencing template, thus producing cell-attached copies of templates. Isothermal amplification through cyclic denaturation, annealing, and extension steps mediated through different buffers generate clonal templates. During amplification cycles, fluorescently labeled dNTPs are incorporated into the growing DNA chain and are imaged and cleaved off for the next cycle. Illumina technology, especially the HiSeq2000 system combining HiSeq control system (HCS) and real-time analyzer (RTA), can be characterized by high-throughput but comparable short-read assembly [1].

Ion Torrent Technology

Ion Torrent technology also enables sequencing by synthesis and facilitates both short run times and read length of up to 400 base pairs. DNA templates are generated on a bead or sphere by emulsion PCR (emPCR). Oil-water emulsion forms small reaction vesicles containing one bead or sphere, one library molecule, and reagents necessary for amplification. One oligoprimer is present in emulsion bound to the templates, while the other primer is bound to the sphere. During sequencing steps, library molecules are amplified and can be detected while bound to the beads. The Ion Torrent chip contains a flow compartment and a pH sensor on micro-wells enabling detection of incorporated nucleotides through release of H+ during extension of each nucleotide during amplification of template DNA. Individual dNTPs are applied in a consecutive order, and change in pH indicates incorporation into the growing DNA chain allowing identification of each base. Ion Torrent system presents a sequence technology with high and rapid throughput, while the main point criticism is relative high error rate at 3.5% [1].

Pacific Bioscience Technology (PacBio)

The sequencing technology offered by PacBio is single-molecule detection; thus, there is no need for template amplification. Sequencing steps take place at the bottom of zero-mode waveguide (ZMW) wells containing the template molecule, primers, and DNA polymerase bound to the bottom. While technologies described above use linear library molecules, adaptors applied by PacBio are circular molecules with hairpin structure. Sequencing does not require cycles of extension and imaging like other technologies. Instead, single fluorescently labeled nucleotides in the growing DNA chain can be detected in real time by very sensitive optics. Furthermore, fluorescent labels are terminally phospholinked to the nucleotides and cleaved off during chain extension. Importantly, unlike other sequencing technologies showing increasing errors toward end of reads, PacBio possesses distributed errors independent of read length. Advantages are long read length and absence of GC bias realized by using phi29 DNA polymerase, while the main disadvantage presents relatively low output [1].

Molecular Signatures of Primary Prostate Cancer

Prostate cancer (PCa) is characterized by structural genetic alterations such as chromosomal rearrangements comprising copy number alterations as well as translocations eventually resulting in gene fusions [3]. In contrast, point mutations or small insertions or deletions (indels) occur less common in prostate cancer. Mutation frequencies of several genes in primary PCa are summarized in Table 12.1.

In general, gain-of-function and loss-of-function mutations of oncogenes and tumor suppressor genes, respectively, are defined as driver mutations, which are causally implicated in oncogenesis. The resulting growth and survival advantage of cancer cells harboring driver mutations contribute to selection and clonal expansion. These driver mutations must be distinguished from neutral (passenger) mutations in cancer, which do not contribute to cancer development or progression [4]. Common mutations in oncogenes and tumor suppressor genes in various cancers show relatively low mutation frequencies in PCa. Multiple pathways and gene regulation are altered during PCa initiation, metastasis, and development of resistance, but mainly through chromosomal aberrations. While mutations affecting the androgen receptor regarding activity and ligand specificity play a crucial role in the progression to castration resistance, mutated AR does not predominantly contribute to malignant properties in primary PCa cells.

Speckle-Type POZ Protein (SPOP)

The speckle-type POZ protein (SPOP) gene is located at chromosome 17, and despite the N-terminal MATH domain (amino acids 28–166) and the BTB domain (amino acids 190–297), SPOP contains a C-terminal nuclear localization sequence

Table 12.1 Summary of mutation frequencies in primary PCa

Gene	Mutation frequency in primary PCa	Literature
TNK2	17%	18
SPOP	6–14%	6–10
PTEN	5–10%	3, 6, 36, 56
p53	6–13%	6, 23, 26
CHD1	2–42%	3, 10
NCOR	2–23%	18
NCOA2	2–8%	6, 18
c-myc	4%	6
Rbbp	4%	6
PI3KCA	4%	3, 6, 48
FOXA1	3%	6, 11, 23
EP300	3%	18
MLL	1–4%	3, 6
Rb	<1%	12, 37
AKT	<1%	6
ILK	<1%	6
GSK3B	<1%	6
SHC1	<1%	6
HRAS	<1%	6
RAF	<1%	6
LSD1	<1%	6
NSD1	<1%	6

(amino acids 365–374) [5]. SPOP serves as an E3 ubiquitin-protein ligase adaptor to recruit substrates for Cu13-based ubiquitination. The enzyme Cu13 belongs to the Cullin-RING ligases, a subclass of ubiquitin-protein ligases that are responsible for covalent conjugation of ubiquitin to specific substrates. SPOP is one of more than hundreds of BTB (Bric-a-brac–Tramtrack–Broad complex) domain-containing proteins that bind Cu13. Additionally, the MATH (meprin and TRAF homology) domain of SPOP recruits diverse substrates by protein interaction [5]. Thus, the MATH-BTB protein SPOP selects targets for ubiquitylation by recruiting them to Cu13, which are subsequently degraded. Genetic alterations of SPOP influence efficiency or specificity of substrate binding resulting in differential degradation of proteins with oncogenic or tumor-suppressive roles.

SPOP mutations represent the most common point mutation in PCa showing recurrent mutations in 6–14% and are thought to be an early event in PCa development [6–8]. With the objective to characterize systematic patterns of mutations across different cancer entities, sequencing of 441 tumors including PCa revealed for the first time mutations in the SPOP gene in prostate tumors [9]. One year later, SPOP mutations were observed in 2/7 high-risk primary prostate tumors using paired-end sequencing [10]. The first comprehensive analysis of SPOP mutations in a large number of PCa tumor samples was performed by Barbieri et al. in 2012 [6]. In this study, exome sequencing identified recurrent somatic SPOP mutations in 13% of 112 treatment-naïve radical prostatectomy samples. Among other novel mutated genes, SPOP emerged as the most frequently mutated gene. Sequencing of additional, independent

cohorts comprising 300 primary tumors confirmed recurrent heterozygous SPOP mutations in 6–13% of cases. In contrast, benign prostate tissues and prostate stroma showed no mutations in the SPOP gene. The observation that SPOP mutations occur in intraepithelial neoplasia (HG-PIN) adjacent to invasive carcinoma gives evidence that SPOP mutations represent an early event in prostate tumorigenesis. However, mutational analyses from tissues at an advanced or castration-resistant stage show discordant results. While SPOP mutations in advanced tumors from patients with metastatic PCa have been observed in 14.5% of cases [6], sequencing of castration-resistant tissues revealed only low SPOP mutation rates [11, 12].

All mutations initially found in PCa were missense mutations affecting the substrate-binding domain of SPOP at the amino acid positions Y87, F102, S119, F125, K129, W131, F133, and K134 [6]. The following sequencing studies confirmed mutation rates of SPOP ranking from 4% to 18% and discovered additional mutations (F104 and K135) in the MATH domain [7, 8] (Fig. 12.1).

Sequencing of prostate tumors from international cohorts including Caucasian, African American, and Asian patients revealed no significant association between the SPOP mutations with ethnicity, clinical, or pathologic parameters [7].

SPOP-mutated prostate cancer is a distinct molecular subtype. On one hand, SPOP mutations have been found to be mutually exclusive or inversely correlated to ERG rearrangement status (see Chap. 10), suggesting that ETS fusions and SPOP mutations possess divergent driver events in PCa initiation [6, 7]. Furthermore, SPOP mutations in primary tumors are inversely associated with TP53 lesions as well as mutations and/or deletions of PTEN and PIK3Ca [6]. On the other hand, recurrent deletions at 5q21 and 6q21 correlate significantly with the presence of SPOP mutations [6, 7]. Both loci encode putative tumor suppressor genes as CHD1, FOXA1, and PRDM1 and have been partly described to be disrupted in PCa. This mutual relationship between the loss of tumor suppressor genes and SPOP mutations may collaborate to drive tumorigenesis.

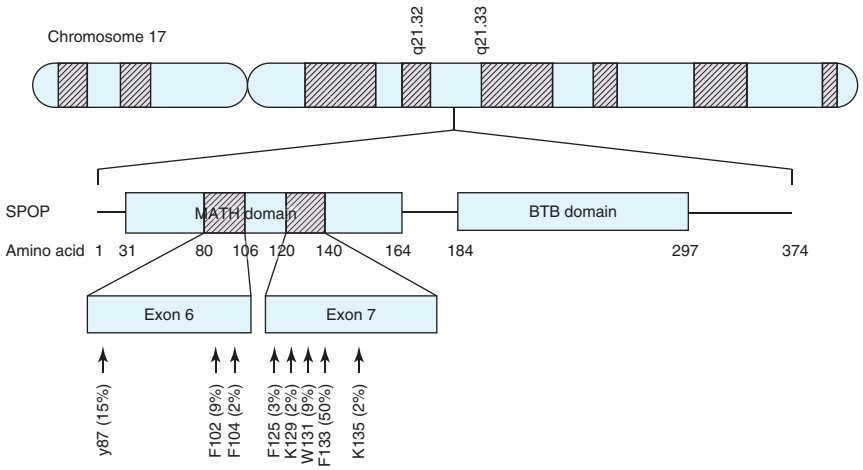

Fig. 12.1 Localization and frequency of SPOP mutations in primary prostate cancer

In PCa, all mutations in SPOP affect specific amino acid residues within the substrate-binding domain [6]. These mutations may lead to impaired or increased ubiquitylation rates of SPOP substrates or to SPOP-mediated recruitment of new substrates by modifying its specificity. Structural analysis of SPOP uncovered several amino acid residues within the MATH domain to be essential for substrate binding [5]. In vitro studies showed that SPOP mutations affecting these residues disrupt substrate binding. SPOP mutations found in PCa affect residues including Y87, W131, and F133, which are PCa specific and lead to loss of function of SPOP. These mutations are typically somatic heterozygous missense mutations with a retained wild-type allele. SPOP mutants are able to dimerize through the BTB or BACK domain with the wild-type SPOP leading to repression of wild-type SPOP activity (dominant-negative effect).

The SPOP-CUL3 complex is responsible for the regulation of diverse substrates that impact different pro-malignant pathways. Evidence for a tumor-suppressive role of SPOP in PCa cells has been shown by increased invasion after inducing SPOP F133V mutation or SPOP knockdown [13]. All commonly used PCa cell lines including androgen-sensitive LNCaP and VCaP as well as metastatic castration-resistant PC3 and DU145 cells express wild-type SPOP [3]. Analysis of the differences in the ubiquitin landscape between PCa cells expressing no SPOP, wild-type SPOP, or mutant SPOP (SPOP-F133L or SPOP-Y87N; SPOP-MT) revealed 12 proteins as potential substrates affected by SPOP mutation [13]. While most substrates were upregulated by SPOP mutations by dominant-negative effect, the abrogated degradation of only two substrates was caused by loss-of-function SPOP mutations. Two more proteins with oncogenic potential, the AR coactivator TRIM24 as well as the putative oncogene DEK, have been shown to be consistently upregulated by SPOP mutation. Consistently, reduced DEK degradation conferred by SPOP mutation increased invasive potential in PCa cells [13]. Recent published data support that wild-type SPOP has a tumor-suppressive character by promoting the degradation of the androgen receptor coactivator "steroid receptor coactivator 3" (SRC3) [14]. In contrast, cells expressing SPOP with PCa-associated mutations are not able to bind SRC3 and thus to further mediate its ubiquitination. While unchanged SRC3 levels in SPOP F133V mutant cells suggest a loss-of-function effect, increased SRC3 levels above baseline in F102C, F125V, and W131G mutant cells may be explained by a possible gain-of-function "dominant-negative effect" [14]. Further in vitro studies are needed to investigate potential gain-of-function mutations or altered SPOP substrate-binding specificity.

Myc

The gene encoding myc is located on chromosome 8 in the human genome. The myc family includes c-myc, N-myc, and L-myc, which share structural and functional characteristics but also possess distinct expression and activity patterns. L-myc is frequently overexpressed in small cell lung carcinoma, and N-myc plays important roles in solid tumors of neural origin. However, most studies have focused

on identifying the impact of c-myc in cell transformation, differentiation, and proliferation in blood-borne and solid tumors. Myc is involved in a large number of pro-oncogenic signaling pathways as well as maintenance of stem cell properties of cells. Thus, deregulated myc expression against the background of other genetic alterations drives tumorigenesis, induces genome instability, and contributes to tumor maintenance and metastasis.

Following transcription, translation of myc mRNA starts at an internal AUG codon generating a polypeptide with an N-terminal transcriptional regulatory domain, a nuclear localization signal domain, and a C-terminal DNA-binding region. Additionally, translation of myc, which initiates at a CUG codon upstream from the start codon, produces a longer myc protein with distinct functions. Following activation, myc interacts with several transcription factors such as TRAP and TBP by its N-terminal domain, dimerizes with Max protein to bind DNA, and regulates a large number of genes. The myc E box region on DNA is regulated by many transcription factors and activates a gene signature promoting cell proliferation and growth [15].

In normal cells, the proto-oncogene myc is tightly regulated by many processes that constantly inhibit myc activity. In contrast, in malignant cells, myc receives oncogenic potential mainly through mechanisms that lead to myc overexpression and its disconnection from regulatory signalings. Myc overexpression arises through constitutive activation of pathways such as Wnt and PI3K signaling or through amplification and chromosomal translocation affecting the gene encoding myc. Importantly, amplification of the wild-type myc protein is sufficient to promote tumorigenesis, while alterations of the coding sequence by mutations occur frequently in Burkitt lymphoma in nearly 50%, but rarely in other cancers. The most common point mutation in myc affects its phosphorylation sites, which are recognized for ubiquitin-mediated proteolysis. However, the impact of cancer-related myc mutations remains unclear and requires further investigation [16].

Amplification of chromosome 8q including the myc gene occurs in 30–70% of prostate tumors, thus presenting one of the most common genetic alterations in PCa [17]. Previous sequencing studies reported myc amplification at low frequency in castrate-resistant prostate cancer (CRPC), while no localized PCa tissue harbored myc gene alterations [12]. Furthermore, N-myc amplification and overexpression is associated with CRPC with small cell carcinoma-like clinical features. This highly aggressive clinical variant is referred to as neuroendocrine phenotype of PCa (NEPC), “anaplastic” PCa, or small cell carcinoma [18, 19]. In contrast, mutations affecting myc activity in PCa are rare events, identified by Barbieri and colleagues in only 4 out of 112 primary tumors [6]. Interestingly, three mutations affected the regulator myc binding protein (mycbp), which binds to the N-terminal domain of c-myc and thereby enhances c-myc-mediated gene expression. Additionally, a single mutation was found in the gene encoding N-myc. Most recent data from preclinical models demonstrate that N-myc overexpression drives an aggressive variant of PCa that molecularly mimics the so-called NEPC [20]. The potential clinical implication is that N-myc overexpression also sensitizes cells to Aurora kinase A inhibitors and EZH2 SET domain inhibitors.

The PCa cell lines LNCaP and PC3 harbor amplified c-myc, and c-myc has been shown to be upregulated during androgen deprivation. Transgenic mice overexpressing c-myc develop PIN lesions followed by progression to invasive adenocarcinoma. However, studies investigating effects of myc mutations are rare and need further investigation.

p53

The gene encoding p53 is located on chromosome 17p13 and was discovered to serve as tumor suppressor 30 years ago [21]. Amino acids 1–62 constitute the N-terminus containing the transactivation domain of p53, which allows interactions with regulatory proteins and components of the transcription machinery. Residues 63–93 contain SH3-domain-binding motifs with regulatory function followed by the central core domain, which binds specifically to double-stranded DNA. P53 recognizes DNA response elements of genes involved in cell cycle arrest, apoptosis, as well as other cell features. The C-terminal region includes the tetramerization and negative autoregulatory domain, both with regulatory functions [21].

Inactivation of p53 belongs to the most common cancer-related genetic alterations showing mutation rates of more than 50% across different cancer types. p53 serves as transcriptional activator of genes involved in cell cycle regulation, apoptosis, and senescence, thus allowing adaptive processes in response to cellular stress, changing cellular environment, as well as DNA damage and oncogenic signaling. The tumor-suppressive impact of p53 is mainly given by its ability to eliminate irreparably damaged and malignant cells. The majority of p53 mutations found in cancer are missense mutations of the central DNA-binding domain and thus are unable to transactivate genes upregulated by the WT protein. Interestingly, the proportion of missense mutations (75%) is much higher than in other tumor suppressor genes, suggesting that expression of the p53 mutants may confer some selective advantage to the cells expressing the mutant protein over cells null in p53 or expressing the WT form [22, 23].

Furthermore, the thermodynamic stability and thus the proportion of folded/unfolded protein are regulated by the core domain of p53. Therefore, mutations affecting this ability reduce functional p53 despite accumulation of p53 protein in malignant cells.

Loss of p53 by chromosomal deletion has been observed in 25–40% of PCa, while recurrent missense mutations in the p53 gene occur in about 6–13% of primary prostate tumors [6, 24]. In 2006, a study reported that most mutations are located in exons 5–8 presenting the most commonly mutated region in human cancer, but also in exons 4 and 10 [22]. TP53 lesions were generally absent in tumors harboring SPOP mutation but correlated significantly with ERG rearrangement [6]. Several studies supported the frequency of p53 alterations but could not confirm somatic mutation frequencies above 1% of tumors [17]. The frequency of p53 lesions increases during PCa progression as shown by several studies. Exome

sequencing revealed that CRPC harbors 18% and 42% of deletions or mutations affecting p53, respectively [11, 25]. Comparison of mutation frequencies between low-grade and high-grade or metastatic PCa foci indicated that p53 gene alterations occurred exclusively in advanced tumors [26]. Recently, Kluth et al. performed a comprehensive analysis of p53 gene and expression status and its clinical relevance [27]. Sequencing of 96 PCa samples revealed potential dominant negative mutations in 18 cases, which was significantly associated with strong p53 immunostaining. Importantly, high p53 expression resulting from dominant negative or oncogenic mutation correlated strongly with the risk of PSA recurrence [27].

Single mutations have been identified in regulatory components of p53 activity. The polycomb protein member RYBP directly interacts with and thereby decreases MDM2-mediated p53 ubiquitination. Therefore, RYBP exhibits tumor-suppressive function by increasing p53 stability and accords with reduced RYBP expression in cancer tissues [28]. Barbieri et al. found RYBP to be mutated in a single tumor out of 112 primary PCa [6]. However, the frequency and importance of RYBP mutations have not been further investigated and require broader analysis for any conclusions.

While androgen-sensitive LNCaP cells express wild-type p53, the metastatic cell line DU145 harbors two different mutations on both alleles and in the PC3 cell line, each allele exhibits a mutation or a deletion in p53 [29]. Several in vitro studies show that the p53 activity status is critically involved in determining chemosensitivity, cell response to radiation, as well as castration-resistant growth [30–32]. Transgenic induction of mutant p53 resulted in the development of prostatic intraepithelial neoplasia, while simultaneous inactivation of p53 and retinoblastoma (Rb) leads to rapid formation of aggressive, metastatic PCa [33] (Fig. 12.2).

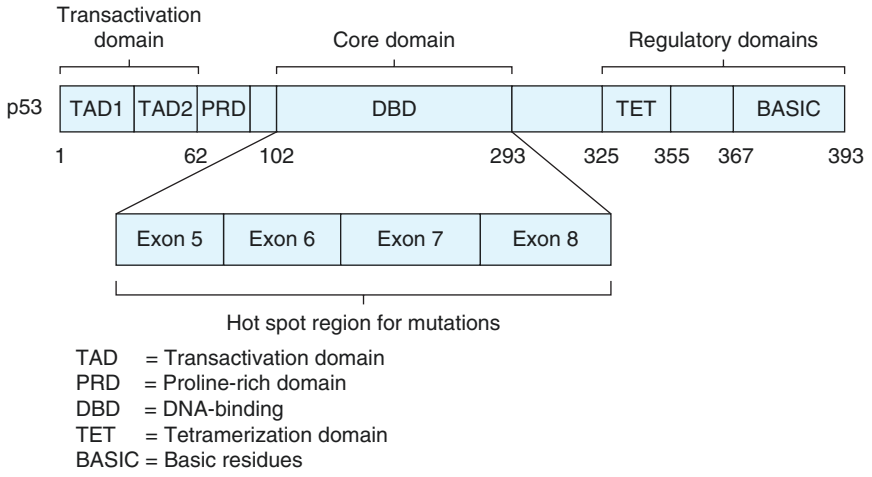

Fig. 12.2 Localization of p53 mutations on primary PCa

Forkhead Box A1 (FOXA1)

The gene encoding FOXA1 is located on chromosome 14q21.1, and translation of the coding region including 5300 base pairs leads to expression of a 473-amino-acid-long protein. All members of the Fox gene family harbor a highly conserved forkhead DNA-binding domain that consists of three α[alpha]-helices, three β[beta]-sheets, and two "wing" regions flanking the third β[beta]-sheet. DNA transactivation domains are located on the N- and T-terminus, while the DNA-binding winged helix spans amino acids 168 to 268 in the middle of the gene. Furthermore, there are 11 putative acetylation and phosphorylation sites as well as interaction regions for USF2, SMAD3, NKX2.1, and the androgen receptor (AR). FOXA1 belongs to the forkhead box (Fox) gene family defining evolutionary conserved transcription factors which can based on phylogenetic analysis be subdivided into 19 subclasses ranking from FoxA to FoxS. Fox genes are involved in multiple molecular cell processes such as proliferation, cell cycle, differentiation, and embryogenesis. Different Fox proteins exhibit distinct functions mainly through defined interaction with modifying enzymes and coregulators as well as diverse tissue and differentiation-dependent expression patterns [34].

FOXA1 is an essential interaction partner of hormone receptors driving estrogen and androgen receptor-mediated gene expression. While several studies revealed tumor suppressor functions of FOXA1 in different cancer types, its implication in PCa remains controversial. Some studies showed that high expression of FOXA1 in PCa tissues correlates with poor survival of patients, whereas other studies found reduced FOXA1 levels in association with castration resistance and poor prognosis. Also in vitro studies showed controversial results regarding the effect of FOXA1 upon PCa cells [34, 35].

Alterations of Fox genes have been described in several human diseases, including breast and prostate cancer. In primary treatment-naïve prostate cancer, FOXA1 has first been identified to be mutated in 4 of 111 cases using WES as well as on 63 transcripts of tumor RNA using RNA sequencing [6]. Eight different non-silent missense or frameshift mutations found were located within the forkhead domain near to the DNA-binding domain. Thus, it has been suggested that these mutations might influence the binding FOXA1 to DNA. Another study identified novel somatic insertion of two base pairs in two localized PCa samples and two PCa cell lines [11]. Including increasing sample size, Grasso et al. found FOXA1 to be mutated in 4 out of 101 localized PCa as well in 1 out of 46 CRPC samples (3.4% in total). Most mutations and both indels were located in the C-terminal transactivation domain, and all mutations found were missense or frameshift mutations. Collectively, there are two mutational hotspots in the FOXA1 forkhead gene domain recurrently identified in five prostate and four breast tumors [6, 11, 34].

In vitro experiments demonstrated that overexpression of wild-type or mutated FOXA1 increased proliferation and xenograft growth [11]. While AR signaling is not fully impaired in the absence of FOXA1, mutations in FOXA1 have been suggested to influence AR binding specificity and the transcriptional program. Recent studies revealed that wild-type FOXA1 inhibits PCa cell motility,

epithelial-to-mesenchymal transition (EMT), and metastases by AR-independent signaling. These results give evidence that mutations might abolish the ability of FOXA1 to act as repressor of tumor growth and metastases [11, 35].

Retinoblastoma Protein (Rb)

The gene encoding retinoblastoma protein (Rb) is located on chromosome 13q14 and was the first tumor suppressor gene identified in human cancers. Rb is inactivated by chromosomal deletion or inactivating mutation in multiple cancer types, thereby contributing to deregulated cell cycle, apoptosis, and reduced genome stability [36].

Loss of chromosome 13q12.3 presents one of the most common copy number alterations in PCa and affects a broad range of known genes. In primary tumors, Rb loss mostly results from gene deletion at frequencies between 11% and 40% [37]. During disease progression and development of castration resistance, the frequency of Rb gene alterations significantly increases and is associated with reduced recurrence-free survival of patients. Several studies identified single-point mutations and indels in the Rb gene occurring at low frequency in primary tumors [38]. Although exome sequencing of 112 primary PCa revealed no mutations in the Rb gene itself, 4 somatic missense and a single mutation affecting splicing of Rb binding proteins (Rbbp) have been reported [6]. Interestingly, simultaneously an independent study found that tumor-suppressive function of Rb in PCa requires interaction between Rb and E2f with Rbbp properties. However, this relationship has not yet been further investigated and needs functional evidence for drawing conclusions [39].

Targeted sequencing of commonly affected oncogenes and tumor suppressor genes supported previous findings by showing Rb gene truncation in a single localized PCa sample compared to two truncations, one point mutation, as well as four gene deletions out of 25 CRPC samples examined [12].

While androgen-sensitive LNCaP cells express wild-type p53, metastatic DU145 harbor mutant p53 and PC3 lack p53 [40].

Interestingly, a recent study reported a significant frequency of Rb loss in 90% of prostatic small cell neuroendocrine carcinomas compared to high-grade and metastatic adenocarcinomas. However, Rb loss detected by FISH and IHC resulted from allelic loss, while no mutations occurred in any prostatic tumors examined in this study [41].

Mutations Affecting Distinct Components of Signaling Complexes

Mutations in Genes Involved in Chromatin Regulation

Among transcriptional regulation, gene activation or repression is epigenetically modulated by packaging of coding DNA, underlying the control of chromatin remodeling proteins. Alterations of the chromatin structure lead to deregulated gene activation and inefficient gene silencing physiologically maintaining cell homeostasis.

Therefore, molecular changes affecting the activity of chromatin remodeling complexes including oncogenic pathway activation as well as genetic alterations in genes encoding chromatin remodeling proteins have been broadly investigated. While copy number variations cause altered expression of chromatin remodelers, mutations have been identified to influence its activity and distinct properties enabling chromatin modification [42].

Until now, four families of chromatin remodelers have been identified, which share general features but also exhibit unique properties to influence selectively the epigenetic profile of cells. Chromatin remodeling families comprise the SWI/SNF, ISWI, IN080, and CHD/NuRD/Mi-2 proteins. Among the common features of these proteins are the interaction with the nucleosome core and binding to nucleosomal histone tail residues, as well as the presence of regulatory and interaction domains. Each family is characterized by distinct motifs. The CHD/NuRD/Mi-2 protein family contains a chromodomain (CHD) with core-ATPase, DNA-binding, and helicase activity as well as chromatin interaction domains and acts as ATP-dependent chromatin remodeler [42].

Chromodomain-helicase-DNA-binding (CHD) protein 1 located on chromosome 5q21 presents an important tumor suppressor in PCa and ranks as the second most homozygous deleted gene in primary and metastatic tumors. It is associated with additional copy number losses predominantly in 2q, 5q, and 6q but negatively correlated to the TMPRSS2-ERG fusion, suggesting CHD1-deficient tumors as novel molecular subclass of aggressive PCa [35, 41]. Thus, CHD1 might play a role in preserving genomic stability or is involved in the accumulation of genetic alterations driving PCa [43]. Additionally, previous studies implicated CHD1 in the regulation of embryonic stem cell pluripotency and gene regulation [10]. Next-generation sequencing revealed somatic mutations in the gene encoding CHD1 with varying frequencies. Out of seven high-risk primary PCa, the CHD1 gene exhibited somatic mutation and intragenetic breaks in three cases resulting in a truncated protein [10]. Barbieri et al. identified a single missense mutation in CHD1, but 14 mutation affected genes encoding several CHD isoforms [3]. Lower mutation frequencies about 2% have been reported by other studies supporting the current assumption that loss of CHD1 through gene deletion is the predominant mechanism promoting PCa. Further functional studies are required to investigate whether potential damaging mutations in CHD1 result in loss of its tumor-suppressive function in PCa.

Other genes involved in histone modification by methylation and identified to be mutated in PCa comprise KDM6A/UTX, MLL2, and MLL3 [3]. These regulators are able to modify methylation of the histone variant H3 known to be critically involved in gene regulation. Missense and nonsense mutations in MLL2 exhibiting H3K4-specific histone methyltransferase activity were found in 4 out of 112 primary tumors using exome sequencing [6]. Further studies supported these findings by showing recurrent mutations of MLL2 in 8.6% of prostatic tumors predominantly occurring in CRPC and rarely in primary PCa. Additional genes involved in chromatin and histone modification and partly member of the MLL family were deleted or mutated at higher frequency in CRPC but absent in primary localized PCa [11].

Epigenetic modulators of the AR signaling axis have been shown to be rarely mutated in primary PCa. Lysine-specific demethylase 1 (LSD1, KDM1A) functions epigenetically as AR coactivator through histone methylation modification [44] and was mutated in a single primary tumor out of 112 exomes [6]. Further, a single missense mutation has been identified in the nuclear receptor-interacting domain 1 (NSD1) that directly binds the AR with activation or repressor function [6, 45].

PI3K-AKT-mTOR Signaling

The PI3K signaling is one of the most common altered pathways in cancer whose activation results in enhanced tumor growth and progression. Copy number alterations and mutations in genes involved in the PI3K-AKT-mTOR signaling network lead to increased proliferation, survival, and invasion of cancer cells. The major mechanism for PI3K pathway activation is the inactivation of the tumor suppressor activity of PTEN resulting in enhanced production of PIP3, a second messenger involved in multiple signaling cascades with oncogenic potential [46].

While numerous studies showed genetic alterations affecting the PI3K axis in approximately 25–70% of primary PCa, mutations contribute rarely to the activation of PI3K signaling [3]. An integrative analysis considering copy number alterations (CNA), the transcriptome as well as mutations of prostate tumors, PCa cell lines, and xenografts identified frequent alterations in common core pathway. Almost half of primary prostate cancers and all metastases examined in this study showed dysregulation of the PI3K signaling. This study confirmed previous observations by showing that downregulation of the PI3K regulators PTEN, INPP4B, PIK3R1/3, and PHLPP phosphatases and upregulation of the PI3CA itself are the main molecular alterations in PCa and that mutations in these components occur infrequently [17].

Phosphatidylinositol 3-Kinase (PI3K)

The PIK3CA gene is located on chromosome 3q26.3 and consists of 20 exons and encodes the kinase domain p110α[alpha]. Multiple isoforms of PI3Ks can be subdivided into three different classes which all function through phosphorylation of membrane inositol lipids resulting in the generation of phosphatidylinositol 3-phosphates. Subsequent recruitment of effector proteins results in different cascades with distinct roles of each PI3K [47]. Until now, there is no evidence that mutations in class II and III PI3Ks contribute to the pathophysiology of human diseases. In contrast, many growth factor pathways underlie PI3K class I activity mediating cell growth, proliferation, survival, and metabolism. Genetic alterations of the PI3K class I or components involved in PI3K I signaling have been described in numerous diseases, including cancer [46, 47].

Class I PI3K is composed of heterodimers consisting of a catalytic kinase domain p110 (p110α[alpha], β[beta], δ[delta], or γ[gamma]) and a regulatory

domain (p85α[alpha], p85β[beta], p55γ[gamma], p101, or p84). The regulatory subunit p85 recruits the PI3K to phosphorylated tyrosine in response to the activation of receptor tyrosine kinases (RTKs) and serves additionally as negative regulator of the p110α[alpha] catalytic domain. Furthermore, the p110γ[gamma]-p101/p84 complex is activated by G protein-coupled receptors (GPCRs). The Ras-binding domain (RBD) in the N-terminal region of PI3K I is responsible for the activation of Ras, Rab5, or Rho GTPase family members. Synthesis of the second messenger phosphatidylinositol [3–5]-trisphosphate (PIP3) by the PI3 kinase domain recruits pleckstrin homology (PH) domain-containing proteins such as AKT/PKB kinases, which are subsequently activated by the constitutively active phosphoinositide-dependent kinase 1 (PDK1) and by PDK2 [mammalian target of rapamycin complex 2 (mTORC2). Finally, activated AKT/PKB transduces the signal to downstream targets including Akt, S6K, 4EBP, and GSK3β[beta] [47].

Gene amplification, deletion, and somatic mutations in the PIK3CA gene lead to enhanced catalytic activity gene and have been observed in various cancer types [46, 48]. Mutations affecting the PIK3CA gene are mostly gain-of-function single-nucleotide substitutions in the helical or kinase domain resulting in constitutive activation of downstream targets. Highest mutation rates of around 30% have been found in liver, breast, and colorectal cancers [48]. Indeed, in PCa, gene amplification is the most common genetic alteration found in the PI3K gene, which emerges in 13–40% of cases [37, 49, 50] and is associated with castration resistance [48]. PIK3CA mutations occur in around 4% of primary PCa with higher frequencies in advanced tumors [3, 6, 49]. Using exome sequencing, the downstream target AKT and the regulator PIK3R1 were identified to be mutated in a single tumor, and recurrent missense mutations in the PI3CA occurred in four samples, mainly pathologic stage pT3 tumors [6]. Interestingly, in 33% of prostate tumors harboring PI3K alterations, genetic alterations in the PIK3CA and PTEN genes were mutually exclusive except one tumor [49]. However, other studies observed genetic alterations in the PI3K pathway but did not confirm mutations in the PI3CA gene described before [10, 51].

PTEN

The phosphatase, tensin homologue (PTEN) gene is located on chromosome 10 q23.31 and emerges as the most frequently inactivated tumor suppressor gene in cancer. The discovery of heterogenic allelic losses on the long arm of chromosome 10 in prostate tissues provided first evidence that this region might be involved in prostate tumorigenesis [52]. Together with the observation that wild-type chromosome 10 suppressed oncogenic potential of glioblastoma cells in mice led to the hypothesis that 10q23 encodes a tumor suppressor gene [53]. PTEN has dual phosphatase activity, uses 3′-phosphoinositides as substrates for dephosphorylation, and thereby suppresses PI3 kinase activity. Most mutations found in cancer are indels resulting in frameshift mutations and are located in or near the phosphatase domain

of PTEN [54]. Thus, mutant PTEN proteins lack only the lipid phosphatase function (e.g., PTEN-G129E) or lack both the lipid and protein phosphatase functions (e.g., PTEN-C124S and PTEN-G129R) [55].

In PCa, deletions of the PTEN gene occur in nearly half of primary tumors, while loss-of-function mutations are detected in approximately 5–10% of cases [3, 37]. Non-synonymous base pair substitutions including K128N, R130Q, Y336, G129R, R173H, and R233 have been previously described in other cancers and are enriched in locally advanced tumors. Furthermore, PTEN mutations significantly correlated with TMPRRS2/ERG fusion [6], and functional studies in mice show that ERG promotes prostate tumorigenesis in cooperation with PTEN haploinsufficiency [56]. Further investigation revealed somatic indels in the PTEN gene at low frequency [10]. However, other studies observed high frequencies of PTEN copy number loss, but could not confirm the mutation frequencies found by other studies, eventually caused by using alternative sequencing methods [12, 17, 25]. In early-onset PCa, diverse genetic alterations including deletion, translocation, and upregulation of PTEN-targeting miRNAs result in PTEN inactivation, but no somatic mutation has been observed [57]. However, higher PTEN alterations are observed in advanced and metastatic PCa. Analysis of CRPC revealed PTEN alterations in more than half of the tumors where around 8% of cases harbored a PTEN mutation [11]. Even higher mutation rates have been observed by sequencing multiple metastases from same patients with interfocal heterogeneity among different metastatic sites [58]. Rearrangements of MAGI2, identified by whole genome sequencing of PCa, may also result in a PTEN mutated phenocopy [10] (see below).

Two out of four in vitro cell lines commonly used in PCa research harbor PTEN alterations, and large numbers of in vivo xenografts derived from PCa patients exhibited deletions or mutations [59]. While PTEN is homozygously deleted in the metastatic cell line PC3, androgen-sensitive LNCaP cells show a frameshift mutation in PTEN. In vitro studies presented the functional consequences of PTEN loss in PCa cells by promoting malignant potential, stem cell properties, and drug resistance as well as its implication in various pathways [3, 60, 61]. PTEN wild-type expression in PTEN mutant LNCaP cells induced chemosensitivity, while disruption of the lipid phosphatase activity of PTEN by mutations abolished this effect [60].

While homozygous ablation of PTEN in mice results in embryonic lethality, heterozygous mutation in PTEN caused PIN formation within 8–10 months. Only in cooperation with additional genetic events such as simultaneous loss of other tumor suppressor genes (TSG), PTEN+/− mice developed invasive adenocarcinoma [62].

Other Components of the PI3K Signaling Axis

Mutations affecting genes within the PI3K signaling axis mostly result in inactivation of pathway repressors or activation of coactivators important for signal transduction and occurred at low frequencies. This includes mutations in mTOR, which is essential as hub between AKT and downstream targets, as well as mutations in integrin-like kinase (ILK) and GSK3B serving as regulatory kinase downstream

from PI3K [6, 17]. Moreover, mutations affecting scaffold protein SHC1, which is directly involved in signal transduction from activated receptors to PI3K pathway, are suggested to influence PI3K activity [6]. The epidermal growth factor receptor (EGFR) is an upstream activator of the PI3K and described to be altered in various malignancies including PCa. Direct sequencing of the EGFR tyrosine kinase domain in 100 radical prostatectomy tissues revealed mutations in the EGFR gene in 13% of samples [63].

Deletions in the gene encoding the PI3K-negative regulator ´PH domain and leucine-rich repeat protein´ (PHLPP1) or rearrangement of MAGI2, encoding a PTEN scaffolding protein [10], contribute to PI3K dysregulation. However, until now no mutations affecting these additional coregulators have been identified (Fig. 12.3).

Ras/RAF/MAPK Pathway

The mitogen-activated protein kinase (MAPK) signaling cascade includes the activation of the small G protein Ras by activation of receptor tyrosine kinases (RTK) or non-RTKs, which subsequently activates RAF. The MAPK is phosphorylated by dimerized RAF leading to activation of the extracellular signal-regulated kinase (ERK) that translocates into the nucleus where it transduces the signal to diverse transcription factors resulting in gene expression involved in cell proliferation, survival, and other biological features.

Three different RAF isoforms (ARAF, BRAF, and CRAF) are encoded by RAF proto-oncogenes on chromosomes Xp11, 7q32, and 3p25, respectively. Besides small G proteins, other activators of RAF such as non-RTK Src and Janus kinase, interferon beta, protein kinase C, or the antiapoptotic protein Bcl-2 have been iden-

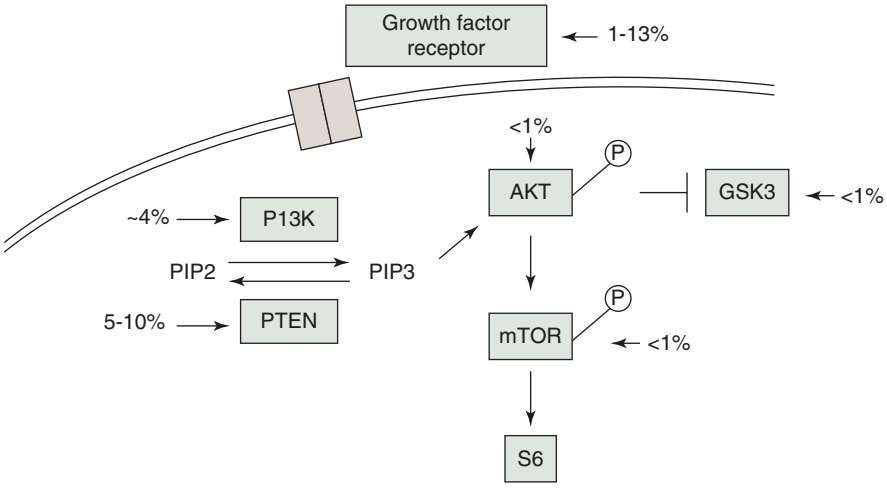

Fig. 12.3 Mutations in genes involved in the PI3K pathway of primary PCa

tified [64]. Activating mutations affecting Ras and RAF present common oncogenic events in multiple cancer types. While Ras is mutated in about 15% of cancers, BRAF mutations occur at even higher frequency in melanoma, thyroid, ovarian, colorectal, and liver cancer. All BRAF mutations found affect the kinase domain, and 80% present a base pair substitution (V600E) resulting in enhanced kinase activity [65].

Activation of the MAPK pathway in PCa results from upregulation of its components and has been associated with development of castration resistance [66]. Moreover, BRAF gene fusions to androgen-responsive genes were found in 1–2% of advanced [67] and a subset of metastatic PCa and in sensitized PCa cells to RAF and MEK inhibitors [68].

However, several sequencing studies could not observe Ras or RAF mutations in primary and metastatic PCa tissues and cell lines. Others reported rare mutation frequencies in KRAS and BRAF in primary and metastatic PCa, respectively [17]. The Ras family member HRAS and RAF were identified to be mutated in a single sample out of 112 primary tumors [6] confirming low mutation frequencies for MAPK signaling members described before [69].

Conflicting results have been shown by Cho and colleagues who identified BRAF mutations in 10.2% and KRAS mutations in 7.2% of 206 radical prostatectomy specimens [70].

Androgen Receptor Signaling Axis

The androgen receptor (AR) gene is located on chromosome Xq11-Xq12, and the protein coding region, comprising 2757 nucleotides, encodes a 110 kDa protein. It belongs to the ligand-dependent nuclear receptor transcription factor family, which exhibits a highly conserved DNA-binding domain flanked by the N-terminal as well as C-terminal ligand-binding region. Upon ligand binding, heat-shock proteins are displaced from the AR, and the receptor is translocated into the nucleus binding to androgen response elements (AREs) in promoters of target genes. Thus, the AR regulates transcription of genes involved in cell proliferation, apoptosis, and other features enabling balanced prostate growth and function. In PCa, increased AR activity through diverse alterations such as amplification, mutation, and altered splicing contributes to malignant features of prostate cells [71].

A broad range of different AR gene alterations have been described to occur during progression to castration resistance and to present the main resistant mechanism against androgen deprivation therapy. Next-generation sequencing studies identified high rates of AR gene alterations, which were completely absent or present in only single cases in localized, primary PCa tissues [6, 11, 12, 17]. Thus, based on large studies investigating the AR status in primary versus advanced PCa, it is currently assumed that AR gene alterations are not implicated in PCa initiation but present an important mechanism to maintain AR signaling in the absence of circulating androgens [3].

In contrast, deregulation of AR coactivators, modulators, and interaction partners has been observed in both primary and with higher frequencies in CRPC. Taylor et al. performed a comprehensive AR pathway analysis and identified that 56% of primaries and 100% of metastases harbored genetic alterations in components involved in AR signaling [17]. Interestingly, while the AR itself was exclusively mutated in metastases, mutations in other AR signaling genes have been observed predominantly in primary tumors.

One of the most affected genes was NCOA2, a nuclear receptor coactivator, which was significantly amplified and observed to exhibit somatic mutations in 8% of primary PCa. Two novel point mutations in the regulatory or transcriptional activation domain of NCOA2 and near to known mutations in melanoma and lung cancer could be identified [17]. Supporting data have been reported by Barbieri et al., revealing point mutations in NCOA isoforms 2 and 3 in single cases [6]. Interestingly, the AR inhibitor nuclear receptor corepressor 2 (NCOR2) was mutated in 23% of primaries [17] supporting previous data from exome sequencing that reported mutations in the NCOR isoform 1 in two PCa samples [6]. NCOR represses target gene expression by directly binding to the AR with its C-terminal receptor-interacting domain [72].

Additionally, AR activators TNK2 and EP300 were mutated in 17% and 3% of primary tumors, respectively [17]. TNK2 is a non-receptor tyrosine kinase (also known as ACK1) shown to phosphorylate the AR and thus promote hormone-refractory tumor growth. Amplifications and point mutations in TNK2 have been observed in different cancer types, suggesting TNK2 to represent an oncogenic kinase [73].

The adenovirus E1A-associated cellular p300 transcriptional coactivator protein EP300 (p300) presents a histone acetyltransferase that regulates gene transcription by chromatin remodeling. It is overexpressed in advanced PCa, is involved in AR transactivation, and promotes PCa cell proliferation [74]. Mutations in p300 were detected in primary PCa tissues at low frequencies by independent sequencing studies (Fig. 12.4).

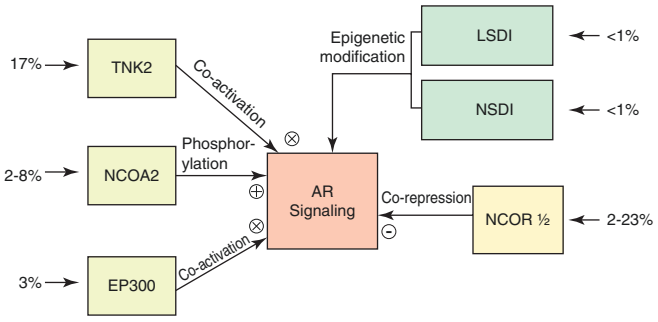

Fig. 12.4 Mutations affecting regulatory components of the AR signaling of primary PCa

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Chapter 13
Epigenetic Alterations in Primary Prostate Cancer

Fátima Valdés-Mora and Clare Stirzaker

Contents

Introduction in Epigenetics

Epigenetics refers to the study of "both heritable changes in gene activity and expression (in the progeny of cells or of individuals) and also stable, long-term alterations in the transcriptional potential of a cell that are not necessarily heritable" (Roadmap Epigenomics project http://www.roadmapepigenomics.org). There are a number of epigenetic processes identified, each of which can work independently or together to modulate gene regulation of single genes or sets of genes or globally across the genome, to form the "epigenome." These processes primarily include DNA methylation, posttranslational histone modifications, the incorporation of

F. Valdés-Mora, B.Sc., Ph.D. (✉)
Histone Variant Group, Genomics and Epigenetics Division,
Garvan Institute of Medical Research, Sydney, Australia

St. Vincent's Clinical School, UNSW Sydney, Sydney, Australia
e-mail: f.valdesmora@garvan.org.au

C. Stirzaker, B.Sc. (Honours), Ph.D.
Epigenetic Deregulation Group, Genomics and Epigenetics Division,
Garvan Institute of Medical Research, Sydney, Australia

St. Vincent's Clinical School, UNSW Sydney, Sydney, Australia

© Springer International Publishing AG 2018

B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*,
Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_13

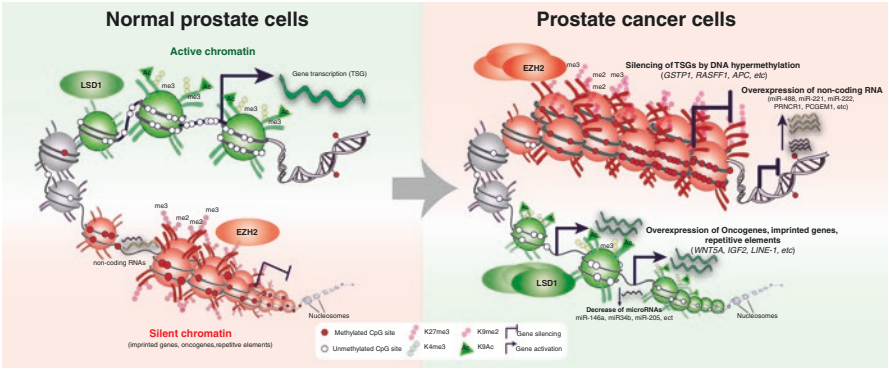

Fig. 13.1 Most prevalent epigenetic changes in prostate cancer. LHS, epigenome of a normal prostate epithelial cell. Active chromatin (*green*) is characterized by unmethylated CpGs (*white circles*) at promoters containing CpG islands of genes that are expressed for normal function of the cell. These actively transcribed genes also have active chromatin marks including H3K9ac (*green triangle*) and H3K4me3 (three green circles). In contrast, inactive chromatin regions (*red*) are characterized by H3K7me3 (three *red circles*), H3K9me2 (two *red circles*), and by DNA methylation at CpG sites of silent or imprinted genes or repetitive elements. Noncoding RNAs, including microRNAs (*yellow*) and long noncoding RNAs (*brown*), are also expressed to epigenetically silent genes. Chromatin remodelers and histone modifier proteins, for example, EZH2 (histone methyltransferase) or LSD1 (histone demethylase), are responsible of maintaining the chromatin homeostasis for normal cell function. RHS, prostate cancer is a model of "epigenetic catastrophe" where the cancer cells undergo a profound alteration of the epigenetic landscape. This is characterized by global DNA hypomethylation (unmethylated CpGs, *white circles*); DNA hypermethylation at CpG islands of TSG promoters (examples are included) causes gene transcriptional silencing. The histone code is also altered to promote TSG silencing, with a gain of H3K9me2 and H3K27me3 marks. Several noncoding RNAs have been found overexpressed and associated with gene silencing (examples provided). In contrast, other genomic areas become abnormally active and are characterized by a loss of DNA methylation and gain of active chromatin marks, like H3K4me3 and H3K9ac. These areas include oncogenes, imprinted genes, and repetitive elements. Decreased levels of miRNAs (examples given) have been reported to cause abnormal gene activation. The modification of the histone code suggests a causative dysfunction of histone-modifying enzymes, where EZH2 and LSD1 have been shown to be upregulated in prostate cancer

histone variants, nucleosome positioning, the expression of noncoding RNAs, and the three-dimensional chromatin conformation (Fig. 13.1) [1].

Epigenetic alterations are a common feature of cancer, including prostate cancer, in addition to genetic alterations (Fig. 13.1) [2]. The cancer epigenome is characterized by global changes in DNA methylation (focal CpG island hypermethylation and global genomic hypomethylation) and changes in histone modification patterns, as well as altered expression profiles of chromatin-modifying enzymes, histone modifier proteins, and noncoding RNAs. These epigenetic changes result in global dysregulation of gene expression profiles leading to the initiation and progression of disease states [3], including prostate cancer.

The molecular mechanisms involved in prostate cancer are diverse and heterogeneous, which include global and gene-specific epigenetic changes that take place at different stages of the disease. Epigenetic changes are influenced by genetic factors

but also by age, diet, and environmental factors [4]. The three major risk factors in prostate cancer are age, geographic ancestry, and the environment [5]; epigenetic aberrations can be attributed to any one of these risk factors [6]. This highlights the importance of understanding the epigenetic changes that take place in the different stages of the disease [7] and, furthermore, what is the main cause of these changes. Thus, epigenetic alterations have the potential to be used as biomarkers for both early prostate cancer detection, monitoring of disease progression, and disease management. In contrast to genetic alterations, epigenetic aberrations can be chemically reversed making them potential therapeutic targets [8].

In this book chapter, we describe the most prevalent epigenetic aberrations identified to date in primary prostate cancer and discuss the biological consequences of these epigenetic alterations. We also review the potential of epigenetic aberrations as biomarkers and therapeutic targets in prostate cancer.

DNA Methylation Changes in Prostate Cancer

DNA methylation (5-methylcytosine: 5mC) is the addition of a methyl group to the carbon-5 position of cytosine at CpG dinucleotides. CpG sites occur at a lower than expected frequency in vertebrates; however, some regions of the genome contain a higher than expected CpG density, termed "CpG islands." While cytosine methylation occurs primarily in the context of CpG dinucleotides, low levels of cytosine methylation in non-CpG contexts have also been identified by methylome sequencing in embryonic stem cells, oocytes, and the brain [9–12]. However, it is still unclear if non-CpG methylation plays any functional role in normal development and cancer. In contrast, a profound alteration of the CpG DNA methylation landscape occurs in the early stages of cancer initiation and continues to change during cancer progression [2]. These alterations are characterized by a global DNA hypomethylation and are thought to promote genome instability and the transcriptional activation of oncogenes and imprinted genes, whereas local DNA hypermethylation, typically at CpG island promoters of tumor suppressor genes (TSG), is associated with gene silencing. Aberrant DNA methylation (hypo- and hypermethylation) is one of the best-characterized epigenetic alterations in prostate cancer (Fig. 13.1).

DNA Hypomethylation in Prostate Cancer

Global DNA hypomethylation occurs in both early [13, 14] and late stages of prostate cancer [15–17] and is linked to chromosome instability and disease progression [18]. IGF2 loss of imprinting by DNA hypomethylation has been also demonstrated in prostate cancer, in nonmalignant adjacent and distant tissue areas from the peripheral zone of matched specimens; however, this pattern of expression is uncommon in benign prostatic hyperplasia (BPH) [19, 20]. This suggests a regional and

tissue-specific pattern of gene expression, which might predispose patients to neoplastic transformation over a long period of time.

DNA hypomethylation at specific gene promoters leads to aberrant oncogene expression in prostate cancer, including CAGE [21], CYP1B1 [22], HPSE [23], PLAU [24], CRIP1, S100P, and WNT5A [25]. For example, WNT5A is hypomethylated in 65% of primary prostate tumors, but not in normal tissues, and this is correlated with aberrant expression of WNT5A gene. WNT5A (wingless-type MMTV integration site family, member 5A) is a member of the WNT family that activates the β[beta]-catenin-independent pathways. Wnt5a regulates a variety of cellular functions, such as proliferation, differentiation, migration, adhesion, and polarity; aberrant Wnt5a signaling is involved in various diseases including cancer [26].

DNA Promoter Hypermethylation in Prostate Cancer

5mC promoter hypermethylation is the best-characterized epigenetic alteration in prostate cancer (Fig. 13.1) [27]. More than 50 genes with common aberrant hypermethylation have now been described to be involved in both early and late stages of the disease [27]. These genes are involved in oncogenic-related cellular pathways, including cell cycle control, hormone response, DNA repair, signal transduction, tumor invasion, and apoptosis. A number of these genes, including GSTP1 [28–34], RASSF1A [35–39], and APC [35, 36, 39–41], are consistently hypermethylated in the vast majority of prostate cancer cases and therefore are potential DNA biomarkers for cancer detection. Furthermore, frequent promoter methylation is also found in high-grade prostatic intraepithelial neoplasia (HGPIN) and morphologically normal prostate tissue (e.g., APC, CCND2, GSTP1, RARB2, RASSF1A, PTGS2) suggesting that some epigenetic alterations are early events in prostate carcinogenesis [36, 42, 43].

Glutathione-S-transferase pi 1 (GSTP1) gene encodes for the glutathione S-transferase enzyme that is involved in reactive chemical species and carcinogen detoxification. Thus, when prostate cells fail to express GSTP1, frequently due to promoter hypermethylation [44], the cells become vulnerable to oxidants and electrophiles causing DNA damage that contributes to neoplastic transformation leading to prostatic intraepithelial neoplasia (PIN) and prostate cancer malignant progression [45]. Notably, promoter hypermethylation of the GSTP1 gene is a promising epigenetic biomarker for early cancer detection [31, 46] as it is found hypermethylated in >90% of prostate cancer cases [47]. Commercialized assays have been developed for the detection of GSTP1 methylation with high specificity (86–100%) for prostate cancer. However, the sensitivity of these assays is variable depending on the methylation assay design and sample type, e.g., 19–83% sensitivity in urine [29, 33, 48–50] and 13–72% sensitivity in urine or plasma [28, 30]. To improve the sensitivity of methylated GSTP1 in detecting prostate cancer, multigene promoter methylation testing has been suggested [33, 40, 51]. For example, in the prostate cancer methylation assay (ProCaM), combining the GSTP1, APC, and

RARB2 promoters demonstrated an increase with respect to GSTP1 alone in the overall predictive power of the test in men with prostate-specific antigen (PSA) levels of 2.0–10.0 ng/ml and was associated with an increased likelihood of having a higher Gleason score in the biopsy [52].

The Ras association domain family 1A (RASSF1A) gene is a TSG that negatively regulates Ras signaling [37]. Inactivation of RASSF1A gene by promoter hypermethylation has been reported in multiple tumor types including prostate cancer, in which 49–99% of prostate carcinomas have RASSF1A promoter hypermethylation [35, 36, 38, 39]. The methylation pattern has also been detected in PIN [36], BPH, and even in histologically normal prostate epithelial cells [53]. However, in independent studies, RASSF1A methylation was not found in BPH, normal prostate stromal cells, or noncancerous prostate tissues [39], or the methylation grade was less in HGPIN and BPH than in the cancerous tissues [35]. Thus, a meta-analysis of the studies on RASFF1A promoter hypermethylation or more quantitative methods are needed to evaluate this gene as a biomarker of early diagnosis. In addition, higher frequency of RASSF1A methylation is associated with increased malignancy [36, 38, 39] suggesting a potential role as a biomarker for tumor progression.

Adenomatous polyposis coli (APC) protein is a negative regulator of the E-cadherin-β[beta]-catenin complex. This complex plays a critical role in cell-cell adhesion, and it is required for the maintenance of normal intercellular adhesion. APC is a TSG as silencing of APC leads to aberrations in cell adhesion, favoring epithelial to mesenchymal transition (EMT) and cell migration in cancer cells [54]. Analyses of the promoter methylation levels of the APC gene in different body fluids and in prostate cancer tissues have shown that the APC promoter is often methylated at a frequency of 27–100% [35, 36, 39–41, 55]. APC promoter hypermethylation status could be used as biomarker when the levels are measured in prostate tissue, as the specificity and sensitivity are very high (~90% and 80%, respectively) [55]. Overall, APC promoter methylation may be a potential biomarker for prostate cancer diagnosis, as well as a prognostic biomarker, since higher APC promoter methylation levels are significantly associated with higher Gleason score and PSA levels [35, 36].

The re-expression of DNA hypermethylated genes through demethylating chemical agents has been proposed as a potential new therapy in prostate cancer, especially in castration-resistant prostate cancer (CRPC) [56, 57]. There are two hypomethylating compounds approved by the US Food and Drug Administration (FDA) for the elective treatment of myelodysplastic syndromes, 5-azacytidine and decitabine [58, 59]. 5-azacytidine and decitabine inhibit DNA methyltransferase enzymes, leading to the reactivation of methylated silenced genes, like TSGs [8]. Although there is extensive experimental evidence proving the efficacy of these compounds in reversing DNA methylation in prostate cancer cell lines, only a few phase II trials of epigenetic drugs combined with chemotherapy treatments have been carried out in CRPC patients [56, 57]. These studies showed limited benefit from these drugs, expanding the progression-free interval by only a few weeks. Furthermore, these compounds exhibit

cytotoxic effects and have mutagenic potential [60], thus indicating there is a clear need for novel demethylating agents with improved pharmacological and clinical profiles.

DNA 5-Hydroxymethylation in Prostate Cancer

In addition to 5mC, the recent discovery of 5-hydroxymethylcytosine (5hmC) [61] provides a new component of epigenetic modification controlling gene expression in the genome. The TET (ten-eleven-translocation) family of proteins, TET1, TET2, and TET3, catalyze the hydroxylation of 5mC to 5hmC, and, as 5hmC is not recognized by Dnmt1, its presence promotes either passive demethylation during replication or active demethylation by a DNA repair system and replacement with an unmodified cytosine [61–64].

In mammalian cells, 5hmC levels are low (~1% of the total 5mC levels) [65] but are reported to be enriched at CpG-rich promoters [66], enhancers, and gene bodies [67, 68]. Notably, the global levels of 5hmC have found to be reduced in cancer, including prostate cancer [69–71]. Furthermore, mutations in the TET2 gene, commonly observed in human myeloid malignancies, display low levels of 5hmC but high levels of 5mC [72, 73]. In prostate cancer tissues, the presence of 5hmC and Ki67 protein (a proliferation marker) has shown to be mutually exclusive, suggesting that the combined analysis of Ki67 and 5hmC levels could be developed into a biomarker for prostate cancer diagnosis [71]. The involvement of 5hmC in tumor development has been further validated in a mouse model of prostate cancer where gene-independent 5hmC reduction during tumor development was observed [71].

Noncoding RNAs in Prostate Cancer

Noncoding RNAs (ncRNAs) comprise short (<200 nucleotides, nt) and long (>200 nt) transcripts [74]. Short ncRNAs, in particular microRNAs (miRNA), have well-evidenced roles in cancer [75]. Long noncoding RNAs, on the other hand, are emerging as novel players in carcinogenesis [76], and the understanding of their functional capabilities in normal development and disease is still in its infancy.

MicroRNAs and Prostate Cancer

MiRNAs are a class of small noncoding RNAs (18–25 nt) that bind and inhibit target sites in the 3′UTR of specific mRNAs. Each miRNA can potentially bind and inhibit 200 or more different mRNAs simultaneously, and furthermore, each mRNA

can be targeted by multiple miRNAs. MiRNA expression is altered in cancer cells by both genetic and epigenetic mechanisms, potentially affecting both oncogene and TSG regulation (Fig. 13.1) [77].

More than 50 miRNAs have been reported to be abnormally expressed in prostate cancer and have been widely associated with local invasion [78] and with the early stages of prostate cancer [79]. Potential miRNA biomarkers in prostate cancer are reviewed by references [75, 80]. MiRNAs are attractive biomarkers as they have been shown to be present and relatively stable in biofluids such as serum, plasma, urine, and saliva, which are easily obtained with minimal invasion [81–83].

Despite the extensive list of miRNAs reported to be dysregulated in prostate cancer, only a few have been experimentally proven to contribute to the disease [84]. MiRNA deregulation affects epigenetic reprogramming, blockade of apoptosis, promotion of cell cycle, migration, and invasion and is an alternative mechanism sustaining androgen-independent growth.

There is broad evidence suggesting that androgen receptor (AR) signaling aberrations and miRNAs are linked, either by miRNA regulation of AR signaling or androgen-independent regulation of miRNAs [84]. For example, overexpression of miR-488 represses the transcriptional activity of AR [84], and loss of function of miR-146a is frequent in hormone-refractory prostate cancer. Interestingly, a TSG role for miR-146a has been further demonstrated through its effect on suppressing ROCK1 expression, a kinase involved in the activation of hyaluronan-mediated hormone-refractory prostate cancer transition [85]. In addition, miR-31 directly targets AR expression and suppresses prostate cancer growth in vivo [86]. The same study also showed that miR-31 is suppressed by DNA hypermethylation in prostate cancer and its downregulation is associated with more aggressiveness of the disease. Moreover, the induced overexpression of miR-221 or miR-222 in LNCaP cells increased androgen-independent growth [87, 88]. The mechanisms of how miR-221 induces androgen independence have been further studied [88], and it appears that it is able to regulate androgen-dependent genes by downregulating HECTD2 and RAB1A in an AR-independent manner. Conversely, androgens may also be involved in miR-221/miR-222 downregulation [87]. Recently, a loss of expression of miR34b was shown to be consistently associated with prostate cancer relapse. Interestingly, in vitro loss of miR34b in prostate cancer cell lines induces the expression of Sox2, an AR-repressed gene [89].

In addition to their role in AR signaling, miRNAs are implicated in the inhibition of apoptosis and also in the facilitation of EMT during prostatic carcinogenesis [84]. For example, the overexpression of miR-21 inhibits apoptosis in prostate cancer cells through targeting the phosphatase and tensin homolog (PTEN) and programmed cell death 4 (PDCD4) genes [90, 91]. Decreased miR-205 expression levels have been observed in prostate tumors with a more pronounced reduction in carcinomas from patients with local regionally disseminated disease [92], and overexpression of miR-205 in prostate cancer cells resulted in a loss of an EMT signature, reduction of cell migration and invasion, and the downregulation of several oncogenes known to be involved in disease progression; this suggests that miR-205 could be used as a potential biomarker for prostate cancer progression [92].

Long Noncoding RNAs in Prostate Cancer

Long ncRNAs (lncRNAs) are non-translated transcripts greater than 200 nt that share many characteristics of mRNAs. LncRNAs have been identified within the introns of protein-coding genes, in intergenic regions, and antisense to protein-coding genes. In the last few years, with the advances in next-generation sequencing technologies, the number of discovered human lncRNA genes has increased from 6000 to over 13,870 [93]. A high number of identified lncRNAs show specific and temporal patterns of expression, suggesting functionally crucial roles [94]; however, only a minority of lncRNAs have been fully characterized. LncRNAs have been implicated in a wide variety of processes, including genomic imprinting, embryonic development, cell proliferation, cell differentiation, apoptosis, cell cycle control, and regulation of EMT [95]. Importantly, lncRNAs are aberrantly expressed in numerous human diseases, including cancer [96].

In prostate cancer, aberrant expression of several lncRNAs has been described (Fig. 13.1), with some being correlated with disease progression [97]. Overexpression of prostate cancer-specific oncogenic lncRNAs promotes tumor cell proliferation and metastasis [76] through enhancer-promoter chromatin looping with AR [98], antisense gene regulation [99], alternative splicing [100, 101], and the inhibition of DNA repair [102].

Recently, Yang et al. identified two highly overexpressed lncRNAs in aggressive prostate cancer, prostate cancer noncoding RNA1 (PRNCR1), and prostate cancer gene expression marker 1 (PCGEM1). These two lncRNAs strongly enhance both ligand-dependent and ligand-independent AR-mediated gene activation programs [98]. Remarkably, the lncRNAs localize to distal androgen response elements, thus facilitating looping between enhancer and promoter sequences and 3D gene activation. Functional analyses in prostate cell lines further validated the oncogenic properties of PCGEM1 [103] and PRNCR1 [104] in promoting cell proliferation [104] and transformation [98, 103].

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) lncRNA interacts with the serine-/arginine-rich family of nuclear splicing factors, modulating their distribution to the nuclear speckles, where it is proposed that they are stored until being recruited to active sites of transcription [101]. MALAT1 is overexpressed prostate cancer and has been associated with markers of poor prognosis, including high Gleason score, late tumor-node-metastasis stage, and serum PSA >20 ng/ml, and CRPC [100]. The mechanism behind MALAT1 upregulation in cancer remains unclear; however, several cancer-associated chromosomal translocation breakpoints exist within the MALAT1 gene [105]. Interestingly, the expression levels of MALAT1 fragments in human plasma were able to distinguish prostate cancer patients from non-prostate cancer patients with higher accuracy than PSA levels [106]. This evidence suggests the use of plasma MALAT1 levels as a potential non-invasive biomarker for prostate cancer diagnosis.

In the context of the use of lncRNAs as biomarkers, urinary measurement of the prostate cancer antigen 3 (PCA3), a highly specific lncRNA for prostate cancer, can

identify the presence of prostate cancer with greater tumor specificity than PSA [107, 108]. Over the course of the past 15 years, PCA3 has been successfully translated into the clinical setting. The Progensa PCA3 test is approved by the FDA and is commercially available to aid in the decision of repeat biopsies [109, 110]. For further discussion on the potential utilization of lncRNAs as biomarkers in prostate cancer (e.g., NEAT1, SChLAP1), please refer to Chakravarty et al. (2014) and Prensner et al. (2013) [111, 112].

Chromatin Alterations in Prostate Cancer

The basic repeating unit of chromatin is the nucleosome, a particle consisting of an octamer of core histones (two copies each of H2A, H2B, H3, and H4), around which ~146 base pairs of DNA are wrapped (Fig. 13.1). Nucleosomes provide a physical support to DNA and are involved in transcriptional regulation, repair, and replication [113]. The histone octamer forms a structure in such a way that the N-terminal histone "tails" protrude through the DNA, enabling posttranslational modifications (e.g., acetylation, methylation, phosphorylation, sumoylation, and ubiquitylation) to occur (Fig. 13.1). The combination of the specific modified amino acids confers local structural changes to the chromatin that affect gene transcription (known as "the histone code") [114]. The most well-characterized chemical histone modification is acetylation of histone 3 lysine 9 (H3K9ac) (Fig. 13.1), which confers a more open or active chromatin conformation enabling gene transcription. Histone methylation, on the other hand, can be associated with either active or inactive transcription depending on which amino acid residues are modified. For instance, methylation of lysines 4, 36, and 79 of histone 3 (H3K4me3 [Fig. 13.1], H3K36me, and H3K79me) is an active chromatin mark, while methylation of lysines 9 and 27 of histone 3 (H3K9me2 and H3K27me3, Fig. 13.1) and lysine 20 of histone 4 (H4K20me) is an inactive mark usually found in silent promoters and heterochromatin [115]. During embryogenesis and development, the histone code forms an epigenetic landscape that determines cell fate decision-making and fine-tunes gene transcription at specific gene loci (Fig. 13.1, LHS) [114].

Advances in next-generation sequencing have enabled genome-wide mapping of chromatin changes that occur during tumorigenesis. Cancer cells display global changes in histone acetylation and histone methylation patterns that lead to inappropriate gene expression (Fig. 13.1, RHS) supporting uncontrolled cell proliferation and invasion [3, 115]. For example, alterations in H3K9 and H3K27 methylation patterns are associated with aberrant gene silencing of tumor suppressor genes in various types of cancer, including prostate cancer [116, 117] (Fig. 13.1). Global, regional, and locus-specific changes in chromatin remodeling in prostate cancer are suggestive of a causative dysfunction of histone-modifying enzymes.

Global Changes in the Histone Code in Prostate Cancer

Global loss of the inactive H4K20me3 mark has been demonstrated across several different primary tumors including prostate cancer and is predictive of prognosis and survival [118, 119]. In addition, global overexpression of the active marks, H3K18ac and H3K4me2, is indicative of recurrence of prostate tumors in patients with low-grade prostate cancer [120, 121]. Prostate cancer patients with high H3K4me1 levels (an enhancer mark) are also more likely to experience recurrence [122]. To date, there is still only one study at this time showing the plausible use of histone modification levels for early detection of prostate cancer, where H3K9ac and H3K9me2 were significantly reduced in prostate cancer compared to nonmalignant prostate tissue. The specificity and sensitivity of these potential biomarkers were >91% and >78%, respectively [122].

Long-Range Epigenetic Changes in Prostate Cancer

While histone and DNA methylation changes have been shown at single genes, epigenetic gene silencing can also encompass large chromosomal domains, by a process termed long-range epigenetic silencing [123, 124]. LRES is a common phenomenon in cancer and involves regional chromatin remodeling, including gain of histone-repressive modifications (H3K27me3 and H3K9me2) and loss of histone-active modifications (H3K9Ac and H3K4me3) (Fig. 13.1). In addition, many CpG island-associated promoters in LRES regions gain DNA methylation, thereby leading to a consolidation of epigenetic silencing and a reduction in transcriptional plasticity. More recently, it has been shown that long-range epigenetic remodeling in cancer is not only associated with gene repression but can promote regional gene activation, termed long-range epigenetic activation (LREA) [125]. LREA regions are typified by a regional adoption of an active chromatin state, with the gain of H3K9ac/H3K4me3 and loss of H3K27me3 (Fig. 13.1). Importantly, several prostate cancer-associated genes are contained in the activated domains, including the most sensitive prostate cancer biomarker, KLK3 (PSA) [126]. LRES and LREA are common in cancer and combine changes in histone modifications and DNA methylation to give rise to concordant change in gene expression.

Histone Modifier Proteins in Prostate Cancer

Histone modifier proteins are enzyme complexes responsible for the different post-translational modifications of histones; these include histone acetyltransferases (HAT), deacetylases (HDAC), methyltransferases (HMT), demethylases (HDM),

chaperones, kinases, etc. [115]. Together, these complexes play an essential role in transcriptional regulation; deregulation of histone modifier proteins in cancer is extensive [127].

Enhancer of zeste 2 (EZH2) is a HMT that forms a polycomb complex directing the trimethylation of H3K27. EZH2 is strongly upregulated in metastatic CRPC, and its overexpression is a marker of poor outcome (Fig. 13.1) [128–130]. Furthermore, a polycomb repression signature, consisting of 13 genes regulated by EZH2, has been associated with metastatic prostate cancer [117]. Additionally, multiple members of the polycomb group family of repressors including EZH2 have been recently found upregulated in neuroendocrine prostate cancer, where this increase mediated a polycomb-aberrant silencing of key differentiation genes [131]. In contrast, recent studies have reported that the oncogenic activity of EZH2 in CRPC is independent of its role as a polycomb transcriptional repressor [132, 133], but rather as an AR coactivator. Increased expression of various epigenetic AR coactivators, such as TIF-2 [134], p300 [135], CBP [136], Tip60 [137], and recently EZH2 [132], is found in CRPC. Thus, upregulation of epigenetic coactivators during androgen ablation could be relevant to the failure of endocrine therapy in patients with prostate carcinoma. This suggests that EZH2 could be a new direct or indirect therapeutic target in CRPC [138].

Lysine (K)-specific demethylase 1A (LSD1) is a HDM that specifically demethylates H3K4me2/me1 or H3K9me2/me1, hence affecting target gene transcription. LSD1 is upregulated in prostate cancer (Fig. 13.1), and this is associated with poor prognosis [139, 140]. It has been shown to be a critical driver of prostate cancer by multiple mechanisms. In fact, LSD1 appears to exhibit distinct functions in hormone-dependent [139, 140] and hormone-independent prostate cancer [141]. LSD1 promotes transcription in cooperation with AR and is required for androgen-dependent proliferation through the demethylation of H3K9, leading to derepression of AR target genes [140]. On the other hand, LSD1 directly controls the transcription of numerous focal adhesion and cytoskeleton-associated genes, controlling the metastatic behavior of androgen-independent cells [141]. The selective and reversible inhibition of LSD1 represents a new strategy to block the activity of AR in androgen-dependent prostate cancer cells and has been shown to impair AR target gene expression, androgen-dependent tumor cell proliferation, and xenograft tumor growth [142].

Histone deacetylases HDAC 1, 2, and 3 are also upregulated in prostate cancer [143, 144], and HDAC 1 and 2 are associated with CRPC [144] and PSA relapse [143], respectively. HDACs play major roles in prostate cancer progression. The function of specific HDAC isoforms in human cancers remain elusive, but it appears that the regulation of AR function could be intimately associated with the activity of certain HDACs [145]. HDAC inhibitors are emerging as a new class of chemotherapeutic agents and have been shown to induce cell growth arrest, differentiation, and/or apoptosis in prostate cancer. The combined use of HDAC inhibitors with other chemotherapeutic agents, radiotherapy, or antiandrogen therapy has shown promising results [146–148].

Conclusions and Future Perspectives

Epigenetic alterations, in particular hypermethylation of CpG island promoters, are a common feature of prostate cancer and play an important role in prostate carcinogenesis and in disease progression.

The implementation of new technologies, such as next-generation sequencing for global epigenomic analyses and integration with genomic and transcriptomic data, will exponentially expand our understanding of prostate tumorigenesis [149] and will also enhance the discovery of the role of unexplored epigenetic mechanisms. For instance, recently, long noncoding RNAs [76], 5 hydroxymethylation [71], and histone variants [150, 151] have been identified as new and critical players in prostate cancer development and progression.

Prostate cancer has been proposed as a model of "epigenetic catastrophe" [39], particularly in relation to the widespread changes potentially observed in DNA methylation patterns. In contrast to genetic alterations, epigenetic changes are reversible. These features make prostate cancer a particularly attractive disease for epigenetic drug targets. However, there are some potential risks associated with the currently available epigenetic drugs, and novel epigenetic-based therapies need to be developed in order to become more useful therapies in prostate cancer.

Acknowledgments We thank Prof Susan J. Clark and Dr. Hui-Ming Lin for their careful review of the manuscript. FVM is a National Breast Cancer Foundation/Cure Cancer Australia Foundation Postdoctoral Training Fellow. CS is supported by the National Health and Medical Research Council (NHMRC1070418). This work is supported by the National Health and Medical Research Council project grants (NHMRC 1063560 and NHMRC1070418).

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Chapter 14
Proteomics in Prostate Cancer Research

Samantha E.T. Larkin, Benjamin Abbott, Michael D. Brown, Thomas R. Jackson, Noel W. Clarke, and Paul A. Townsend

Contents

Introduction

Prostate Cancer

Worldwide prostate cancer (PCa) is the second most commonly diagnosed cancer and the sixth most common cause of cancer death amongst men [1]. Within the USA alone, 238,590 new cases and 29,720 PCa deaths were recorded in

S.E.T. Larkin, B.Sc., Ph.D.
Cancer Sciences Unit, Faculty of Medicine, University of Southampton, Southampton, UK

B. Abbott, B.Sc., M.Res. • M.D. Brown, B.Sc., Ph.D.
T.R. Jackson, B.Sc., M.Sc., Ph.D. • P.A. Townsend, B.Sc., Ph.D. (✉)
Manchester Academic Health Sciences Centre, Faculty of Biology, Medicine and Health, Division of Cancer Sciences, Manchester Cancer Research Centre, University of Manchester, Manchester, UK
e-mail: paul.townsend@manchester.ac.uk

N.W. Clarke, M.B.B.S., Ch.M., F.R.C.S (Urol).
The Christie and Salford Royal NHS Foundation Trusts, Manchester, UK

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*, Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_14

2013 [2]. The principle problem arising from PCa is its propensity to metastasise. PCa preferentially metastasises to the bone marrow of the axial skeleton, and it is these metastases that are the major cause of PCa-associated morbidity and mortality [3, 4].

However, it is clear that not all PCa lesions progress towards life-threatening disease. Early studies by Franks [5] and more recently by Thompson et al. [6] showed a universally high incidence of microscopic PCa lesions in young men. However, this number does not translate in to clinically significant disease and progression towards metastatic disease and death. Autopsy studies have shown that latent disease could be detected in up to 30% of male 50-year-old prostates which increases to 75% in men over 80 [7]. The clinical relevance of this latent disease is currently unknown, and deciding on which tumour will become significant, with a risk of metastasising, remains one of the predominant diagnostic challenges facing urologists today. This variability in PCa leads to a significant level of uncertainty in PCa prognostication with subsequent overtreatment of the disease.

Epidemiological studies have shown clinical incidence, and rate of progression to metastatic disease is greater in western societies compared with developed non-western countries such as Japan [8]. It should be noted, however, that adoption of a western lifestyle by non-western men, usually through migration to a western country, has been associated with increased disease prevalence and an increased risk of aggressive disease [9]. This has led to the proposition that environmental factors, including diet, and not the underlying genetics are the drivers of disease progression.

PCa is usually asymptomatic until it advances with most tumours being found either incidentally or through routine screening and health checks. Currently the most common indication of disease is either an abnormal digital rectal examination (DRE) or a raised prostate-specific antigen (PSA) test. Nevertheless, both of these tests suffer from a lack of specificity and sensitivity. Although it has been reported that approximately 1/5 of all PCa tumours are detected by DRE, DRE only has a sensitivity and specificity of 52% and 81%, respectively [10].

PSA

Prostate-specific antigen (PSA) is 33 kDa protein secreted by the prostate epithelium and is an androgen-regulated serine protease involved in liquefaction and spermatozoa release [11]. PSA is detectable in high concentrations (mg/ml) within the semen and can be measured within the serum although at lower concentrations (ng/ml). Although PSA is the current gold standard for PCa detection, the PSA test has significant problems. The precise threshold at which a biopsy should be triggered is unclear. The Prostate Cancer Prevention Trial (PCPT) reported that up to 33.5% of men with a PSA score of <4.0 ng/ml, a value often used as a cut-off for no disease, actually had PCa [12]. A recent meta-analysis demonstrated 13% of patients had

false positive PSA tests leading to 5.5% having unnecessary biopsies. The reported sensitivity and specificity of the PSA test of 79% and 59%, respectively [13], combined with the fact that conditions such as benign prostatic hyperplasia (BPH) and prostatitis, ejaculation or vigorous exercise up to 48 h prior to testing can raise PSA levels has led to concerns around PSA screening for PCa. Schröder et al. [14] reported on the European Randomised Study of Screening for Prostate Cancer; PSA screening reduced PCa mortality by 20%, but it was associated with a high risk of overdiagnosis such that 1410 men would need to be screened and 48 additional cases radically treated to prevent one death from PCa.

This paucity in markers not only affects detection of disease but also its management. A significant number of men still present with metastatic PCa and will undergo androgen deprivation therapy (ADT). These men will progress to castrate-resistant disease (CRPC) within a median of 11 months [15]. Also a significant proportion of men presenting with high-risk non-metastatic disease will go on to develop CRPC and metastatic disease [16]. Although there are a range of therapies for CRPC metastatic disease, the lack of robust biomarkers has proved problematic in therapy selection, scheduling and disease monitoring. As with initial diagnosis, the reasons for this are multifactorial, including the unreliability of PSA and tumour heterogeneity. A recent expert consensus meeting stated that currently there are no validated predictive biomarkers available for use in daily clinical practice in CRPC [17].

There is, therefore, a clear need to identify new robust clinical biomarkers for the detection, grading, therapeutic management and monitoring of PCa.

The term ‘proteome’ was coined in 1994 by Marc Wilkins at a symposium and appeared in print in 1995 [18]. It was used to define the entire protein compartment within a cell/tissue/biological sample.

Proteomics

Proteomics is, therefore, the study of the proteome and is defined by Anderson and Anderson as ‘the use of quantitative protein-level measurements of gene expression to characterise biological processes and decipher the mechanisms of gene expression control’ [19]. The study of proteomics is not merely a study of protein expression, however. Proteomics also encompasses the study of the function of those proteins, including activity, post-translational modifications, localisation and protein interactions [20].

Proteomics employs a wide range of technologies for its study. Well known, but now outdated, is two-dimensional gel electrophoresis (2DGE). 2DGE is a gel-based method that separates proteins two dimensionally, firstly by isoelectric point (pI) and secondly by molecular weight enabling better resolution than a single-dimensional separation. Interestingly, this technique was first utilised some 20 years prior to the invention of the word ‘proteome’ and was described as a ‘high resolution two-dimensional electrophoretic method’ [19].

Proteomic biomarker discovery generally takes the form of nontargeted relative quantification methods leading to biomarkers being described as up- and downregulated. These methods include the gel-based methods (1D, 2D and DIGE) followed by mass spectrometry (MS) for identification purposes or entirely MS based. Advances in MS have resulted in proteomic tools that can compare and identify proteins implicated in different disease states with no need for an intermediate gel-based step. Many MS-based methods involve protein digestion, and so the protein analysis is actually based on surrogate peptides (bottom-up), but there are some technologies that focus on the whole protein (top-down).

Top-down

2DGE has the ability to resolve thousands of proteins and, coupled with MS for protein identification, became a renowned tool for protein biomarker discovery [21]. 2DGE, however, has several limitations such as an inability to resolve all proteins present due to huge fold differences in protein expression. In addition, there are problems with protein solubility and under-representation of basic proteins [22].

In 1997, 2D differential in-gel electrophoresis (2D DIGE) was highlighted as an alternative that overcame problems with comparing two different gels. 2D DIGE utilises different fluorescent dyes to label three different protein samples (e.g. normal, disease, control) which allows them to be run on the same IPG strip and gel enabling direct comparisons between the experimental samples and a control [21, 23]. Both 2DGE and 2D DIGE result in the identification of protein spots in relation to a particular disease or condition, but MS must be utilised to elucidate the identity of protein spots. Protein spots are excised and trypsin digested to produce peptides. MS techniques used to identify proteins from these peptide fragments include matrix-assisted laser desorption ionisation time-of-flight MS (MALDI-TOF-MS) and electrospray ionisation tandem MS (ESI-MS/MS) [24].

Surface-enhanced laser desorption ionisation TOF-MS (SELDI-TOF-MS) utilises chips with a number of different binding affinities to study subsets of proteins from a sample. Laser ionisation releases bound proteins which are detected by MS. Relative abundance of proteins can then be compared across many samples. Unfortunately, SELDI-TOF-MS fails to result in direct protein identification, often requires sample pre-fractionation and has received some criticism regarding reproducibility due to sample handling variation [25].

MS imaging (MSI) shares similarities with SELDI-TOF-MS utilised for the profiling of proteins in relation to topological information at the tissue and cellular level. Studies have utilised MALDI-TOF-MS (matrix-assisted laser desorption ionisation TOF-MS) to gain knowledge of the carcinogenic alterations that can occur outside of tumour margins highlighting the importance of the tumour microenvironment in tumourigenesis [26].

Top-down proteomics is still trailing behind in terms of sensitivity, but technologies are emerging that are capable of performing these analyses.

Bottom-up

Proteolytic peptides are either labelled with isotopic tags (ICAT25), isobaric tags (iTRAQ [27] or TMT tagging [28]) or non-isobaric tags (mTRAQ [29] or acetylation) or are analysed label-free using methods such as spectral counting [30]. For a review of some of these MS-based methods, see Schulze and Usadel [31].

Isotopic labelling is utilised in ICAT (isotope-coded affinity tags) whereby samples are differentially tagged with stable isotopes that contain a protein-reactive group, a glycol linker and a biotin tag. Linkers (tags) are made from eight hydrogen (light reagent) or eight deuterium (heavy reagent) atoms to enable differentiation between two samples. Once samples have been tagged, they are trypsin digested, fractionated via avidin affinity chromatography and then scanned by MS. Relative quantities of proteins can be determined and then differentially expressed protein peaks identified by MS/MS. The main drawback to ICAT is that the linkers only bind to cysteine and as approximately 10% of proteins do not contain cysteine, these are not labelled or analysed [24].

A similar approach, called isobaric tags for relative and absolute quantification (iTRAQ), allows quantification and identification of differentially expressed proteins in up to ten samples [32]. Isobaric (same mass) reagents are used to differentially label the amine residues of proteins in each sample prior to MS. The main drawback to this method is the potential experimental variation induced from the lengthy sample preparation required [24].

Multidimensional protein identification technology (MudPIT) exploits multidimensional high-pressure liquid chromatography (HPLC) to separate peptides prior to identification by MS/MS. Peptides can either be labelled or label-free. Protein mixtures are digested, and peptide fragments separated using a strong cation exchange column, followed by a reverse phase hydrophobicity column. Peptides eluted from the reverse phase column are then identified by MS/MS. The main benefits of this method are that complex protein mixtures can be separated and protein identification can be carried out rapidly without any pre or post-separation labelling. Although this method is highly sensitive and can be performed label-free (by the use of spectral counting [33]), identification of differential protein expression has been reported to be problematic [24].

These are numerous pre-fractionation methods and MS technologies that are being updated continuously to improve detection and quantification capabilities in bottom-up proteomic analyses. These methodologies have resulted in thousands of potential disease biomarkers.

SWATH MS is a data-independent acquisition (DIA) method which aims to complement traditional mass spectrometry-based proteomics techniques such as those described above. SWATH's main advantage is a complete and permanent recording of all fragment ions of the detectable peptide precursors present in a biological

sample that are produced, therefore allowing reinterrogation of the raw data without the need to repeat 'wet' experiments. Taken together it is high-throughput high reproducibility and consistency.

The method comprises two steps: the data acquisition method and targeted data analysis approach building on the high-throughput SRM (selected reaction monitoring) scoring (using the mProphet approach) developed in the Aebersold lab [34]. SWATH-MS data consists of highly multiplexed fragment ion maps that are deterministically recorded over the user-defined mass precursor mass range and chromatographic separation—by far the most comprehensive MS approach.

Urine Proteomics

Urine represents a logical source of biomarkers to identify disease within the organs and tissues of the urinary tract, with a urine test for PCa potentially offering a far less invasive alternative to blood testing or DRE. Recent studies have, therefore, sought to characterise the proteome of urine in PCa patients to identify potential diagnostic biomarkers. Davalieva et al. [35] used a two-dimensional difference gel electrophoresis (2D DIGE) approach combined with matrix-assisted laser desorption ionisation-mass spectrometry (MALDI-MS) to identify 23 proteins, predominantly secreted enzymes, with statistically significant differences in abundance in the urine of PCa patients compared to those with benign prostatic hyperplasia (BPH). Nine of the proteins were found to be part of the 'acute phase response' signalling pathway, perhaps functioning within the inflammatory tumour microenvironment, and five of these were selected for further validation by immunoturbidimetry. Measurement of haptoglobin and alpha-1-microglobulin/bikunin precursor in combination offered the best diagnostic accuracy, greater than that achieved by measuring serum PSA. Interestingly, previous studies have also highlighted these proteins as being differentially expressed in PCa [36, 37], and, as such, it may be useful to carry out further validation in independent cohorts.

Promising results were similarly obtained in an earlier study by Okamoto et al. [38], who used surface-enhanced laser desorption ionisation time-of-flight mass spectrometry (SELDI-TOF-MS) to identify a peptide panel of 72 peaks which could differentiate those with PCa from cancer-free controls. Hierarchical clustering allowed the discrimination of the two groups with a sensitivity of 91.7% and a specificity of 83.3%. However, urine samples analysed in this study were collected following prostate massage to enhance the detection of prostate-specific proteins and can thus be considered a more invasive approach. Other studies have evaluated the usefulness of panels of proteins or peptides in the PCa diagnosis, rather than single proteins which are typically less reliable. A 12 peptide panel, for example, has been proposed to allow detection of PCa using a sensitive capillary electrophoresis MS approach [39, 40]. The peptides were more readily detected in the first-void urine, which has previously been shown to contain higher levels of prostate-specific biomarkers (e.g. PSA) than the midstream urine [41].

Urine is undoubtedly one of the most convenient bodily fluids to collect for biomarker research and use in the clinic. However, urinary biomarker studies are not completely free from the practical issues associated with large-scale 'omics' studies utilising hundreds or thousands of biological samples. Some common issues for consideration are the time of day at which the sample is collected, the portion of urinary flow to be collected (as discussed previously, PSA is highest in the first-void urine) and degradation of the protein marker over time (e.g. during sample handling or storage). Regardless, future studies of urine biomarkers for PCa are likely to prove fruitful if well designed.

Serum/Plasma Proteomics

Blood contains a huge number of proteins, and being in contact with each organ and tissue, it becomes perfused with proteins secreted from those organs and tissues and with proteins 'leaking' from damaged or diseased cells. Additionally, it is minimally invasive to sample. However, the difficulty with blood as a biomarker pool is that the concentration of proteins covers several orders of magnitude making it incredibly difficult to uncover the lower abundance proteins due to the masking effects of proteins such as albumin and immunoglobulins.

Promising results from SELDI-TOF-MS studies for the detection of new PCa serum biomarkers have been reported. Adam et al. [42] used SELDI-TOF-MS to analyse serum from 167 PCa patients, 77 BPH and 82 healthy controls. They detected nine peaks with a quoted 83% of sensitivity and 97% of specificity. Petricoin et al. [43] analysed sera from a training set of 25 controls and 31 PCa patients, before applying the algorithm to a test set of 266 blinded samples (38 PCa). Seven peaks were detected that could identify 36 out of 38 PCa patients in the test set (95% sensitivity and 78–83% specificity). A further study by Qu et al. [44] analysing 386 serum samples (326 training set, 60 test set) found that 74 peaks could discriminate PCa from healthy samples with a sensitivity and specificity of 100%, but 21 peaks could also do the same with 97% sensitivity and specificity. However, a later study by McLerran [45], using rigorous validation methods, brought doubt on SELDI-TOF-MS as a tool for biomarker discovery. This final study found that the peaks reported by Adam et al. [46] and Qu et al. [44] did not stand up to validation methods and were not capable of differentiating PCa from control specimens. Whilst this was a blow for SELDI-TOF-MS, the authors point out that this does not suggest that the method does not work, but that extensive biomarker validation is vital to the biomarker discovery pipeline.

Another SELDI-TOF-MS study by Pan et al. [47] analysed 178 (83 PCa patients, 95 controls) samples yielding 18 differentially expressed proteins between PCa patients compared to the controls. After the application of a decision tree algorithm, eight proteins were identified that could correctly screen PCa patients with 93% sensitivity and 96% specificity.

An 8.9 kDa peak, identified as an apolipoprotein A-11 (apo-11) isoform, was found via SELDI-TOF-MS [48] in PCa patients whose PSA levels ranged from 0 to 4 ng/ml. Consequently, it has been suggested that apoA-11 could be a marker of PSA negative PCa. However, apoA-11 is an acute-phase protein which may be raised in a variety of clinical conditions due to inflammation and thus of limited use as a biomarker [49] but could be useful in a multivariate biomarker diagnostic. This is a potential limitation of many proteomic methods without immunodepletion or fractionation strategies to span the several orders of magnitude of protein expression in serum.

A further three studies utilised SELDI-TOF-MS for the analysis of serum samples; Le et al. [50] could differentiate PCa patients with and without bone metastases with 89.5% sensitivity using cluster of SAA (serum amyloid A) isoforms, Al-Ruwaili et al. [51] had a panel of 20 peaks capable of distinguishing indolent and aggressive disease (45 vs. 54; based on Gleason score) with 73.3% sensitivity and 60% specificity, and Rosenzweig et al. [52] utilised a high-resolution SELDI-qTOF instrument to identify two predictive markers (complement component 4a and protein C inhibitor) of recurrence in pre-radical prostatectomy serum samples.

A study by Qin et al. [53] utilised anion displacement chromatofocusing chromatography followed by 2D DIGE to analyse sera from 10 PCa and 10 BPH patients. They identified (using MS/MS) three low-abundance proteins: SCCA1 (squamous cell cancer antigen 1), S100A9 (calgranulin B) and haptoglobin-related protein. The authors propose this pre-fractionation method as a way to uncover low-abundance proteins within the serum proteome.

A study by Jayapalan et al. [54] combined 2DE with lectin-based methods to identify O[omicron]-glycoproteins. They found APOA2 (apolipoprotein AII), complement C3 β[beta]-chain fragment, TTR (transthyretin), SERPINA1 (α[alpha]-1-antitrypsin) and KNG1 (heavyweight kininogen light chain) to be significantly differentially expressed. As APOA2 and the complement fragment are acute phase reactants, and SEPINA1 and TTR are not glycoproteins, it is difficult to imagine these are robust biomarkers. KNG1, however, has been implicated in breast, cervical and endometrial cancers.

Highlighting the confounding of inflammation in biomarker proteomics, Bergamini et al. [55] studied biomarkers present with and without inflammation in PCa and BPH. They found that SELDI-TOF-MS and 2DE protein profiles were different depending on whether samples were from patients who had evidence of inflammation and that the presence of inflammation could confound biomarker discovery. SELDI-TOF-MS analysis including inflammation samples found no significant difference between BPH and PCa, but exclusion of the inflammation samples revealed 20 significantly different peaks. 2DE profiles that exclude inflammation samples identified two additional proteins that hadn't been found in the inflammation comparisons.

Utilising immunodepletion strategies followed by 2D DIGE, Byrne et al. [23] identified 13 differentially expressed proteins between PCa patients with Gleason score 5 and Gleason score 7. PEDF (pigment epithelium-derived factor) and ZAG (zinc-α[alpha]2-glycoprotein) have undergone validation, and the group suggests

that PEDF is an accurate marker of early PCa. The same group went on to apply 2D DIGE and metabolomics (using nuclear magnetic resonance) [56] to identify biomarker panels of diagnosis and progression of PCa. They found a three-biomarker panel to distinguish BPH from PCa (apolipoprotein A-IV, serum amyloid P component and glutathione peroxidase 3) with an AUC of 0.926, five biomarkers to distinguish Gleason score 5 from Gleason score 7 disease (kininogen-1, protein AMBP, complement factor H, coagulation factor XIII B chain and glutathione peroxidase 3) with an AUC of 0.549 and another three-biomarker panel to differentiate organ-confined from non-organ-confined disease (protein AMBP, haptoglobin pigment epithelium-derived factor and kininogen-1) with an AUC of 0.742.

Lam et al. [57] used a whole protein top-down MS profiling method, encompassing MALDI-TOF MS, to identify a stage-specific marker in a cohort of 16 PCa vs. 15 healthy individuals. The marker was identified as PF4 (platelet factor 4), and they found it to be significantly decreased in patients with metastatic PCa but not in those with localised or no PCa. This was an interesting approach but on a limited number of samples.

In 2012, Rehman et al. [58] used immunodepleted samples followed by an iTRAQ approach to identify biomarkers that could distinguish BPH, localised PCa, PCa with local spread (biochemically detected) and metastatic PCa. There were several promising biomarkers, but the group specifically highlighted EEF1A1 (eukaryotic translation elongation factor 1 alpha 1) as it progressively increased in expression from BPH through to metastatic PCa.

From all of these serum studies, only two markers have been identified in two separate analyses—APOA2 and KNG1. With APOA2 being an acute phase reactant, it would not be prostate specific. However, KNG1 may prove useful as a diagnostic marker having been highlighted as differentially expressed between BPH and PCa [54, 56].

Tissue

PCa tissue or cell lines seem to be an obvious choice for proteomic analysis as it is a direct way to look at what is happening at the tumour level. Not only can this result in promising biomarkers, but can also yield valuable information on the mechanisms involved in the tumourigenesis of PCa. However, tissue is more difficult to obtain, requiring surgery and associated risks. In addition, the tumour microenvironment is complex, and assaying only tumour cells may not give the full view of what is happening during the course of the disease.

Using 2DGE, Meehan et al. [59] compared normal and cancerous tissue proteomic profiles from 34 radical prostatectomy samples. They identified 20 proteins (via MS) that were lost in the cancerous tissue and validated ubiquitin-like NEDD8 and CNN1 (calponin) using western blotting and immunohistochemistry. The group found that the cellular localisation of ubiquitin-like NEDD8 and CNN1 was altered in the cancerous tissue.

2DGE was again used by Lexander et al. [60] to study the proteomics of fresh radical prostatectomy specimens from 29 malignant (grouped into low and high Gleason score) and 10 benign samples. They identified 39 proteins whose expression differed between the groups of which 15 had differential expression between low and high Gleason score (Gleason 6/7 and Gleason 8/9, respectively). The group then identified 30 proteins by MS including glutathione S-transferases (GST)-π[pi]. GSTs are a well-characterised family of enzymes thought to have a role in the prevention carcinogenesis initiation. Lee et al. [61] used antibodies to show that in 88 of 91 PCa samples, GST- π[pi] was not detectable. They also found hypermethylation of the regulatory sequence for the gene encoding GST- π[pi] (GSTP1) in all PCa samples, distinguishing from PCa. Further, a correlation between methylation of GSTP1 and prognosis has been found [62].

Another group adopting a 2DGE approach found 21 protein spots differentially expressed between two subtypes of the LNCaP cell line [63]. Originally from a lymph node metastasis [64], this cell line is used frequently in biomarker studies due to its hormone sensitivity, and because it expresses PSA [65]. Ten of the 2DGE spots were identified using MS, and the group validated one spot in formalin-fixed paraffin-embedded (FFPE) tissue. This protein was the 60 kDa heat-shock protein (HSP60) whose expression correlated with clinical features of PCa. Cell line studies have advantages over tissue studies as they can reduce variables and sample selection bias, but they are only a model for disease, and therefore results do not always translate.

Alaiya et al. [66] identified a panel of 22 markers capable of not only distinguishing BPH from PCa but also low- and high-grade PCa. They had performed proteomic analysis using 2DGE and MS on fresh tissue from 8 PCa patients and 16 BPH patients, and they found that 15 of their markers overlapped with other studies from different geographical locations, indicating the homogeneity of tissue expression across different ethnic populations.

Lin et al. [67] utilised 2DGE to assess protein expression in tissue taken from prostate biopsies comparing BPH and PCa (14 vs. 9, respectively) and found 52 protein spots significantly differentially expressed between the two groups. Using MS they were able to identify FLNA(7–15) (filamin A) and FKBP4 (FK506-binding protein 4), both androgen receptor co-regulators, and PRDX4 (peroxireduxin 4) which were confirmed by western blotting as being altered in PCa tissue. FLNA(7–15) was decreased in PCa whereas FKB4 and PRDX4 were increased.

A similar study using 2DGE and biopsy tissue, 11 BPH vs. 12 PCa [68], reported 79 differentially expressed proteins including PAP (prostatic acid phosphatase). They went on to study prohibitin at the mRNA and protein level and found it to be upregulated in PCa. The group then went on to study protein expression between normal and PCa tissue in 24 radical prostatectomy specimens [69] using 2D DIGE and MS identifying 79 proteins that were differentially expressed. By western blotting they confirmed overexpression of eIF4A3 (eukaryotic initiation factor 4A-III; thought to be involved in translation), DDAH1 (dimethylarginine dimethylaminohydrolase 1; has a role in NO signalling and possibly androgen-independent cellular growth), ARG2 (arginase 2; involved in polyamine metabolism which is important

in PCa development, has been implicated in small cell lung cancer), Prdx3 and Prdx4 (peroxireduxin 3 and 4; antioxidant agents thought to be involved in cell proliferation, apoptosis and gene expression) in many PCa tissues compared to matched benign samples.

A study of epithelial and stromal cells in normal, BPH, prostatitis and PCa using 2DGE and MS was performed by Khamis et al. [70]. They report a downregulation of cellular retinoic acid-binding protein 2 was in basal cells of benign prostate. Caspase-1 and interleukin-18 receptor 1 were overexpressed in PCa leukocytes. Proto-oncogene Wnt-3 was downregulated in prostatitis endothelial cells, and tyrosine phosphatase non-receptor type 1 was found only in normal and benign endothelial cells. A downregulation of poly ADP-ribose polymerase 14 was reported in myofibroblasts of prostatitis tissue. Finally, an upregulation of integrin alpha-6 was seen in epithelial cells but could not be detected in PCa myofibroblasts.

Han et al. [71] also enlisted the 2D DIGE-MS approach in their study of four radical prostatectomy specimens looking and proteome differences between PCa and adjacent tissue. Analysed alongside gene expression microarray data they went on to identify 60 proteins and from these selected 14 differentially expressed proteins to validate by ELISA in serum from 84 PCa, 35 BPH and 13 healthy patients. Their study resulted in three putative serum biomarkers associated with PCa, methylcrotonyl-CoA carboxylase 2 (beta) (MCCC2), TNF receptor-associated protein 1 (TRAP1) and inosine-50-monophosphate dehydrogenase 2 (IMPDH2).

Another 2D DIGE-MS study comparing 5 BPH and 5 PCa radical prostatectomy samples [72] yielded 39 protein spots with significantly differential expression between the groups. They validated three proteins (with roles in the cell cycle and progression) in a further 28 BPH and 14 PCa samples (UBE2N, PSMB6 and PP1CB) using western blotting.

Rowland et al. [73] used 2D DIGE to study androgen ablation in LNCaP cells. They identified 107 proteins differentially expressed between androgen-supplemented cells and anti-androgen-supplemented cells, the majority of which have not previously been associated with the androgen-responsive network.

Skvortsov and co-workers [74] utilised 2D DIGE with MALDI-MS to compare proteomes from matched benign and tumour radical prostatectomy samples. They were able to identify 19 proteins that were significantly differentially expressed, of which HSP60 was significantly upregulated in PCa (as in the aforementioned subtypes of LNCaP66) compared to benign and lamin A was able to discriminate between low- (Gleason score 6) and high (Gleason score $\geq$ 8)-grade disease.

Pang et al. [75] studied localised PCa (10), lymph node metastatic (LNM) PCa (7) and BPH tissue (10) samples using 2D DIGE with MS. They identified six markers associated with LNM PCa proposing them as candidate biomarkers of aggressive disease. They validated them using real-time PCR, western blotting and immunohistochemistry. These markers are FABP5 (fatty acid-binding protein, epidermal), MCCC2 (methylcrotonyl-CoA carboxylase beta chain, mitochondrial), PPA2 (inorganic pyrophospatase 2, mitochondrial), EZR (ezrin), STOML2 (stomatin) and TAGLN (transgelin).

A SELDI-TOF-MS study by Zheng and colleagues [76] reported a marker they call PCa-24 (*m/z* 24,782.56) to be present in 16 of 17 LCM (laser capture microdissection) obtained PCa samples but not in paired normal cells nor in 12 BPH samples assayed alongside. Another group employed a similar methodology [77] to compare LCM enriched normal, high-grade prostatic intraepithelial (HGPIN; PCa precursor) and PCa cells from 22 radical prostatectomies. They identified a 24 kDa protein with expression in 19/27 PCa, 3/8 HGPIN and in none of the normal cells. This protein was identified as GDF15 (mature growth differentiation factor 15) and the authors claim that it could be a marker of prostate carcinogenesis. Another study [78] utilising SELDI-TOF-MS assessed protein expression and TNM stage of PCa in 43 primary PCa and 26 matched non-cancerous samples. They found that TIMP1 (metalloproteinase inhibitor-1) was differentially expressed between different stages.

An initial technical PCa study utilising ICAT (isotope-coded affinity tags) was an optimisation of the procedure coupled with ESI-MS/MS (electrospray ionisation tandem mass spectrometry) [79]. The group assessed the proteomes of non-tumourigenic (P69) and highly tumourigenic (M12) PCa cell lines to identify two overexpressed and four under-expressed proteins in the tumourigenic cell line. A further study utilised a similar approach to label specific membrane proteins (PSCA and c-ErbB2) and study expression levels in PCa cell lines [80]. Subsequent work utilising ICAT with LNCaP cells have compared androgen-depleted and androgen-stimulated differences in protein expression [81, 82] and cell surface and secreted proteins [83]. Several well-characterised PCa-associated proteins were identified in addition to other proteins with unclear roles in PCa. Further validation studies are required to ascertain their utility as diagnostic and prognostic markers.

iTRAQ has been used to examine tissue and cell line proteomes. Garbis et al. [84] studied of BPH and PCa snap frozen tissue from 20 patients (10 BPH and 10 PCa) utilising iTRAQ alongside LC-MS/MS to identify 825 proteins. They found that 30 were upregulated, and 35 were downregulated in PCa compared to BPH. These markers included the well-characterised PCa markers AMACR, PAP and PSMA.

Sun and colleagues [85] also studied BPH and PCa using iTRAQ and 2D LC-MS/MS. From 50 biopsy samples (20 BPH, 20 PCa and 10 BPH with local PIN), they identified 46 differentially expressed between BPH and PCa and 33 between PCa and BPH with local PIN. Their markers included PSA and PAP, and the authors went on to validate PSTN (periostin), which they claim to be a promising diagnostic marker.

Another iTRAQ study compared the poorly metastatic cell line LNCaP with its highly metastatic variant, LnCaP-LN333. Ten proteins were shown to be over- and four under-expressed in the highly metastatic cell line. Gp96 and GRP78 were validated using 2DGE and western blotting demonstrating their overexpression in the variant cell line. Immunohistochemistry of benign and malignant prostate tissue further validated Gp96. GRP78 was previously identified as differentially expressed in isogenic prostate cell lines and Gp96 in IFNγ[gamma]-treated isogenic cell lines [86].

Sardana et al. [87] identified four PCa markers (follistatin, chemokine ligand 16, pentraxin 3 and spondin 2) using an MS-based top-down approach. For their initial

discovery, they analysed the secretome of three different cell lines, and these biomarkers were subsequently validated on serum samples.

Geiger et al. [88] analysed the proteome of 11 cell lines, including LNCaP, using an LTQ Orbitrap Velos mass spectrometer with a 'high field' Orbitrap mass analyser and detected 10,369 proteins from the LNCaP cell line.

Using the newer SWATH-MS method, Liu et al. [89] searched for glycopeptides associated with aggressive PCa. They compared 10 normal prostate, 24 non-aggressive PCa, 16 aggressive PCa and 25 metastatic PCa tissues. They reported 220 glycoproteins with differential expression associated with PCa aggressiveness and metastasis. They validated two biomarkers associated with aggressive PCa in an independent cohort, NAAA (N-acylethanolamine acid amidase) and PTK7 (protein tyrosine kinase 7). They report that these biomarkers could pinpoint aggressive disease and help minimise overtreatment of indolent tumours.

Semen Proteomics

As stated, prostate-specific antigen, PSA, is the current gold standard biomarker for PCa and is currently used in the clinic in spite of its documented issues with specificity and sensitivity. PSA was first isolated from seminal fluid and is found at much higher concentration within the ejaculate (1.2 mg/ml) than in the blood serum (<4 ng/ml) [13, 90]. However, even though the seminal fluid is known to be rich in proteins, there has been little published regarding its utility for PCa biomarker discovery.

Seminal fluid is a complex mixture arising from multiple organs and plays multiple roles in the ejaculate. The seminal fluid consists of secretions from the seminal vesicles, prostate gland, tests and epididymis, bulbourethral gland and the periurethral gland (see Table 14.1), with each gland providing components for the multiple functions of the seminal fluid.

Table 14.1 Major source and components of the seminal fluid

Organ	Percentage contribution to seminal fluid	Major secretions
Seminal vesicle	65	Cytokines (including TGFβ[beta] [91], prostaglandins, fructose [92], semenogelins, fibronectin, protein C inhibitor, mucin 6 [93])
Prostate	25	Proteolytic enzymes (kallikreins, PAP) citrate, lipids, zinc α[alpha]-2-glycoprotein, β[beta] microseminoprotein [94–96]
Testes and epididymis	9	Clusterin, prostaglandin D2 synthase, human epididymal protein E4, glutathione peroxidase 5 [97]
Bulbourethral and periurethral glands	1	Galactose, sialic acid, mucus [98]

The seminal fluid also contains non-sperm cells collectively known as ‘round cells’. Round cells are a mixture of leukocytes, developing spermatids, sertoli cells, epithelial cells, lymphocytes, neutrophils and macrophages, and there number varies from man to man, with age, health, sexual activity and fertility [99].

The main role of the seminal fluid was thought to be the liquefaction of the semen and providing nutrition for the spermatozoa. However, the role of the seminal fluid is much more complex. The seminal fluid interacts in with the female urogenital tract to modulate both the local microenvironment and the female immune system to aid sperm survival. High levels of basic polyamines such as spermine, spermidine and putrescine make the seminal fluid alkaline in nature, which helps neutralise the normally acidic female urogenital tract [98]. Cytokines such as the immunosuppressive TGF-B secreted by the seminal vesicles with levels reaching 150–200 mg/ml [91] help to reduce the female host immune response to the ‘invading’ and foreign male sperm. Secretions from the bulbourethral glands help lubricate the semen aiding spermatozoa motility and thereby fertility [98].

Although the first published study of seminal proteomics was in 1888, describing the discovery of propeptone as a seminal contamination of urine, it is only recently that large-scale proteomic studies have been conducted. Starita-Geribaldi et al. [100] isolated >100 proteins from seminal plasma by 2D-MALDI-TOF-MS from men with azoospermia. This has been superseded by Batruch et al. [101] using 2D-LC-MS/MS who isolated >2000 proteins in seminal fluid from pre- and post-vasectomy men or men with azoospermia. However, this may not represent the entire proteome.

The data clearly demonstrates that the seminal fluid is a highly complex proteome which, like blood plasma, contains large amounts of secreted high-abundance proteins such as kallikreins and semenogelins, which can account for >80% of the total protein content [97]. The dynamic range of detected proteins is also large, spanning 9 orders of magnitude, with total concentrations 40–60 mg/ml for the top secreted proteins to <10 pg/ml for pro-inflammatory interleukins. Over 97% of these proteins are soluble with the remaining 3% being found within microvesicles, many of which are secreted by the prostate gland, known as prostasomes [102]. Prostasomes are membrane bound vesicles between 40 and 500 nm in diameter with membranes predominantly composed of cholesterol. Prostasomes contain a sample of the interior of the prostate epithelial cells and potentially have functions in spermatozoa motility [103], seminal liquefaction [104], antibacterial activity [105], growth inhibition [106], protection from the acidic milieu of the vaginal tract [107] and immunomodulation of the vaginal tract [108]. Prostasomes have the advantage for proteomic analysis in that there is no single dominating protein complicating downstream analysis [109].

Even though the introduction of PSA significantly increased the numbers of PCa cases detected, it has failed to make an impact on the number of PCa-associated deaths [110]. There is, therefore, a clinical need for new and robust markers. Global proteomics of the seminal fluid benefits from the collection being non-invasive and from being a proximal fluid, being in contact with any potential prostate tumour, as

compared with circulating blood specimens. To date, there have only been a limited number of proteomic studies specifically studying PCa.

A study [111] using 2DGE identified pigment PEDF to be an early tumourigenesis biomarker in PCa. Weak expression was observed in some HGPIN samples and all PCa samples; the weak expression seen in the HGPIN was associated with subsequent PCa diagnosis. This supports the study by Byrne et al. [23] mentioned previously who also found PEDF to be important in PCa progression.

Neuhaus et al. [112] took a top-down approach to analyse the seminal plasma from 125 patients (70 PCa, 21 BPH, 25 chronic prostatitis, 9 healthy controls). Native peptides from seminal plasma were sequenced by LC-MS/MS using a Dionex UltiMate 3000 RSLS nanoflow system prior to analysis using an LTQ Orbitrap hybrid mass spectrometer. Using this approach, Neuhaus et al. [112] were able to show that seminal plasma proteomics could generate biomarker profiles able to detect PCa and to discriminate between high- and low-grade disease. A stepwise application of a 21- and a 5-peptide biomarker profile was shown to detect PCa with a sensitivity and specificity of 83% and 67%, respectively. A second 11-peptide marker profile was able to differentiate between Gleason score 7 organ-confined (stage ≤T2c) and advanced Gleason score 7 tumours (stage ≥T3a) with a sensitivity and specificity of 80% and 82%, respectively. Whilst this approach enables the researcher to directly detect combinations of post-translational modifications, sequence variants and degradation products, the technique's ability to detect post-translational modifications can affect the ability to sequence the peptides. Therefore only eight of the peptides were definitively identified: semenogelin 1–4, stabling-2, PAP, N-acetyllactosaminide beta-1,3-N-acetylglucosaminyltransferase and GTPase IMAP family member 6.

An alternative approach to seminal plasma is to analyse the proteome of the prostasomes. An earlier study by Renneberg et al. [113] found approximately 80 peptide spots on 2D gels representing the normal prostasome proteome. Utleg et al. [109] took this further using μ[mu]LC-ESI-MS/MS coupled with an iterative gas-phase fractionation (GPF) approach to identify 139 proteins. Purified prostasome peptide fragments were then analysed by μLC-ESI-MS/MS along with gas-phase fractionation to achieve maximum coverage. A total of 139 proteins were confidently identified, of which 119 proteins had a probability score of 0.9, with the remaining scoring between 0.5 and 0.7. The proteins identified fell into six groups: (1) enzymes (including PAP, PSA, TMPRSS2, fatty acid synthase), (2) transport and structural proteins (six members of the annexin family, actins, ezrin, corfilin, tubulins and profilins 1 and 2), (3) GTP proteins (Rab family), (4) chaperone proteins (HSP 27, 70, 71, 90 and grp 7), (5) signal transduction proteins (including 14–3-3 protein β[beta], γ[gamma], ε[epsilon], ζ[zeta], σ[omega], clusterin, calmodulin, zinc-alpha-2-glycoprotein) and (6) unannotated (see Utleg et al. [109] for complete listing). Many of the enzymes identified within this study are exclusively present in prostasomes and coupled with the fact that many of the proteins identified are also related to PCa and the lack of a dominant protein; prostasomes are an attractive target for proteome biomarker discovery.

Several of the proteins identified by Utleg et al. [109] have also been observed by galectin-3 binding of proteasomal proteins [114] and from seminal plasma proteins [115]. Galectin-3 is a 30 kDa carbohydrate-binding protein found on the surface of prostasomes and is a proteolytic substrate of PSA [116]. MS/MS identified candidate galectin-3 binding ligands such as PSA, PAP, zinc-alpha-2-glycoprotein, CD26, CD13, neprilysin, clusterin, antibacterial protein (FALL-39) and alpha1-acid glycoprotein [114]. Hassan et al. [115] also identified PSA, PAP and zinc-alpha-2-glycoprotein, along with progastricsin and PIP as being differentially expressed in seminal plasma proteomics. Interestingly 2D electrophoresis of pooled normal or cancer patient's seminal fluid both gave rise to the same 917 spots. By examining 2D gels loaded with decreasing amounts of protein, five differentially expressed spot clusters were identified and sequenced following tryptic digestion.

However, to date there has not been a large-scale proteomic analysis of seminal fluid prostasomes in PCa patients [117]. This may be due to the potential limitations in seminal fluid sampling due to religious, societal, physical and emotional issues and the age of the subjects.

These limitations would apply to both prostasome and global proteomic biomarker screening of seminal fluid. Combined with the highly standardised protocols and associated infrastructure, it is currently unlikely that seminal fluid proteomics will be taken up by the clinical fraternity. However, it remains a valid biofluid for biomarker discovery [117].

Exosomes

Exosomes are small (50–100 nm in diameter) vesicles which are endocytic in origin and secreted from the cell surface [118]. Exosomes are known to contain proteins and thought to act as an additional route of cell-cell signalling. Importantly, it is becoming increasingly apparent that exosomes are released by cancer cells (including PCa cells) into the bodily fluids and may therefore warrant further investigation as a potential source of biomarkers. In cancer, exosomes are proposed to function as shuttles for proteins, which act on surrounding cells to facilitate tumour growth and metastasis [119, 120]. In PCa, an increasing body of research is beginning to characterise exosomal proteins as potential diagnostic markers and markers of disease progression or drug response/resistance.

PCa exosomes have been isolated from a number of fluids including the blood, seminal plasma, expressed prostatic secretions and urine, as well as primary prostate tissue and cell lines. A recent study by Øverbye et al. [121] to identify urinary exosomal markers of PCa found that 246 proteins were differentially expressed between the exosomes of PCa patients and healthy controls, with the majority showing upregulation in the cancer samples. A total of 37 proteins found to be significantly enriched could distinguish PCa with 100% specificity and a sensitivity of 50% or higher. These included claudins, Ras-related proteins and various enzymes and regulatory factors. The protein showing the highest sensitivity (94%) and level of enrichment in PCa (140-fold) was TM256, a potential candidate for further validation.

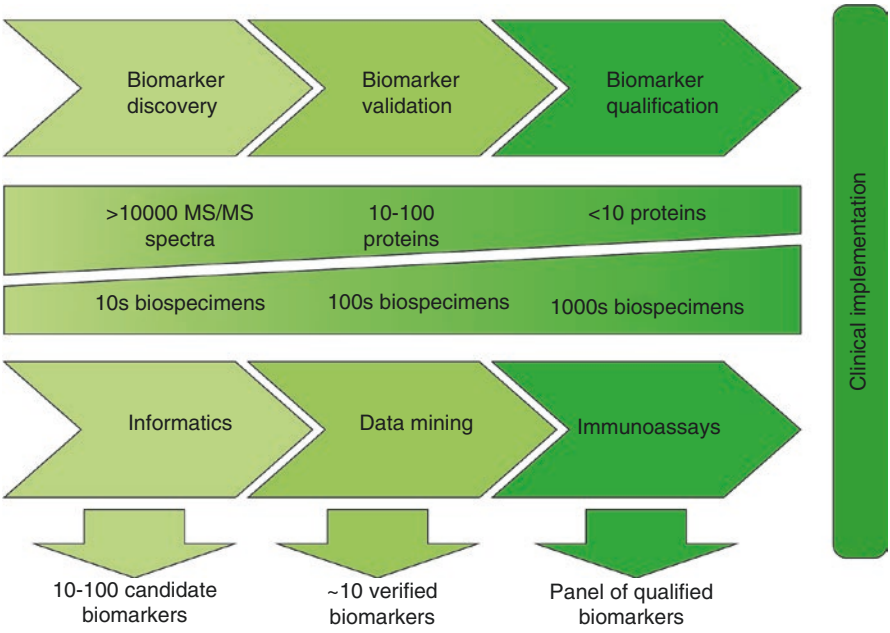

Fig. 14.1 Diagrammatic representation of the stages involved in biomarker discovery through to clinical implementation giving an indication of the number of analytes and samples used at each stage

Concluding Remarks

A simple Pubmed search to identify studies with the search term 'proteomics' and 'prostate cancer' anywhere in an article yielded 607 hits. This is quite an achievement for just 21 years since the inception of proteomics. Indeed, these results demonstrate biomarkers that should revolutionise PCa diagnosis, prognosis and treatment. Yet, the roadblock is that since these studies first started being published, few markers have even come close to becoming a clinically applicable test. Only two markers were identified in more than one study in serum, APOA2, an acute phase reactant, and KNG1 which may prove valuable as it has also been identified in other cancers [54, 56]. Additionally, PEDF was identified in both serum and semen [23, 111].

This lack of clinical applicability is almost certainly due to study design. Ransohoff [122] has described the phenomenon of overfitting; the differences between groups in MS peak patterns are due to chance rather than biological difference, and as such the results are not reproducible. This occurs as the number of features analysed (typically thousands of peaks) are greater than the number of samples (see Fig. 14.1). This can be detected by attempting to validate results in an adequately sized independent sample. This means that until a validation has been performed, high-throughput study results are fairly meaningless.

Group selection, sample collection and sample storage are all times where bias can be introduced, and this represents a further potential pitfall for any proteomic study.

Indeed when McLerran et al. attempted to minimise bias, they found that SELDI-TOF-MS became unable to differentiate PCa from biopsy-negative controls [45, 123]. It is true that with such international centres such as those in the USA, Switzerland and the UK, the application of proteomics to stratifying medicine will become a reality. It has to. We now have the ability to manage and mine huge amounts of live 'big data'. A focus on a precision medicine will allow us to help patients, when they need it and in real time.

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Chapter 15
Metabolomic-Based Stratification in Prostate Cancer

Stefano Cacciatore, Giorgia Zadra, and Massimo Loda

Contents

S. Cacciatore, Ph.D.
Department of Oncologic Pathology, Dana-Farber Cancer Institute, Boston, MA, USA

Institute of Reproductive and Developmental Biology, Imperial College London, London, England, UK

G. Zadra, Ph.D.
Department of Oncologic Pathology, Dana-Farber Cancer Institute, Boston, MA, USA

Department of Pathology, Brigham and Women's Hospital, Boston, MA, USA

M. Loda, M.D. (✉)
Department of Oncologic Pathology, Dana-Farber Cancer Institute, Boston, MA, USA

Department of Pathology, Brigham and Women's Hospital, Boston, MA, USA

The Broad Institute, Boston, MA, USA

Division of Cancer Studies, King's College London, London, England, UK
e-mail: massimo_loda@dfci.harvard.edu

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*,
Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_15

Background

Prostate cancer (PCa) is the fourth most common cancer in the world and the second most commonly diagnosed cancer in men [1]. In 2017, PCa will be diagnosed in 161,360 men in the United States alone and 26,730 will die fo the disease [2]. Multiple genetic and demographic factors contribute to the high incidence of PCa including age, family history, genetic susceptibility, and race [3].

Most patients are diagnosed as a result of prostate-specific antigen (PSA) screening or, less commonly, with a positive digital rectal examination (DRE). Diagnosis is usually confirmed by biopsy, which may miss lesions due to the multificality of the disease while imaging techniques have still poor resolution [4].

Even when a tumor is sampled by the biopsy, the portion of the tumor obtained for histopathologic analysis may not be representative of the patient's disease, a fact reflected by the relatively common upgrading and upstaging of disease that occurs at the time of prostatectomy [5].

Nearly 90% of PCas are clinically localized at the time of their diagnosis [6] and their clinical behavior is highly variable. While some remain indolent and can be completely eradicated with prostatectomy or if left untreated and safely observed ("active surveillance"), others progress to aggressive cancer leading to metastases and lethal disease. Unfortunately, locally advanced or metastatic PCa treated with androgen deprivation therapy or last-generation agents targeting androgen receptor (AR) signaling, such as abiraterone and enzalutamide, eventually develop resistance, a state known as castration-resistant PCa (CRPC) [7]. In a meta-analysis of 11 large clinical cohorts, Chou et al. concluded that PSA screening has no effect on PCa-specific mortality. Therefore, new approaches are urgently needed to distinguish individuals with aggressive PCa from those with indolent disease [8].

The capability to accurately classify into biologically and clinically meaningful subtypes is the first step toward personalized cancer treatment. Traditionally, the diagnosis and staging of PCa is based on the histopathologic assessment of a core needle biopsy, the extent of the cancer in the biopsy, and the histologic grading based on the degree of differentiation of the tumor as defined by the Gleason score. Each specimen is assigned a Gleason score based on its most prevalent and second most prevalent histologic grades. The combination of the two grades gives the total score, which is tightly associated with prognosis. However, a large group of patients presents with intermediate differentiation and organ-confined disease. In these patients, traditional morphologic parameters are insufficient to predict the tumor behavior and thus unable to guide adeguate therapeutic strategies, specifically to identify patients that will benefit from therapeutic intervention such as surgery or radiation therapeutic intervention, from those for which active surveillance would be preferable.

Molecular and genetic profiling has been increasingly used to subtype cancers and guide targeted therapeutic interventions. Several studies have investigated the genomic basis of primary PCa and identified multiple recurrent genomic alterations including mutations, DNA copy number changes, rearrangements, and gene fusions

[9–16]. The most common alterations in PCa are fusions of ERG and other members of the ETS family of transcription factors to genes with androgen-regulated promoters. Between 40% and 50% prostate tumor foci harbor TMPRSS2-ERG fusion [17, 18]. In a population-based cohort, this fusion was associated with PCa-specific death, suggesting a more aggressive phenotype in untreated PCa [19]. However, patients with fusion-bearing tumors do not appear to have a significantly different prognosis than those without, following prostatectomy [20, 21]. PCa also shows different degrees of DNA copy number alterations. Indolent and low-Gleason tumors are characterized by few alterations, while more aggressive primary and metastatic tumors have an extensive burden of alterations genome-wide [14, 22, 23]. In contrast, somatic point mutations are less common in PCa. The most frequently mutated genes in primary PCa are SPOP, TP53, FOXA1, and PTEN [10]. Next-generation sequencing studies have provided evidence for distinct molecular subtypes of PCa categorized by specific alterations such as CHD1 deletions, ERG rearrangements, and SPOP mutations [9–11, 14, 24–26]. Recently, as part of The Cancer Genome Atlas (TCGA), a comprehensive molecular analysis of primary PCa was performed, integrating data from exome sequencing with genome-wide DNA copy number, DNA methylation, mRNA and microRNA expression, and targeted protein profiling [26]. The results revealed a molecular taxonomy in which 74% of these tumors fell into one of seven subtypes defined by specific gene fusions (ERG, ETV1, ETV4, FLI1) or mutations (SPOP, FOXA1, IDH1). Extensive molecular diversity at the level of copy number alterations, gene expression, and DNA methylation was observed within and between the subtypes. The AR activity of these tumors also varied widely and in a subtype-specific manner with SPOP and FOXA1 mutant tumors having the highest levels of an AR transcriptional signature. Although the current therapeutic strategies for PCa are focused on targeting the AR, the study showed that 25% of PCa harbors alterations in PI3K or MAPK signaling pathways, while 19% of them are characterized by inactivation of DNA repair genes. These observations revealed new and actionable levels of molecular heterogeneity among primary PCa and supported the possibility of novel therapeutic opportunities [7]. In metastatic PCa, genomic studies demonstrated additional alterations in AR [27] and in the androgen signaling pathway [28–30]. Genomic alterations were also identified in PIK3CA/B, R-spondin, BRAF/RAF1, APC, beta-catenin, and ZBTB16/PLZF. Moreover, aberrations of BRCA2, BRCA1, and ATM genes were observed at substantially higher frequency compared to those in primary PCa [25]. Gene expression analysis of primary PCa has been largely utilized to predict clinical phenotypes and behaviors [14, 26, 31]. In 2011, Penney et al. [31] identified a molecular signature of Gleason grade that improves the prediction of lethal disease among men with moderate Gleason 7 tumors, the most common grade, and the most indeterminate in terms of prognosis. The signature has clinical implications by further estimating the risk of lethal PCa and thereby guiding therapy decisions to improve outcomes and reduce overtreatment [31].

Proteomic approaches have also been used to stratify PCa patients and identify novel targets [32]. Recently, Shipitsin et al. utilized a quantitative proteomic approach and identified 12 biomarkers that predict PCa aggressiveness and lethal

outcome robustly in both high- and low-Gleason areas of the tumor, thus developing a sampling error-resistant clinical biopsy test for prediction of PCa aggressiveness [33]. For a detailed discussion on proteomic techniques and their potential application in PCa, please refer to Chap. 14.

However, despite their critical importance, genomic, transcriptional, and proteomic studies cannot provide a direct assessment of the tumor biochemical activity, or of the influence of environment, which more closely reflects the actual tumor phenotype. On the contrary, the metabolome offers a window to interrogate how biochemistry relates to cellular phenotype. In this sense, metabolomic profiles represent the integration of genetic regulation, enzyme activity, and metabolic reactions in a dynamic profile of the biological state of a tissue. Furthermore, because the total complement of metabolites is likely to be considerably smaller than the number of genes, transcripts, or proteins, metabolomics may be able to more clearly characterize altered cellular networks and activity associated with disease states. The development of innovative high-throughput metabolomic platforms thus allows the identification and quantification of new specific and sensitive biomarkers for PCa detection, stratification, and treatment, as discussed below.

Why Metabolomics?

The application of genomic analysis has contributed enormously to the developments in precision medicine, an emerging approach for disease treatment and prevention that takes into account individual variability in genes, environment, and lifestyle. The publication of a reference human genome sequence [34] by the Human Genome Project represented a milestone in biomedical research and in cancer in particular. However, genomics is not sufficient to account for individual susceptibility, pathway activation, cellular context, and environment. In contrast, metabolomics provides a tool to measure biochemical activity directly by monitoring the substrates and products transformed during cellular metabolism while at the same time capturing “exposome” factors, which include but are not limited to diet, lifestyle, and prescription drugs [35]. This is particularly important for PCa since diet, lifestyle, and metabolic diseases are known to affect its development and progression [36, 37]. Although data associating obesity and PCa risk have been inconclusive, obesity has been definitely linked to the risk of developing an aggressive disease [38–41]. Metabolic syndrome, a metabolic dysregulation state characterized by hyperglycemia, insulin resistance, hypertension, and predisposition to type II diabetes, has been also associated with increased risk of PCa [42]. In this context, metformin, a biguanide utilized as mainstream therapy for type II diabetes for its insulin-sensitizing effects, has shown antitumor properties in preclinical models [43], and its exposure after PCa diagnosis has been recently associated with decreases in both all-cause and PCa-specific mortality among diabetic men [44]. However, the mechanism of action responsible for metformin’s antitumor effects is pleiotropic and not completely

understood and has been only in part attributed to the activation of the energy-sensing serine/threonine kinase AMPK [45]. Thus, a full appreciation of the metabolic alterations and the perturbation of cancer metabolome by drugs, the tumor microenvironment, and other external factors like diet is of paramount importance, particularly if analyzed alongside other high-throughput analyses, such as gene expression profiling and mutational status of neoplastic cells. The integration of metabolomics with other "omics" provides a system biology approach to the discovery of clinically relevant cancer biomarkers and pathways and may enable us to develop new approaches in medicine that will be predictive, preventive, and personalized.

Akin to the Human Genome Project, the Human Metabolome Project was launched in 2004 as an inventory of 2500 small molecules described in human tissues and biofluids [46]. The publication of the third version of the Human Metabolome Database (HMDB) [47] and following updates include a vast and freely available electronic database containing quantitative physical, chemical, clinical, and biological data on 41,933 experimentally detected and biologically expected human metabolites (version 3.6).

While genomic and proteomic studies focus on molecules that are chemically similar or at least comparable, metabolomics deals with structurally heterogeneous and physicochemically different molecules, including lipids, oligopeptides, nucleotides, amino acids, sugars, and metabolic intermediates.

The enormous chemical and structural variability of the metabolites together with the difference in concentration (the range of concentration can span up to nine orders of magnitude [48]) is the biggest challenge in metabolomics. Metabolomics is driven by a continuous development of technology as well as new methods of data analysis [49, 50]. More sophisticated instruments with higher sensitivity, specificity, and reproducibility have been essential for the remarkable discoveries obtained in cancer research. Nowadays, different techniques are available to investigate the metabolome in untargeted or targeted approaches.

Techniques Commonly Used in Metabolite Profiling

Metabolomics has developed with an exponential speed over the last years as confirmed by the increasing number of scientific publications. The innovation is driven by the development of more sensitive and robust analytical instrumentation and new methods of data analysis. The platforms that have been developed to profile metabolites in biological samples include nuclear magnetic resonance (NMR) spectrometry, high-performance liquid chromatography (HPLC), mass spectrometry (MS), and Raman spectroscopy. Due to the chemical complexity of the overall ensemble of metabolites, no single analytical technique can provide a comprehensive coverage of the entire metabolome. NMR spectroscopy and MS are recognized as the most powerful techniques used for the high-throughput investigation of the metabolome, with specific advantages and limitations (Table 15.1).

Table 15.1 Advantages and limitation for NMR and MS

	NMR	MS
Analytic reproducibility	Very high	Low
Sample preparation	Minimal	Extensive
Sensitivity	Low	High
Cost per sample	Low	High
In vivo measurement	Possible	No

MS represents the most sensitive technique allowing the detection and quantification of thousands of metabolites in a single-run experiment. NMR spectroscopy is able to extract information in a nondestructive manner from a range of molecules not accessible with MS, such as lipoproteins, but it suffers of limited sensitivity. Thus, NMR techniques and MS can be considered complementary approaches to investigate the metabolome.

Mass Spectrometry

Among all the techniques used to detect and quantify the metabolites, MS-based approaches represent the most sensitive. MS platform consists in a mass spectrometer that converts metabolites in ions and can pilot them with external electric and magnetic fields. Ions are subsequently separated according to their mass-to-charge (m/z) ratio and quantified in a mass analyzer. The metabolites can be identified by their mass and/or their characteristic pattern of fragmentation. Mass accuracy is essential to confirm metabolite identities in a complex mixture, although it is not sufficient [51]. Multiple compounds with different empirical formulas can share the same nominal molecular weight. For example, purine ($C_5H_4N_4$) and acetophenone (C_8H_8O) have the same nominal mass of 120 Da. Mass accuracy is usually expressed in parts per million (ppm) and indicates the deviation of the instrument from a known monoisotopic calculated mass. Thus, a putative metabolite assignment must be also confirmed by comparing the retention time and MS/MS data of a model compound to that from the feature of interest in the research sample.

Triple quadrupole (QqQ), QTrap, and ion trap mass analyzers have been largely utilized for metabolomic studies, but they may present some limitations in identifying metabolites by mass accuracy and mass resolution (the degree of separation between two adjacent ions in the mass spectrum). They offer higher sensitivity when used in selective ion-scanning modes to detect specific metabolites or metabolite classes, thus representing the gold standard for the absolute quantification of single metabolites.

With the advent of ultrahigh accuracy mass spectrometers, new possibilities for quantitative analysis of ion species opened up. There are two categories of mass spectrometers that can achieve high resolution with good accuracy (<5 ppm): the quadrupole time-of-flight (qTOF) geometry-MS and Fourier transform MS (FT-MS) including the Fourier transform ion cyclotron resonance (FT-ICR) and the Orbitrap.

Recently, utilizing an atmospheric pressure photoionization (APPI) FT-ICR MS, a mass accuracy of <0.05 ppm was achieved, allowing the detection of more than 85,000 mass spectral peaks in a single mass spectrum from an organic mixture sample [52].

MS can be coupled with chromatographic techniques such as gas chromatography (GC) or liquid chromatography (LC) to increase the resolution in the detection of the metabolites. GC-MS is the most robust and widely used technique in MS-based metabolomics to profile volatile, thermally stable, low-molecular-weight metabolites (<500 Da). Relatively nonvolatile metabolites can be derivatized with nonpolar silyl groups and separated in the gas phase at temperatures of up to 300 °C. GC-MS is very reproducible and precise, and it is a standardized technology [48] to profile organic acids, amino acids, nucleic acids, sugars, amines, and alcohols [48]. On the other hand, LC offers several advantages over GC including the possibility to analyze polar metabolites without chemical derivatization. LC-MS techniques are typically more sensitive, and they also measure masses much more accurately over a much larger mass range (from 800 to 2000 Da) than GC-MS. MS can be also coupled with flow cytometry enabling the simultaneous quantification of masses in single cells. The use of this hybrid technology (mass cytometry) allows both the quantification of metabolites [53] and the replacement of fluorophore reporters with isotopically pure heavy metal ions. With metal ions conjugated to antibodies or affinity reagents is possible the detection of up to 40 parameters [54–56].

Recent developments in MS technologies have allowed the spatial localization of metabolites in tissue samples. Selected tissue sections can be analyzed through an array of spots in which MS spectra are acquired at spatial intervals. MS spectra are plotted to form a 2D image where the colors represent the concentration of the metabolites. Different techniques have been applied to achieve this goal [57], such as matrix-assisted laser desorption/ionization (MALDI) and secondary ion mass spectrometry (SIMS). SIMS is conceptually similar to MALDI but can achieve higher spatial resolution (~500 nm) than MALDI-MS imaging (5–30 μm) and detect small molecules <500 Da, but it cannot detect peptides, proteins, and most lipids [57]. Nanostructure-initiator mass spectrometry (NIMS) [58] is a newly developed MS-based tissue imaging technique that addresses several of the issues related to SIMS and MALDI. NIMS offers several advantages including higher resolution (150 nm) and sensitivity, no sample preparation, and reduced molecule fragmentation allowing the direct mass analysis of single cells.

Nuclear Magnetic Resonance

NMR is based on the detection of electromagnetic radiation emitted by nuclei of some isotopes (e.g., ^{1}H, ^{13}C, and ^{31}P) when placed in a static magnetic field. It is a powerful approach to profile compounds less tractable by GC-MS and LC-MS, such as sugars and amine, as well as volatile and nonchemical compounds amenable to derivatization.

NMR-based metabolomics is a straightforward and high-throughput technique for the qualitative and quantitative analysis of a wide range of components [59], including low-molecular-weight metabolites, lipids, and proteins. Moreover, NMR can quantify the size and the composition of the classes of lipoproteins, e.g., high-density lipoproteins, low-density lipoproteins, and very low-density lipoproteins. The development of the high-resolution magic-angle-spinning (HR-MAS) technique has led to the measurement of metabolite concentration in intact tissues without extraction protocol requirements [60, 61].

NMR instruments can provide spatial information of molecules as well. Magnetic resonance imaging (MRI) is an imaging technique used primarily in medical settings to produce high-quality images of human organs and tissues. Its important role in the anatomic evaluation, detection, and staging of cancer is well established [62]. Other techniques, such as magnetic resonance spectroscopic imaging (MRSI), combine both spectroscopic and imaging methods to produce spatially localized spectra of tissues inside a patient. In vivo MRSI has been applied for scientific investigations and to characterize pathological states correlating anatomic imaging with metabolite concentration [63]. Localized biomolecular profiles of tissue structures in the context of cancer can be potentially used to detect lesion sites, to guide biopsy, and to assist in prognostic evaluations.

The drawback of NMR is the sensitivity, which is several orders of magnitude lower than MS. Thus, only abundant chemicals can be detected by NMR spectroscopy due to a detection limits of 0.1 mM [64]. One common approach for increasing the sensitivity is the application of a higher magnetic field. As an alternative, hyperpolarization can offer a potential strategy to overcome the sensitivity limitation allowing the development of new imaging techniques without using radioisotopes. Despite the large increase in sensitivity afforded by hyperpolarization in MRSI, positron emission tomography (PET) with the use of positron-emitting radioisotopes is still a more sensitive approach. The key advantage of hyperpolarization is that both the injected substrate and its metabolite products can be simultaneously detected, thus allowing real-time observation of multiple metabolites. Hyperpolarization techniques are very promising in preclinical studies with a large range of possible applications including metabolic fluxes [65–67] (using [1–^{13}C]-pyruvate, [2–^{13}C]-fructose, and [1–^{13}C]-glucose), cell death [68] (using [1,4–$^{13}C_2$]-fumarate), assessment of the tumor pH [69] (using [^{13}C]-bicarbonate), and the redox status [70] (using [^{13}C]-vitamin C).

Metabolic Profiling of Prostate Cancer

Metabolic Hallmarks of Normal and Neoplastic Prostate

One of the first metabolic changes observed in cancer cells was the increased oxidation of glucose to lactate even in normoxic conditions (phenomenon known as aerobic glycolysis or Warburg effect from the name of his discoverer) [71]. Although the

full oxidation of glucose in the Krebs cycle and oxidative phosphorylation is energetically more efficient, cancer cells often prefer to use aerobic glycolysis as the privileged mechanism of glucose oxidation. This is because it is rapid and provides intermediates for amino acids, lipids, nucleotides, and NADPH production, which are all necessary to support highly proliferating cancer cells [72]. Although the Warburg effect is very common in several cancers, it is not often present in PCa, except perhaps in tumors purely driven by alteration in phosphatidylinositide-3-kinase (PI3K)/Akt pathway [73]. PI3K is an upstream activator of Akt that has been implicated in the regulation of glucose uptake in non-transformed cells [74, 75] and in promoting aerobic glycolysis and glucose dependence in cancer cells [76, 77]. In most human prostate cells, the glycolytic product pyruvate, rather than being converted to lactate, enters the mitochondria and is oxidized to acetyl-CoA. Next, a two-carbon acetyl group from acetyl-CoA is transferred to the four-carbon acceptor compound (oxaloacetate) to form a six-carbon compound called citrate. In normal prostate cells, citrate cannot continue to be oxidized during Krebs cycle due to the high concentrations of zinc, which is usually present at 10–20-fold concentrations compared to other organs. Zinc inhibits the mitochondrial enzyme m-aconitase, which catalyzes the first step of the citrate oxidation (citrate to isocitrate). Thus, citrate is accumulated and secreted in the prostatic fluid. Replenishment of four-carbon intermediates in the Krebs cycle is accomplished by the uptake of aspartate and subsequent transamination of aspartate to oxaloacetate [78, 79]. However, because of inhibition of citrate oxidation truncates the Krebs cycle, citrate accumulation has dramatic energetic consequences for the cell, which generates approximately 60% less ATP from glucose oxidation. When prostate cells undergo neoplastic transformation, they lose the capacity to accumulate zinc, which leads to restoration of m-aconitase activity and citrate oxidation with consequent loss of citrate accumulation and increased generation of ATP. Thus, restoration of citrate oxidation results in a large bioenergetics gain for malignant prostate cells. An additional metabolic change associated with malignant transformation is the need for increased lipid biosynthesis for cellular proliferation, membrane formation, and intercellular signaling. This requires conversion of citrate to acetyl-CoA in the cytosol, which is a precursor for lipogenesis and cholesterogenesis, as discussed below. NMR spectroscopy has thus been used to detect lower concentration of citrate in seminal/prostatic fluid of patient with PCa [80, 81]. Low citrate concentrations were also directly measured in prostatic tissue by MRS [82] and higly correlated with PCa Gleason score when used in association with other metabolic biomarkers (e.g., choline, creatine, or spermine) by using *ex vivo* High-Resolution Magic-Angle Spinning (HR-MAS) NMR spectroscopy [83, 84] or by *in vivo* MRSI [84, 85]. Recently, hyperpolarized [1–^{13}C]-pyruvate has been used in MRSI to characterize metabolic alterations in prostate tumors [86]. The distribution of [1–^{13}C]-pyruvate and its metabolic products (i.e., lactate, alanine, and bicarbonate) was evaluated in a time range of seconds and showed elevated [1–^{13}C]-lactate/[1–^{13}C]-pyruvate ratio in regions of biopsy-proven cancer. Metabolic reprogramming also supports the invasive properties of PCa cells. In 2009, sarcosine, an N-methyl derivative of the amino acid glycine, was identified as a differential metabolite that is highly increased

during PCa progression to metastasis and can be detected noninvasively in urine. Sarcosine levels were also increased in invasive PCa cell lines relative to benign prostate epithelial cells. Knockdown of glycine-N-methyl transferase, the enzyme that generates sarcosine from glycine, attenuated PCa invasion, whereas addition of exogenous sarcosine or knockdown of the enzyme that leads to sarcosine degradation, sarcosine dehydrogenase, induced an invasive phenotype in benign prostate epithelial cells [87], suggesting sarcosine as a potentially important metabolic intermediary of cancer cell invasion and aggressiveness. Although the use of sarcosine as biomarker was criticized due to the problem of distinguishing it from alanine in GC-MS [88], Khan et al. have recently reproduced earlier findings that sarcosine in post-DRE urine sediments is a biomarker of PCa [89], that the enzymes that produce and catabolize sarcosine are dysregulated in aggressive PCa, and finally that modulation of the sarcosine metabolic pathway results in concordant modulation of PCa aggressiveness, both in vitro and in animal models.

Lipid Metabolism and De Novo Lipogenesis

Altered lipid metabolism is a common feature of both primary and advanced PCa [90]. Lipids play several important roles both as bioenergetic sources through fatty acid oxidation [37, 91] and as structural components of cell membranes [92]. The relatively low level of aerobic glycolysis and the very weakly expressed glucose transporter GLUT-1 [93] and the overexpression of beta-oxidative pathway enzymes [94, 95], such as alpha-methylacyl-CoA racemase (AMACR) and pristanoyl-CoA oxidase (ACOX3), support the hypothesis that fatty acid oxidation is a dominant bioenergetic source in primary PCa [91]. Moreover, although normal prostate cells utilize preferentially circulating dietary fatty acids, increased de novo lipogenesis and cholesterogenesis characterize the fingerprint of malignant PCa phenotype to support the synthesis of phospholipids, oncogenic signaling lipids (e.g., lysophosphatidic acid), and steroid hormones, known to fuel cancer cell pathogenicity.

Overexpression of enzymes and transcriptional factors involved in the synthesis of fatty acids is observed at early stages of the disease, and drugs targeting this process result in cell cycle arrest and apoptosis [96]. The final step in the de novo lipogenesis of fatty acids is catalyzed by fatty acid synthase (FASN), a “bona fide metabolic oncogene” [97, 98]. High levels of FASN protein and mRNA levels have been found [90, 99] associated with aggressive biological behavior [99] and androgen-independent bone metastatic disease suggesting that alterations in lipid metabolism can be involved in the progression to androgen resistance [90]. Recently, de novo lipogenesis has been shown to be essential for mitosis completion [100]. Therefore, several efforts have currently made to evaluate the potential of de novo lipogenesis as a therapeutic target [101–103]. Moreover, lipid profiling by MS-based lipidomics holds great potential as a companion diagnostic for anti-lipogenic drugs. Recently, lipid biomarkers of PCa were identified by using electrospray tandem spectrometry [104]. The expression and activity of choline kinase, the enzyme

required for the synthesis of phosphatidylethanolamine and phosphatidylcholine (the major phospholipids found in the cellular membranes), is increased in PCa and correlates with poor prognosis [105–107]. Choline kinase has oncogenic activity when overexpressed, suggesting that the synthesis of phospholipids is rate limiting for neoplastic transformation [108, 109]. Choline- and ethanolamine-containing metabolites are increased in PCa and have been positively correlated with Gleason score [110–112].

As mentioned above, unlike most of solid tumors, PCa does not show the characteristic glycolytic switch. Thus, primary PCa is not efficiently detectable with analogs of glucose like ^{18}F-fludeoxyglucose (FDG) in PET. Alternative tracers for PET imaging using lipid-based tracers or precursors such as ^{11}C-acetate and ^{11}C-choline have been successfully used in the clinic [42].

Cholesterogenesis

Similarly to de novo lipogenesis, the pathway leading to de novo synthesis of cholesterol is of paramount importance in PCa. Cholesterol is an important component of biological membranes as it modulates the fluidity of the lipid layer. Moreover, cholesterol may be used as precursors for de novo biosynthesis of androgens [113]. Primary PCa and castration-resistant tumors are characterized by an altered steroidogenesis consistent with either a promotion in the conversion of adrenal androgens to dihydrotestosterone or, in the case of CRPC tumors, de novo synthesis of androgens from cholesterol and/or progestin precursors. Adrenal androgens have been detected at significant level in both locally recurrent and metastatic castration-resistant tumors [114]. Accumulation of cholesterol has been also reported in PCa, while deregulation of mevalonate pathway has been associated with transformation [115]. Moreover, the metabolome of PCa bone metastasis shows an increased concentration of cholesterol [116].

Oncogene-Driven Metabolic Reprogramming in Prostate Cancer

Metabolism reprogramming is necessary to sustain high proliferative rates of cancer cells. Different oncogenic events can lead to specific metabolic reprogramming to support energy demand and synthesis of biomass. The overexpression of MYC and the activation of the Akt pathway are the most prevalent alterations in PCa. Enhanced PI3K/Akt signaling promotes metabolic transformation through several mechanisms, including increased nutrient uptake (e.g., overexpression of glucose and amino acid transporters at the cell membrane), increased glycolysis and lipogenesis, and enhanced protein translation through Akt-dependent mTOR activation [117–119]. The proto-oncogene MYC plays a

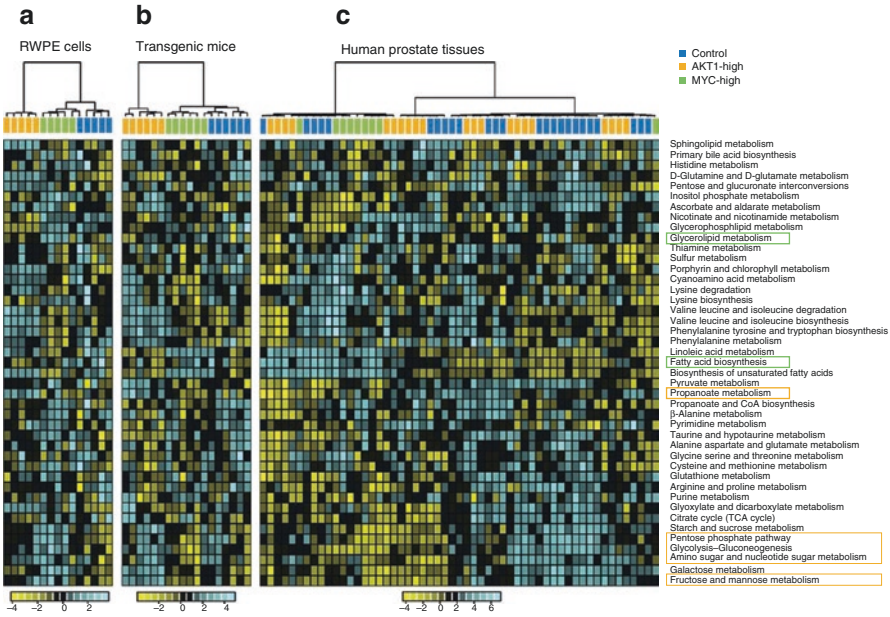

Fig. 15.1 Metabolic pathway analysis in phosphoAKT1-high or MYC-high samples relative to controls. (**a**–**c**), three different datasets (RWPE-1 cells, MPAKT and Lo-MYC mice, and human prostate tissues) of KEGG pathways are represented by heatmap. *Light blue*/*yellow* colors are used to denote high/low enrichment, as in the respective color scales. The phenotypic labels of the samples (control, phosphoAKT1-high, and MYC-high) are indicated as a *colored band* on *top* of the heatmap. From Priolo C, Pyne S, Rose J, Regan ER, Zadra G, Photopoulos C, et al. AKT1 and MYC Induce Distinctive Metabolic Fingerprints in Human Prostate Cancer. Cancer research. 2014 Oct 16. PubMed PMID: 25322691, with permission

significant role in both PCa initiation and progression. MYC protein is overexpressed at early stages of the disease [120], whereas the MYC locus is commonly amplified in advanced and recurrent disease [121]. MYC induces cell transformation and increased cell growth/proliferation by transcriptionally reprogramming central carbon and one-carbon metabolism (e.g., glutaminolysis, glycolysis, mitochondrial oxidation, synthesis of nucleotides, proteins, ribosomes, lipids, proline, etc.) [122–124]. Recently, using an integrated metabolomic approach in PCa (i.e., integration of metabolic profiling from cell, mouse, and human samples), we showed an association of Akt 1 activation with aerobic glycolysis, and of MYC overexpression with dysregulation of lipid metabolism, suggesting that PCa exhibits specific metabolic reprogramming that reflects their molecular phenotypes [73] (Fig. 15.1). Other groups confirmed MYC-induced de novo lipogenesis [125–127], which suggests the potential use of drugs targeting lipogenesis in MYC-driven PCa and ^{11}C-acetate-PET to image these tumors. Moreover, sarcosine levels were associated only with MYC-driven tumors, suggesting that it may be possible to stratify PCa on the basis of the different metabolic profiles, with implications for the development of diagnostics and targeted therapeutics. Further

studies also unraveled that the metabolic adaptation of tumors depends on both the genotype and the tissue of origin [128]. Thus, metabolomics represents an extraordinary tool to profile tumors and to guide in the choice of treatment and noninvasive imaging.

Metabolic Profiling of Formalin-Fixed Paraffin-Embedded Tissue

Metabolomic analysis of biological tissues has been conducted thus far in frozen material, which is not routinely used in diagnostic pathology. Because of the widespread use, the long-term storage as well as clinical and pathological annotation associated with formalin-fixed and paraffin-embedded (FFPE) tissues, this represents an invaluable source of biological material for interrogation in the metabolic space. FFPE material for histological analyses is stable even when stored at room temperature, but the stability of its content at the molecular level is not well understood. The preservation of antigenic sites including some phosphoproteins, DNA, and RNA is variable and may depend on pre-analytical variables such as, but not limited to, the age of the archived tissue block and storage temperature [129]. Tissues with long-term follow-up are available almost exclusively in retrospective FFPE databases [50, 98, 130, 131], which thus represent an invaluable source for extraction and quantitation of metabolic biomarkers, their validation and association with outcomes.

Frozen tissues are currently preferred in metabolomic studies because FFPE tissues are treated with formalin and solvents, such as xylene, during pre-analytical procedures associated with fixation and processing. However, it has recently been suggested that FFPE material could be utilized for metabolic pathway analysis and tumor classification [132]. In this study, MS was used to quantify the concentration of polar metabolites, following methanol extraction. The technical feasibility and reproducibility of the methodology were confirmed using target LC coupled with tandem MS (LC-MS/MS) [133]. Interestingly, we recently showed that in fact there is a significant overlap in metabolite content when matched frozen and FFPE cell lines and tissues are compared [134]. About 60% of metabolites present in frozen samples are conserved in FFPE samples, including lipids, amino acids, and nucleotides that cover most of the common metabolic pathways including lipogenesis, glycolysis, Krebs cycle, nucleotide, and amino acid metabolism. Conversely, some metabolites were not detectable in FFPE. Indeed, specific functional group present in some metabolites, such as carboxamide, can react with formalin [134]. Peptides characterized by the presence of carboxamide functional group are thus rarely detected in FFPE samples. Moreover, it has been estimated that for the same amount of tissue, the concentration of metabolites in FFPE samples is about 20 times inferior to that in the corresponding frozen material [134]. The sensitivity of the mass spectrometer is therefore crucial for the detection of metabolites at very low concentrations in FFPE samples.

Recently, the feasibility of MS-based imaging in FFPE tissues has been demonstrated on large cohorts of different tumor tissues (including breast, gastric, renal, and esophageal tumors) by using in situ MALDI-FT-ICR MS [135]. Due to a less than optimal resolution of the MALDI instruments as well as heterogeneity and multifocality of PCa, a MALDI-FT-ICR may have limited ability to delineate the tumor and normal tissue areas. The application of NIMS technology to the analysis of FFPE material could potentially solve the limitation of MALDI in term of resolution. However, NIMS technology has still limitations since it can easily destroy the tissue morphology, making histological and molecular evaluations after the imaging process difficult.

Future Directions

Metabolomics represents a remarkable tool for the investigation of alterations in the metabolic networks that depend on the genotype, tissue, and cellular context in cancer cells. In addition, metabolic profiling provides additional and nonoverlapping information regarding pathway activation and putative new enzymatic targets, underpinning oncogenic alterations. Thus, the integration with other high-throughput approaches allows a stratification of PCa with increased precision. Finally, the fact that metabolic profiling can be performed noninvasively in serum specimen opens up the possibility of inferring genomic alterations from metabolic profiling. The utilization of large retrospective and clinically well-defined FFPE sample collection will be an invaluable opportunity to discover novel diagnostic, prognostic, therapeutic, and predictive biomarkers needed to move the concept of precision medicine into everyday clinical practice.

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Chapter 16
Tumor Microenvironment: Prospects for Diagnosis and Prognosis of Prostate Cancer Based on Changes in Tumor-Adjacent Stroma

Zhenyu Jia, Chung Lee, Xiaolin Zi, Michael McClelland, and Dan Mercola

Contents

Z. Jia, Ph.D. • C. Lee, Ph.D. • D. Mercola, M.D., Ph.D. (✉)
Department of Botany and Plant Sciences, University of Califorania, Riverside, CA 92521 and Department of Pathology and Laboratory Medicine, University of California, Irvine, CA 92697-4800, USA
e-mail: dmercola@uci.edu

X. Zi, M.D., Ph.D.
Department of Urology, University of Calinfornia Irvine, Orange, CA 92868, USA

M. McClelland, Ph.D.
Department of Microbiology and Molecular Genetics, University of California Irvine, Irvine, CA 92697, USA

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*, Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_16

Introduction

Prostate cancer is the most common non-cutaneous malignancy and the third leading cause of male cancer-related death in the United States in 2016 [1, 2]. The standard procedure for a positive diagnosis of prostate cancer is the histological detection of the presence of cancer in prostate biopsy specimens. However, about 65% of initial biopsies (about 2–300,000 cases per year) are negative [3, 4] of which about 30–50% are false negative results requiring additional evaluation and biopsies [5–11]. Therefore, there is a considerable need for improved tools for guiding patients who have had a negative initial biopsy of the prostate but are considered of high clinical suspicion of having prostate cancer. Equally important is an urgent need for tests that reliably provide a risk assessment for asymptomatic but newly diagnosed prostate cancer. Numerous molecular changes in the microenvironment of prostate cancer have been recognized and some of these changes are the basis of newly available and emerging tests which may help solve these pressing clinical issues.

Stromal-Epithelial Interaction

There are complex interactions between the epithelia and the stroma components that influence all stages of development. It has been known that stroma cells play a pivotal role in the maintenance of cellular homeostasis in the prostate since the landmark discovery that urogenital mesenchyme is required for prostate organogenesis [12–16]. This homeostasis is maintained through tightly regulated signaling events of both the stroma and epithelial cells (see below). An imbalance in these signaling events will result in abnormal growth of the prostate [12–16], such as benign prostate hyperplasia and prostate cancer. In prostate cancer, the stromal cells can be morphologically altered by nearby tumor and consist of an increased population of reactive fibroblasts, myofibroblasts, and inflammatory cells [17]. These reactive fibroblasts/myofibroblasts are able to induce cancer in the adjacent epithelial cells. When human carcinoma-associated fibroblasts (CAFs) are recombined with SV40-immortalized but non-tumorigenic human prostate epithelial (BPH-1) cells and inserted into immunodeficient mice under the kidney capsule, poorly differentiated tumors result, whereas recombination of BPH-1 cells with fibroblasts from normal stroma does not lead to tumors [17, 18]. This striking result highlights the significance of stroma in prostate cancer. Besides prostate cancer, the role of stromal cells in bidirectional communication with the tumor epithelial component is common in all solid tumors [19].

Autocrine/Paracrine Factors in Stromal-Epithelial Interaction

Rowley and colleagues [20] have characterized histological changes in the stroma that accompany prostate cancer, which are collectively termed "reactive stroma." The nature of the communication between cells of stroma and tumor cells is complex but may be derived from autocrine and/or paracrine mechanisms and from the so-called "field cancerization" mechanism. Autocrine and/or paracrine factors that are secreted from one cell may affect the secreting cell (autocrine) or neighboring cells (paracrine) by receptor-mediated stimulation [21–23]. An archetypical example of a prostate stroma-derived paracrine and autocrine mechanism is the androgen dihydrotestosterone (DHT) DHT is generated by stromal fibroblasts using as substrate the 5–9 times less potent testosterone, derived from the serum. Continual production of DHT promotes the expansion of both glandular and stromal components. Other autocrine/paracrine candidates have been identified by expression analysis of isolated prostate stromal fibroblasts [24](Table 16.1). These studies compared RNA expression of stroma-derived fibroblasts to expression of CAFs [17, 18, 33, 34] (Table 16.1).

Factors derived from autocrine/paracrine mechanisms are expected to be effective only within a very short range on the order of 1 mm from the source of the factor [35, 36], although gradients of small molecules like glucose, lactate, and ATP can extend further [37]. Examples of proposed autocrine/paracrine factors are illustrated in Table 16.1. Additional factors have been reviewed [38]. Gene expression changes in stroma due to cancer cells or precancerous lesions may, therefore, be detectable within a zone of approximately 1 mm away from the lesion.

Table 16.1 Examples of paracrine factors identified in prostate

Factors	From	To	Receptor	References
TGF-β[beta]	E, S	E, S	TGF-β[beta]RI + RII + RIII	[25]
VEGF	E, S	E, S	VGFR1/2	[26]
IL-6	E, S	E, S	IL-6r/sIL-6R	[27, 28]
bFGF/FGF2	S	E	FGF-2R	[26]
HGF/SF	S	E	c-MET	[29]
IGF-1	S	E, S	IGF-1R	[30]
KGF	S	E	KGF-R	[31]
Wnt ligands	S	E	Frizzled receptors, LRP5/6	[32]

E epithelial cells of normal or PIN (prostatic intraepithelial neoplasia) or prostate cancer acini, *S* mesenchymal stromal cells

Stroma-Secreted TGF-β[beta], IGF-1, and Wnt Ligands in Prostate Homeostasis, Carcinogenesis, and Cancer Progression

Among the autocrine/paracrine factors (Table 16.1), transforming growth factor β[beta] (TGF-β[beta]), insulin-like growth factor-1 (IGF-1), and wingless (Wnt) ligands, referred to collectively as Wnt, are particularly important. TGF-β[beta] may act as a master regulator and IGF-1 and Wnt acts downstream of TGF-β[beta] signaling during tumorigenesis and metastases [39]. An imbalance in TGF-β[beta] signaling within this normal stromal-epithelial interaction will result in abnormal growth of the prostate. Perhaps, the best example is the report in which a dominant negative TGF-β[beta] type II receptor gene was specifically expressed in prostate stromal cells of transgenic mice resulting in the loss of TGF-β[beta] responsiveness in the stromal cells and the development of prostate cancer [40, 41]. These genetically altered fibroblasts produce elevated TGF-β[beta] [42] and produce an increased level of IGF-1 [43] and Wnt [44–46]. Under the combined influence of elevated TGF-β[beta], IGF-1, and Wnt, the adjacent epithelial cells may eventually become malignant. CAFs with aberrant TGF-β[beta] signaling owing to expression of a dominant negative TGF-β[beta] type II receptor can also interact with the neighboring unaltered stromal cells to participate in prostate carcinogenesis [41]. Moreover, CAFs are able to produce TGF-β[beta]1 in response to IGF-1 [47]. Such an interaction may be part of a positive feedback loop to stimulate the adjacent epithelial cells to undergo proliferation and carcinogenesis [48]. The relevance of these studies to prostate cancer is illustrated by the observation that TGF-β[beta] type II receptor is not detected in 70% of a series of 77 prostate cancer cases [49].

Field Cancerization

The term “field cancerization” refers to a process in which large areas of the tumor environment are affected at the molecular level by a carcinogenic alteration and was introduced in 1953 in a study of a multifocal oral squamous carcinoma [50]. Examples of molecular changes in tumor-adjacent tissue in colon and other cancers are well known [51]. In the case of prostate cancer, field cancerization may extend over the entire prostate gland and consist of molecular changes that precede histologic changes [52]. Examples of molecular alterations in prostate cancer in tumor-adjacent stroma include altered telomere DNA content and elevated expression of the transcription factor EGR-1 (early growth response gene) [53], PDGF-A [54], MIC-1 (macrophage inhibitory cytokine 1), SPOCK (testican), ZFP36 (tristetraprolin), FAS (fatty acid synthase [55]), and GSTP-1 (glutathione *S*-transferase) [55]. Field cancerization provides an important opportunity to identify early detection markers that may indicate the presence of tumors in patients with negative prostate biopsies. The examples cited here and other tumor-adjacent

stroma markers considered below are potential markers of diagnosis and prognosis that may be present and therefore detectable in negative biopsies.

Current Available Stroma-Based Tests

Reactive stroma has been observed in association with high-grade intraepithelial neoplasia (HGPIN) [56]. Saeter et al. [57] used an established grading system of reactive stroma and compared grade with outcome for 318 prostate cancer patients drawn from the Cancers Registry of Norway and observed a concordance index of 0.81 indicating that the grade of reactive stroma is predictive of outcome, independent of other variables.

However, most human prostate cancer is not associated with histologically evident stromal changes, although changes in gene and protein expression occur in this normal-appearing stroma [58, 59]. Gene expression changes of reactive stroma also correlate with biochemical failure following prostatectomy [60]. These observations indicate the potential to derive biomarkers for early detection, diagnosis, and prognosis from gene expression and other molecular alterations of tumor-associated stroma. Such changes should be identified in most or all samples of tumor-adjacent stroma.

Among the molecular alterations in tumor-associated stroma is methylation of CpG sites in the promoter regions of many genes [53, 61]. The extent of methylation of three genes, GSTP1 (glutathione *S*-transferase pi 1), APC (adenomatous polyposis coli), and RASSF1 (Ras association (RalGDS/AF-6)) domain family member 1, is highly correlated with the presence of tumor. These molecular correlates in tumor-adjacent stroma are thought to result from a combination of paracrine interaction between tumor and stroma and by the field cancerization effect [5, 50, 62, 63]. A high degree of methylation in tumor-associated stroma may signal the presence of tumor. The combined degree of methylation of the three genes listed above has been used to develop a test for the diagnosis of prostate cancer [64]. The test has been validated on a series of multicore human formalin-fixed and paraffin-embedded (FFPE) biopsy tissues and has a negative predictive value of 90% for the identification of biopsies with no detectable tumor. A clinical test utilizing existing clinical FFPE biopsies has been developed by MDxHealth Inc. (formerly Veridex Inc.) of Irvine CA as ConfirmMDx™. The major result provided by the test is to identify which patients with a negative initial biopsy and high clinical suspicion may not need a repeat biopsy. ConfirmMDx™ is not FDA-approved. In May 2017, the company announced the results of a retrospective study indicating that the test improved the identification of African American men at risk for aggressive cancer that was missed by a prostate biopsy [65].

The Prostate Core Mitomic Test™ of MDNA Life Sciences Inc., Pittsburgh, PA (acquired from Mitomic Inc., formerly Genomics, Inc.), also utilizes changes in the microenvironment of tumor-bearing prostates to identify the presence of tumor. In this case mitochondrial DNA of biopsy tissue is assessed for the presence or absence

of a 3.4 kDa DNA segment. The segment is deleted in the mitochondria DNA of tumor-adjacent tissue. The mechanism of this effect is thought to be through field cancerization of otherwise normal-appearing tissue [66]. Preclinical studies of human FFPE prostate biopsy specimens demonstrated negative predictive values of 92%. Thus, this test has utility for identifying true negative biopsy cases that may not require repeat biopsies [67]. The preclinical studies also revealed a sensitivity of 85% indicating moderate ability to identify men at a risk of harboring prostate cancer that was not observed in the histologically negative biopsy. The test was introduced in 2011 and is applied to FFPE tissue obtained from existing patient biopsy specimens. The Prostate Core Mitomic Test is not FDA-approved. Recently MDMA with Helomics Corp. of Pittsburgh, PA, launched a liquid biopsy form of the test for the detection of mitochondrial DNA in blood and is performed by Helomics.

Prospects for Improved Analysis of Histopathology Negative Initial Biopsies

Recent studies of prostate cancer and tumor-adjacent stroma derived from prostatectomy specimens indicate that there are hundreds of genes that show a difference in RNA expression compared to normal glandular or stromal components of normal prostate tissue [68]. As noted (Sect. "Autocrine/Paracrine Factors in Stromal-Epithelial Interaction"), there is a "halo" or zone of potentially diagnostic stroma around tumor foci [69] which, when sampled in a multicore biopsy, may indicate the presence of tumor even when the tumor epithelia is missed and not observed in the biopsy tissue. The altered expression in the effected stroma likely extends at least one and likely several mm from tumor [68]. Measurement of gene expression changes due to field cancerization may greatly extend this zone. The accuracy of the detection of tumor declines with distance from tumor and is essentially random at >13 mm [68]. Thus, the effective volume of diagnostic tissue of a biopsy is increased. In contrast, typical 12-core biopsies sample only about 0.04–0.1% of the average size prostate gland likely contributing to high false-negative rate of ~30–50% of histopathology-based analyses [5–7, 9, 10, 70].

We examined tumor-adjacent stroma from a series of patients which were microdissected in order to obtain and analyze highly enriched stroma and found over 2200 age-corrected significantly altered gene expression values compared to normal prostate tissue [68] (Table 16.2). This pool of genes was filtered to remove confounding effects, including all genes that were also expressed in epithelial cells at 10% or more of the expression observed in the stroma of tumor-bearing glands. We further employed a powerful bioinformatics technique called tenfold cross validation to identify the most reliable genes associated with the stroma of tumor-bearing glands. One hundred fourteen genes formed the final profile. In order to form a test for individual patients, the RNA measurements of all 114 genes were used to classify cases as tumor-bearing or not, together with the output of probabilities for the classification of being tumor-bearing or not, using the program PAM (Prediction

Table 16.2 Multigene classifier developed and validated for application to primary prostate tumor-adjacent stroma

Test	Output	Use	Accuracy (%)	References	Remarks
Diagnosis classifier Stroma-based diagnosis 114 gene panel	Patient probability of "presence of tumor"	Detection of presence of tumor in negative clinical biopsy	96	Jia et al. [68]	Positive result in otherwise histopathology negative biopsy may obviate need for follow-up biopsy. Negative result reinforces histopathology result
Prognosis classifier Stroma-based prognosis 15 gene panel	Patient probability of risk of tumor recurrence following prostatectomy	Apply test to stroma component of clinical diagnostic biopsy	88	Jia et al. [71]	Low risk for recurrence may indicate patient suitable for prostatectomy with intention to cure. High risk for recurrence may indicate need for adjuvant treatment such as in refs. [72–76]

Analysis for Microarrays) [77]. The test was validated by application to 364 independent stroma samples of known diagnosis. The sensitivity for detection of presence of tumor was 98% while the specificity was 88%. An overall accuracy of 96% was obtained [68].

A major potential application of these biomarkers is for the evaluation negative biopsies in order to minimize the false negative rate. Stroma from multiple cores of the original biopsy would be combined and the purified RNA used for determining the expression of the genes of the diagnostic classifier. The diagnosis of "presence of tumor" for patients with high clinical suspicion of cancer may identify patients that can avoid repeat biopsies. An attractive further prospect is to utilize the same patient RNA for a complementary test that provides a prognosis for the risk of recurrence following prostatectomy as considered below (Sect. "Prognosis Based on Tumor-Adjacent Stroma").

The assay may be extended from RNA to proteins. Six of the stroma-based biomarkers (CAV1, COL4A2, HSPB1, ITGB3, MAP1A, and MCAM) of the diagnosis classifier were tested in Chinese subjects and were validated at the RNA level, and four were also validated at the protein level on tissue microarrays of prostate cancer and normal prostate gland tissues [78].

A major challenge for further development of the diagnosis classifier is application to formalin-fixed and paraffin-embedded patient biopsy blocks and especially blocks of histologically negative biopsies from patients of high clinical suspicion based on family history and/or PSA values, imaging, and/or physical examination

findings. These studies are underway. Moreover, FFPE patient biopsy blocks that do contain tumor tissue could be used for a stroma-based prognosis of indolent or aggressive disease. Such a test would be very complementary to a tumor-based prognosis tests. Thus, there are multiple goals and potential gains of converting the highly accurate diagnostic and prognosis classifiers to utilize FFPE patient tissue.

Prognosis Based on Tumor-Adjacent Stroma

Devising the best treatment for newly diagnosed and asymptomatic prostate cancer patients poses major challenges. One challenge is the lack of reliable guidance for the risk of recurrence of prostate cancer following possible radical prostatectomy or other radical treatments. The use of biomarkers derived from tumor-adjacent stroma offers advantages over the traditional approach of using tumor tissue itself with or without combinations of clinical parameters. The DNA heterogeneity of many solid tumors, including prostate cancer [79–82], as well as the cell type heterogeneity of most prostate tumors, makes the identification of robust biomarkers or multigene profiles for prognosis very difficult and has hindered progress [82–84]. Tumor-adjacent stroma, whether exhibiting histological change or not, exhibits a much narrower range of DNA alterations [82, 85–88] indicating that gene *expression* changes are much more uniform in the tumor-adjacent stroma for prostate cancer of similar outcome. Stroma DNA alterations that do occur are being exploited to find new biomarkers [54, 89, 90]

We have used tumor-adjacent stroma of fresh frozen prostatectomy tissue of patients with known recurrence and disease-free survival periods following prostatectomy in order to develop multigene profiles [71] (Table 16.2). We sought to develop a generally applicable profile independent of the presence or grade of reactive stroma. First, 115 genes with RNA expression values that exhibit a highly significant correlation with disease-free survival were identified. Second, 131 genes (most of which are different from 115 genes aforementioned) with significantly different RNA expression values between late recurrence (>48 months) and early recurrence 1–12 months were also identified. Finally, a panel of 15 genes common to each set was utilized with PAM to create a new prognostic test. The test provides a classification of patients that are at risk of recurrence or not together with a probability ratio for the classification. When validated on a series of 47 independent patients, the test classified the cases that recurred following surgery with a sensitivity of 88.1%. The positive predictive value is 97.9% [71]. This performance is improved compared to most current nomograms and prognosis tools [91]. Moreover, the results are based on the patient's own gene activity levels [71] thereby providing a personalized result.

Determining the risk of recurrence following prostatectomy may provide important new prognostic information for patient guidance. Patients with a low- or

intermediate-risk clinical profile but with a stroma-based molecular profile indicating a high probability of recurrence following prostatectomy may not be suitable for active surveillance programs. In their review of ten trials of active surveillance, Thomsen et al. [92] found that 14–40% of patients withdraw by 5 years and 40–59% withdraw by 10 years. The most common reason was an increased Gleason grade on re-biopsy and/or increasing PSA kinetics. Mesic et al. [93] recently compared the Prostate Cancer Specific Mortality (PCSM) for low- and intermediate-risk patients on various active surveillance programs. Intermediate-risk patients, generally with Gleason 3 + 4 disease, progressed more frequently and suffered worse PCSM but by small and/or insignificant margins consistent with Thomsen et al. [92]. Carefully selected patients, with intermediate-risk disease especially low-volume Gleason 3 + 4 disease, may be candidates for active surveillance [93]. Masic et al. [93] note that multiple improvements in risk assessment based on clinical, imaging, and genomic criteria are being developed and that in combination may improve selection of Gleason 3 + 4 patients.

Patients with a low risk for recurrence following prostatectomy and otherwise favorable clinical features may elect prostatectomy with intent to cure (Table 16.2). Patients with a stroma-based molecular profile indicating a high probability of recurrence following prostatectomy have had limited treatment options until recently. The STAMPEDE trial has shown the benefits of early chemotherapy [94]. A recent meta-analysis of five trials including STAMPEDE supports the use of early docetaxel with androgen deprivation therapy (ADT) [72]. For high-risk patients that do elect prostatectomy, the use of docetaxel with ADT has also be considered for the adjuvant setting [73, 95–97]. In addition, the clinical trial by Messing et al. [98] with 98 patients compared androgen deprivation therapy (ADT) immediately following surgery to surgery alone and showed improved overall survival, improved cancer-specific survival, and improved disease-free survival at a median follow-up of 11.9 years. The treatment group was composed patients with lymph node metastases discovered at surgery. The Messing et al. [98] results are consistent with the results of a phase II chemo-ADT trial at UCI [74]. The more extensive ongoing SWOG trial (SWOG S9921) of adjuvant ADT therapy with 983 men with high-risk features at prostatectomy (such as Gleason >8, preoperative PSA) 15 ng/ml, stage > T3b, N1 disease, positive surgical margin, or Gleason = 7 with PSA > 10 ng/ml) that have been randomized to either adjuvant ADT or adjuvant ADT plus mitoxantrone chemotherapy is due to be reported in 2017 [75]. A preliminary account was provided in a poster presented at the 2017 ASCO meeting [73] which concluded that overall survival was greater than anticipated in both arms. There is no evidence that adding mitoxantrone improves PCa-specific survival when added to 2 years of adjuvant ADT and in fact the addition of mitoxantrone increased the risk of leukemia.

The improving range of options of early treatment for newly diagnosed prostate cancer with a high probability of recurrence following surgical treatment alone emphasizes the need for highly accurate tools for prognosis.

It is important to consider whether the prognostic profile we have identified contains genes that influence the fate of tumor and favor the formation of circulating tumor cells with the capacity of seeding distant sites. Although the vast majority of patients clinically classified as low risk (T1c or T2a, PSA < 10; Gleason grade < 6) have a very high probability of remaining disease-free following surgery, a significant number, ~10%, do recur [99–101]. Are these failures fated in part by abnormal gene activities of the tumor microenvironment? The contribution of the tumor microenvironment, especially reactive stroma, has been discussed in detail [45, 56, 102, 103]. These studies indicate that several stroma-based mechanisms may contribute to metastasis. Of particular interest is TGF-β[beta] (Sect. "Stromal Secreted TGF-β[beta], IGF-1, and Wnt Ligands in Prostate Homeostasis, Carcinogenesis, and Cancer Progression", above).

Analysis of the expression of genes in the microenvironment of prostate cancer may reveal many additional results important for the practice of urology and especially for understanding prostate cancer. For example, comparison of gene expression for biopsies of normal volunteers with an average age of 52 years with those of normal prostates with an average age of 84 years revealed over 8000 genes with significant age-related differences in the steady-state levels of gene expression [68]. As another example, we recently examined the differences in gene expression between prostate cancer of African Americans and European Americans [104]. In that study, pairs of African American and European American patients were carefully matched for age, tumor-cell content, and stroma-cell content. The two groups were not significantly different in the distribution of Gleason scores or stage. The analyses revealed over 700 significant gene expression differences. The majority of differences were associated with tumor-adjacent stroma rather than tumor tissue. Extracellular matrix, integrin family, and signaling mediators of the epithelial-to-mesenchymal transition (EMT) pathways were all downregulated in stroma of African Americans compared to European Americans. Using software-assisted literature analysis, we observed that 35% of significant ($p < 10–3$) pathways identified EMT and 25% identified immune response pathways such as those including interleukins −2, −4, −5, −6, −7, −10, −13, −15, and −22 ($p < 10–3$). These studies reveal that altered immune processes and decreased expression of extracellular matrix constituents are potentially new factors that may play a role in the more aggressive nature of prostate cancer in African Americans.

Challenges

The diagnostic and prognostic stroma-based profiles summarized here have high accuracy but must be converted to FFPE tissue for general and economic applicability. In addition, no test, whether tumor cell or stroma based, which has been developed to date has been validated in a prospective setting where the guidance suggested by the test was compared to the subsequent patient outcome. Prospective tests like this would demonstrate clinical utility. These challenges lie ahead for the stroma-based test described here.

Summary

- The microenvironment of prostate cancer exhibits a complex relationship with tumor that leads to alterations in histology and molecular mechanisms within the microenvironment that likely begin with the earliest changes associated with tumor formation.
- Tumor-adjacent stroma exhibits gene activity changes that signal the presence of tumor nearby. These gene activity changes, when adapted for use with clinical FFPE biopsies, may be important in detecting false-negative biopsies. Similarly, gene activity alterations associated with field cancerization in tumor-bearing glands may extend over the whole gland and therefore be present in most biopsies.
- Tumor-adjacent stroma exhibits gene activity changes which have been used to develop and validate a prognosis classifier which gives the probability of recurrence following prostatectomy.
- The microenvironment further exhibits changes associated with age. The relationship of these alterations and the response to tumor and possible influence of age-related gene activity changes on tumor formation or progression represent potentially important and largely unexplored areas of investigation.
- Gene expression differences between races (which are known to have distinct natural histories of progression) may shed new light on the mechanisms of aggressive disease and is another potential area of new research.

The microenvironment of tumors and normal glands is a new frontier of research for better understanding tumor progression and for the development of clinically useful tools.

Acknowledgments This work was supported by the NCI Early Detection Research Network (EDRN) Consortium grant U01 CA152738, the NCI Strategic Partner for the Evaluation of Cancer Signatures (SPECS) consortium U01 CA114810, and by bridging funds from the UCI Chao Family Comprehensive Cancer Center Support Grant NCI CA P3062203. This work was also supported in part by Department of Defense Congressionally Directed Medical Research Programs grants W81XWH-08–1-0720, W81XWH-11–1-0312, and W81XWH-14-PCRP-HDRA and by support from the California Women's Auxiliary to the Veterans of Foreign Wars.

Disclosures

Michael McClelland and Dan Mercola are the founders of a company, Proveri, Inc., which has an interest in biomarkers for prostate cancer, including diagnosis and prognosis. The participation of MM and DAM in this work and supporting grants has been disclosed to and approved by the UCI Conflict of Interest Committee.

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Part III
Molecular Signatures of Advanced Prostate Cancer

Chapter 17
Metastatic Prostate Cancer

Verena Sailer

Contents

Introduction

The majority of patients (81%) diagnosed with prostate cancer each year have localized disease, 12% of all patients present with lymph node metastasis (i.e., locally advanced disease), and 4% have distant metastatic disease [1]. The skeletal system is the most common site of distant metastasis (90%), followed by the lung, liver, pleura, and adrenals [2, 3]. Metastatic disease, despite its relatively low frequency at time of diagnosis, is responsible for most of the approximately 30,000 disease-related deaths each year [4]. The optimal screening protocol for distant metastasis remains as yet unclear. Some recommendations aim to identify high-risk patients taking into account PSA level and Gleason score [5]. Tumor stage, lymph node metastasis, PSA level, and Gleason score are still the most powerful predictors of aggressive disease [6, 7]. Prognostic biomarkers are emerging that may aid in risk

V. Sailer, M.D.
Department of Pathology and Laboratory Medicine, Weill Cornell Medicine, New York, NY, USA

Englander Institute for Precision Medicine, New York-Presbyterian, Weill Cornell Medicine, New York, NY, USA
e-mail: verenasailer63@gmail.com

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*, Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_17

stratification in the future [8]. The finding that metastatic disease is a monoclonal event, but intraprostatic tumor material available for biomarker analysis is very heterogeneous with diverse somatic mutations, hampers the latter approach. However, there is some evidence that the index (usually the largest) tumor focus gives rise to metastatic disease [9–11]. To identify the most prevalent clone, a biopsy from a metastatic site might be obtained, usually when the patient shows signs of castration-resistant disease. At time of initial disease recurrence, rising PSA levels after curative therapy serve as confirmation of metastatic or relapsed disease, and a biopsy is rarely pursued. This chapter will summarize the currently available knowledge about molecular alterations in untreated metastatic prostate cancer.

Lymph Node Metastasis

Clinical Significance and Mechanism of Lymphatic Spread

Lymph node metastasis in prostate cancer impacts adversely biochemical recurrence and cancer-specific survival [12]. Sacral and iliacal lymph nodes are the nodes draining the prostate and are therefore the first to be affected [13]. It is still unclear whether lymphadenectomy, either limited or extended, should be performed during radical prostatectomy. However, lymphadenectomy seems to provide a benefit for patients who present with high-risk disease as assessed by nomograms, mostly because early androgen deprivation therapy improves survival in patients with lymph node metastasis [14, 15]. Before tumor deposits in lymph nodes arise, the tumor cell has to enter the lymphatic vessel in order to be transported to the regional lymph node. This happens either through permeation or through cytokines secreted by the lymphatic vessel. Tumor-induced lymphangiogenesis can also be the cause of lymph node metastasis [16–18]. The latter is promoted via the expression of vascular endothelial growth factor (VEGF)-C and VEGF-D by tumor cells resulting in lymphangiogenesis from surrounding preexisting lymphatic vessels [19]. VEGFR-3 is the corresponding receptor on lymphatic endothelial cells but is also present on malignant prostate epithelial cells [20]. Increased expression of VEGF-C and VEGFR-3 is associated with lymph node metastasis in prostate cancer [21, 22]. Congruously, blocking both VEGF-C and VEGFR-3 is associated with reduced tumor lymphangiogenesis and subsequently a lower rate of lymph node and distant metastasis in a mouse model [23]. Lymph node metastasis also promotes tumor cell spread to distant organs [24].

Molecular research on hormone-naïve lymph node metastasis generally uses lymph node tissue resected during prostatectomy, as these patients will not routinely have received antihormonal treatment before surgery. Research using the LNCaP cell line, the most frequently studied cell line of metastatic prostate cancer, had to be excluded in this chapter in order to focus on previously

untreated metastasis. LNCaP cells derive from a supraclavicular lymph node metastasis in a patient with hormone-refractory disease [25–27].

PTEN

A variety of mutations, including genomic rearrangement and epigenetic silencing, can result in PTEN loss in prostate cancer [28]. A more aggressive phenotype is associated with PTEN deletion [29, 30]. Lymph node metastases harbor an overall high frequency of copy number alterations, including PTEN loss [31]. In a small immunohistochemical study, complete loss of PTEN expression was found in 25 (59%) of 42 lymph node metastases [32]. Mutation of PTEN also contributes to the development of lymph node metastasis in mouse models [33]. Additionally, deletion of a cell cycle regulator, CDKN1B (p27), which is influenced by PTEN, is found in 30% of lymph node metastasis [34, 35].

TMPRSS2:ERG

The prostate cancer-specific TMPRSS2-ERG gene fusion is present in lymph node metastasis. Fusion-positive lymph node metastases are found in many cases, e.g., in three studies with a frequency of 30%, 41.2%, and 56%, respectively [36–38]. In the event of multiple fusion-negative and fusion-positive cancer nodules in the prostate, metastasis is likely to originate from the fusion-positive tumor [39]. One study with 19 cases was able to demonstrate that lymph node metastases harbor the same gene fusion pattern as the index (largest) tumor nodule in the prostate [11].

MYC

Deregulation of the proto-oncogene MYC, which is located on chromosome 8, is a very frequent event in human cancer. In prostate cancer, increased MYC expression is associated with a more aggressive phenotype [40, 41]. FISH analysis shows a broad range of chromosomal aberrations from simple gain of a whole chromosome 8 to substantial amplification. The latter is present in the majority (96%) of previously untreated lymph node metastases. Interestingly, the same FISH anomaly is found in one or more foci of the primary tumor and matched lymph node metastases, underlining the concept that metastatic disease stems from a single focus [42]. Copy number alterations in form of gain at 8q (MYC) are also frequently present in lymph node metastases [31]. Immunohistochemical analysis shows an overall high expression of MYC protein in lymph node metastases [43].

TP53

Mutation of the TP53 tumor suppressor gene is a frequent event in human cancer [44, 45]. In prostate cancer TP53 mutation is a step toward tumor progression [46]. The shift toward androgen resistance can be meditated by loss of TP53 and RB1 accompanied by increased expression of the transcription factor SOX2 [47]. A TP53 knockout mouse model results in metastatic disease in almost all animals [48]. Aberrant TP53 staining is seen in a higher frequency in primary tumors and their matched lymph node metastases when compared to expression patterns in organ-confined disease [49–52]. Deletion of TP53 and a mutant PTEN pathway are found in half of metastatic samples, suggesting these mutations trigger a more aggressive phenotype [53, 54].

Androgen Receptor (AR)

The expression of the androgen receptor is significantly decreased in hormone-naïve lymph node metastases [55, 56]. Likewise, 5α[alpha]-reductase, an enzyme that converts testosterone to its biological more active form dihydrotestosterone, is completely absent in previously untreated lymph node metastases [57]. Both findings might suggest the very early initiation of a hormone-independent disease once tumor spread to lymph nodes has occurred. Loss of AR is also found in metastatic disease in a transgenic mouse model [58].

microRNA

Small non-coding RNAs (microRNA/miRNA) contribute to the regulation of cancer growth, invasion, and metastasis [59]. For instance, miRNA-205 and miRNA-203 have a tumor-suppressive function and are downregulated in a variety of human tumors, including prostate cancer [60–64]. Compared to the primary tumor, miRNA-205 and miRNA-203 are even further downregulated in lymph node metastases of hormone-naïve patients [65, 66]. Conversely, miRNA-21, which has been reported to act as an oncogene [67, 68], is upregulated in primary prostate carcinoma tissue with lymph node metastasis when compared to node-negative tumors [69].

Other Aberrations

The signal transducer and activator of transcription (STAT) family is a family of transcription factors that mediates proliferation and cell cycle progression. Particularly Stat3 has been implicated in the progression of a variety of malignant

tumors, including prostate cancer [70, 71]. Stat3 can induce a migratory phenotype and is expressed in the majority of prostate cancer lymph node metastases. Furthermore, Stat-3 contributes to the formation of lung metastasis [72]. Likewise, active Stat-5, which also contributes to the metastatic potential of prostate cancer cells, was found in 81% of lymph node metastasis in one study [73]. The most frequent non-synonymous mutation in prostate cancer is a mutation in the speckle-type POZ protein (SPOP) gene, found in approximately 12% of all prostate tumors and in 14.5% of metastases [74, 75]. Interestingly, in one patient with lymph node metastasis at time of radical prostatectomy, the index genomic alterations (PTEN, SPOP, TP53) were not present in the lymph node metastasis, but were subsequently found in several other metastatic sites [76] along with alteration in ATRX.

Bone Metastasis

The skeletal system, especially the vertebral column, is the most common site of prostate cancer spread [3]. Bone metastases cause a high morbidity due to pathological fractures, spinal cord compression, and the resulting pain, thus severely reducing quality of life in afflicted patients [77].

Metastasis Formation

Metastatic disease is preceded by a complex process that involves loss of adhesive growth in tumor cells, local invasion and invasion of blood vessels, survival in the blood stream, extravasation, and subsequent development of secondary tumors in distant organs [78]. In order to escape the primary site and become a disseminated tumor cell, the neoplastic cell has to convert from pure epithelial to a more mesenchymal phenotype, losing cell-cell adhesion and cell polarity thus acquiring the propensity to move in an extracellular matrix (ECM) that has been extensively remodeled. This epithelial-mesenchymal transition (EMT) is heavily influenced by integrins and proteases [79, 80]. For instance, overexpression of the serine protease hepsin leads to disorganization and disruption of the basement membrane of prostate glands in transgenic mice, thus contributing to progression and metastasis [81]. Matrix metalloproteinases (MMP) also play a critical role in tumor-promoting ECM remodeling. MMP-9 adds to the degradation of ECM and is regulated by Notch-1. Notch-1 is overexpressed in TRAMP mice and aggressive prostate cancer cell lines, suggesting it plays a role in invasiveness [82]. Notch-1 knockdown results in a less invasive phenotype [83]. The EMT shift in phenotype is characterized by a loss of E-cadherin expression. E-cadherin usually promotes cell-cell contacts [84, 85]. Downregulation of E-cadherin by small interfering RNA (siRNA) in vitro results in decreased tumor cell adhesion and increased tumor cell mobility [86].

In many tumors hypermethylation of the promoter region of the E-cadherin gene is frequent, albeit in prostate tumors, E-cadherin is rarely subject to mutation and rather regulated on a transcriptional level, suggesting the necessity of a reverse mesenchymal to epithelial phenotype shift—i.e., cellular plasticity—once tumor cells have settled in their preferred environment outside the prostate. Cellular plasticity is most likely induced by soluble factors stemming from stromal cells [87, 88]. Conversely, in a "cadherin switch," N-cadherin is upregulated in aggressive prostate cancer cells, adding to the production of new blood vessels [89]. There is also evidence that platelet-derived TGF-ß[beta] induces an EMT-like shift in circulating tumor cells [90].

Src kinase, a non-receptor tyrosine kinase, belongs to the Src family kinase group and was the first discovered proto-oncogene [91]. It mediates a number of signal transduction pathways and is implicated in the progression of prostate tumors and subsequent EMT, possibly through the MAPK pathway [92, 93]. Inhibition of Src kinase by dasatinib results in decreased tumor growth and lymph node metastasis in a mouse model [94].

The "seed and soil" theory in bone metastasis envisions the metastatic cancer cells as the seed that plants itself in the fertile soil of the bone microenvironment [95]. Disseminated tumor cells occupy the same niche as hematopoietic stem cells; they benefit from the provided vasculature, growth factors, and cytokines that support the self-renewal of hematopoietic stem cells [96, 97]. Tumor cells prefer bone marrow rich in adipocytes and active hematopoietic tissue and bone remodeling, like the axial skeleton or long-bone metaphyses. When young nude mice are inoculated with prostate cancer cells, they develop significantly more skeletal metastasis than their older counterparts in the same setting, thus underlining the importance of active bone remodeling in the establishment of metastatic lesions [98]. There is in vitro evidence that bone marrow adipocytes may be able to modulate growth, cytokine expression, and morphology of tumor cells and even contribute to their preferred ß[beta]-oxidation metabolic method by direct translocation of lipids from adipocytes to tumor cells [99–101]. Bone metastases from prostate cancer display both an osteoblastic and osteolytic behavior [102, 103]. It is their predilection for the skeletal system that indicates aggressive prostate cancer cells have osteomimetic properties rather than just being subjected to backward venous spread [3, 104].

RANKL

The RANK/RANKL/OPG—members of the tumor necrosis factor (TNF) family—signaling is a critical pathway in bone homeostasis and prostate cancer metastasis. Activation of the receptor activator of the nuclear factor kappa-B receptor (RANK), a transmembrane receptor located on mature and precursor osteoclasts, by its osteoblast-derived ligand RANKL results in osteoclast differentiation, cell survival, and bone resorption [105, 106]. The antagonistic osteoprotegerin (OPG) binds to

RANKL, which is in turn sequestered, thus inhibiting osteoclastogenesis and bone resorption. Tumor cells can stimulate the production of RANKL either via secretion of soluble factors like parathormone-related protein (PTHrP) or produce RANKL and OPG themselves [107].

In previously untreated skeletal metastasis from prostate cancer, a high expression of RANKL, RANK, and OPG is found with a high OPG/RANKL ratio, probably reflecting the osteoblastic character of the lesions [107]. RANKL is rendered soluble by MMP-7, as demonstrated in an animal model, thus conveying its osteolytic ability. MMP-7-deficient mice show less tumor-induced osteolysis [108]. When activated by RANKL, Src kinase is also necessary for the cytoskeletal organization of osteoclasts. It mediates the formation of the ruffled border of the osteoclasts, thus attaching them tightly to the bone and enabling resorption. Furthermore, Src kinase is implicated in osteoclast survival via the PI3K-Akt pathway. It also negatively regulates osteoblast activity [109–111]. Src kinase inhibition by dasatinib results in reduced osteoclast differentiation and activity [112]. Src kinase is a direct target of miRNA-1, the latter is regulated by AR and reduced in prostate cancer metastasis, suggesting the onset of a hormone-independent disease [113]. Preclinical data shows that targeting osteoclasts through the RANK or its downstream c-MYC/c-Met signaling network diminishes progression of skeletal metastasis without influencing visceral tumor burden, thus providing a rationale for this treatment approach in human cancer (see below) [114, 115].

Endothelin Axis

Endothelins and their receptors are a group of proteins that play a key role in maintaining vascular homeostasis. Endothelin-1 (ET-1) was first identified as a potent vasoconstrictor, mediating its function through the G-protein-coupled endothelin A and B receptors (ETA/ETB). More importantly, a number of different signaling pathways are activated as well contributing to the development and progression of cancer [116]. ETA expression increases in aggressive prostate tumors [117]. Prostate cancer patients show elevated serum levels of ET-1 [118]. ET-1 also stimulates osteoblasts and prostate cancer cells [119]. In nude mice ET-1 release from ET-1-producing tumors derived from WISH tumor cell lines resulted in significantly more new bone production than in control animals [120]. Bone formation may be due to ET-1-mediated decreased transcription of dickkopf homolog-1 (Dkk-1), a Wnt signaling inhibitor, that impairs osteoblastic activity [121]. Conversely, overexpression of Dkk-1 in a mouse model results in a decreased osteoblastic phenotype in prostate cancer bone metastases [122]. There is evidence that the paracrine signaling network involving ET-1 is unique to the skeletal system. Blocking of ETA in a mouse model leads to inhibition of tumor growth in bone metastasis, but not in visceral tumor deposits [123].

CXCR4

The chemokine receptor CXCR4 and its ligand CXCL12 (also known as stromal-derived factor-1) play a role in homing of hematopoietic stem cells to the bone microenvironment. In CXCR4 knockout mice, hematopoietic stem cells fail to engraft in the bone marrow. It has been suggested that prostate cancer cells also use this pathway to establish bone metastases [124]. Higher CXRC4 expression in prostate cancer tissue is also correlated with metastatic disease [125]. Targeting CXCR4 reduces the formation of skeletal metastasis in vivo [126]. While CXCR4 is expressed on the tumor cells, the ligand is found in abundance in bone marrow stromal cells, thus contributing to the localization of prostate tumor cells to the bone marrow. Since ERG activates CXCR4, it may also influence skeletal metastases [127].

Other Aberrations

As described previously, loss of PTEN and TP53 results in a more aggressive prostate cancer phenotype. When PTEN-/TP53-deficient prostate cancer cells are injected into mouse tibia, osteoblastic metastases occur [128].

In previously untreated skeletal metastases, miRNA-143 and miRNA-145, believed to have a function as tumor suppressors, are significantly downregulated when compared to primary and nonmetastatic tumor samples [129].

Interestingly, though intratumoral synthesis of testosterone by steroidogenic enzymes is believed to contribute to hormone-resistant disease, in a direct comparison of a small number of bone metastases from hormone-naïve and hormonally treated patients, no difference in enzyme expression was found [130].

Bisphosphonates and Denosumab in Prostate Cancer

Both androgen deprivation therapy (ADT)-related bone loss and osteolysis can be treated by bisphosphonates. Bisphosphonates share a structural similarity with pyrophosphates and bind to mineralized surfaces. They reduce osteoclast activity, thus decreasing bone resorption. Furthermore, the second generation of nitrogen-containing bisphosphonates exhibit in vitro antitumor activity through their ability to block the enzyme farnesyl diphosphatase (FFP) [131, 132].

Denosumab is a fully human monoclonal antibody with an overall low incidence of side effects that binds and degrades RANKL, thus increasing bone mass and reducing skeletal-related events (SRE) associated with ADT in patients with non-metastatic disease [133]. In metastatic disease denosumab delays the appearance of the first SRE as direct comparison with zoledronic acid in a phase III trial has shown [134]. Furthermore, denosumab delays the onset of bone metastasis in patients with

castration-resistant prostate cancer (CRPC) by 4.2 months [135]. However, due to side effects, routine treatment with denosumab in patients with high risk of bone metastasis from prostate cancer has not been recommended in current guidelines [136]. This underlines the urgent need for biomarkers to identify patients who are likely to profit from a more aggressive therapeutic approach.

Visceral Metastasis

Miscellaneous Aberrations

The most common sites for the overall rare extraskeletal spread of prostate cancer cells are the lung, liver, pleura, and adrenal glands [3]. Molecular data from hormone-naïve prostate cancer metastasis, mostly obtained from synchronous tumors at primary diagnosis, is rare. Genomic alterations found in primary tumors are also found in visceral metastasis. For instance, loss of TP53 in a knockout mouse model results in metastatic disease with lung and liver tumors [48]. Transduction of a single oncogene (c-Myc, Ha-Ras, v-SRC) results in increased lung metastasis in a mouse model [137].

In lung metastasis cell lines derived from mouse prostate cancer, TGF-ß[beta] and MMP-9 are significantly increased when compared to primary tumors [138].

Even though Ras and Raf mutations are infrequent events in prostate carcinomas, restoration of the frequently inactivated Raf kinase inhibitor protein (RKIP) results in 70% less lung metastases in mice [139].

Loss of miRNA-205, which is regulated by p63, correlates with EMT shift and is seen in aggressive prostate tumors. Conversely, miRNA-205 inhibits in vivo incidence of lung metastases in a mouse model [140].

Inflammation

Up to 20% of malignant tumors are associated with chronic inflammation, e.g., *Helicobacter pylori* infection and gastric cancer [141]. It seems likely that chronic inflammation contributes to prostate carcinogenesis as well. For instance, loss of PTEN in a prostatitis mouse model results in a trend toward invasive disease in the presence of inflammation [142]. In a prevention study involving more than 8000 men, chronic inflammation was found in nearly 80% of prostate biopsies [143]. Interleukin-6 (IL-6) has been identified as a potential link between inflammation in the prostate gland and prostate carcinogenesis. As a potent cytokine, it is involved in B-cell activation and acute-phase inflammatory response [144]. Besides association of IL-6 with prostate cancer morbidity, high levels of serum IL-6 are found in patients with metastatic prostate cancer [145]. There is strong evidence that IL-6 contributes to aggressive behavior of prostate tumors: IL-6 mediates MMP-9

upregulation through the PI3K-Akt signaling pathway, thus enabling ECM remodeling [146]. Osteoblasts can secrete IL-6, which in turn activates the androgen receptor, possibly supporting a vicious circle in bone metastases [147]. Therefore, a rationale exists for targeting IL-6: Treatment with a monoclonal anti-IL-6 antibody results in tumor growth inhibition in xenograft models. Clinical trials however have provided mixed results [148].

Proliferative inflammatory atrophy (PIA), characterized by atrophic epithelium that shows enhanced proliferation in the presence of inflammation, shows reduced expression of the tumor suppressor PTEN and NKX3.1 [149]. Conversely, PIA lesions show the same expression of m-TOR, which is involved in the PI3K-pathway, as high-grade PIN lesions [150]. However, chemopreventive trials with nonsteroidal anti-inflammatory drugs have provided inconsistent results [151].

Conclusion

Overall, the same molecular changes that are found in in primary prostate cancers are also found in hormone-naïve lymph node and visceral metastases. Due to the unique bone microenvironment, the formation of bone metastases relies on different mechanisms.

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Chapter 18
Castration-Resistant Prostate Cancer

Alastair H. Davies, Jennifer L. Bishop, and Amina Zoubeidi

Contents

The Molecular Pathology of Castration-Resistant Prostate Cancer

Androgens are the primary regulators of normal prostate as well as prostate cancer cell growth and proliferation. When testosterone enters the cell, it is converted to its active metabolite, dihydrotestosterone (DHT), by the enzyme 5α[alpha]-reductase. In turn, DHT binds the AR in the cytoplasm leading to phosphorylation, dimerization, and subsequent nuclear translocation. In the nucleus the AR associates with DNA sequence motifs known as androgen-response elements (AREs), resulting in upregulation or downregulation of target gene transcription [1]. Although androgen deprivation therapy (ADT) functions in depriving cells of androgens (usually 90–95% reduction in serum testosterone) [2], AR and AR-dependent transcriptional programs are thought to remain functional. This is the basis of castration-resistant prostate cancer (CRPC), which is the recurrence of aggressive, lethal prostate cancer in an androgen-depleted setting. Genome-wide expression analysis revealed that CRPC is more similar to hormone-naïve

A.H. Davies, Ph.D. • J.L. Bishop, Ph.D. • A. Zoubeidi, Ph.D. (✉)
Department of Urologic Sciences, Vancouver Prostate Centre, Vancouver, BC, Canada
e-mail: azoubeidi@prostatecentre.com

© Springer International Publishing AG 2018

B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*,
Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_18

primary cancers than to tumors undergoing ADT [3]. Many genes regulated by the AR that initially respond to ADT, such as FKBP5, are re-expressed in CRPC, suggesting a reactivation of the AR signalling axis under androgen-depleted conditions.

Clinicopathologic Characteristics

Men with advanced prostate cancer are typically treated with ADT, which results in tumor shrinkage. However, despite its initial response rate of 80–90%, ADT is palliative but not curative [4]. Many men experience only short-term regression, with nearly 20% of patients eventually progressing to a clinical castration-resistant state within 5 years of follow-up [5]. Compared with patients who are diagnosed with early, localized disease, the prognosis for patients with CRPC is poor, and survival is reduced. Mean survival is approximately 14 months from CRPC diagnosis [5].

The poor survival associated with CRPC is due to metastatic progression of the disease, most frequently to the bone. There is no clear temporal relationship between the emergence of metastases and the development of castration resistance—either can occur first—and this may be dependent on treatment practice. Bone metastases are present in over 84% of CRPC patients [6], and of those patients with no metastases present at diagnosis, 33% develop them within 2 years [7]. Accordingly, bone pain occurs in many patients, and fractures, spinal cord compression, and vertebral collapse are common [5]. Circulating tumor cells (CTCs), which are "seeds" for metastasis, have been accepted by the Food and Drug Administration as a prognostic tool in advanced prostate cancer. Patients with ≥5 CTC/7.5 mL of blood have a shorter overall survival (11.5 months versus 21.7 months) and higher frequency of metastatic disease [8, 9].

In addition to CTC enumeration, a number of biomarkers have been used to prognosticate CRPC patient survival, including prostate-specific antigen (PSA), lactate dehydrogenase (LDH), hemoglobin, albumin, and alkaline phosphatase [10]. A phase III clinical trial of patients receiving treatment for CRPC revealed that CTC number and LDH level are the most effective markers for discrimination between high-risk and low-risk patients. Patients with <5 CTC/7.5 mL of blood are classified as low risk, and those with ≥5 CTC with LDH >250 U/L are classified as high risk with a 2-year overall survival of 46% and 2%, respectively [9].

Identifying patients with CRPC may seem straightforward; however, it has been hindered by a lack of consensus regarding the clinical parameters for diagnosis. To address this issue, the European Association of Urology recently published a set of guidelines aimed to standardize the definition of CRPC [11], the key defining factors of which are listed in Table 18.1.

Table 18.1 Definition of castration-resistant prostate cancer

• Castrate serum levels of testosterone (<50 ng/dL or <1.7 nmol/L)
• Three consecutive rises of PSA, 1 week apart, resulting in two 50% increases over nadir with PSA >2 ng/ml
• Antiandrogen withdrawal for at least 4 weeks (flutamide) or 6 weeks (bicalutamide)
• PSA progression, despite consecutive hormone manipulation
• Progression or appearance of two or more osseous lesions on bone scan or soft tissue lesions using response evaluation criteria in solid tumors (RECIST) with nodes >2 cm in diameter

PSA prostate-specific antigen

Gene Expression Signatures of Castration-Resistant Prostate Cancer

Gene expression profiling has provided much insight into identifying molecular signatures that can define and stratify patients with CRPC. A paradigm-shifting study using microarray-based profiling of isogenic prostate cancer xenograft models reported that increased androgen receptor (AR) mRNA is consistently associated with the development of CRPC [12]. This was the first indication that castration-resistant progression remains dependent on persistent AR signalling. Since then, many studies have described in detail the transcriptional programs and pathways downstream of the AR that are active in CRPC. A transcription-based AR activity signature of 250 genes was developed that could accurately predict patients with CRPC from those with local, hormone naïve prostate cancer [13]. In addition, microarray analysis of genes co-dependent on AR and serum response factor (SRF) identified a 158-gene signature that correlated with aggressive disease, poor outcome, and biochemical recurrence [14]. As expected, a disproportionate number of genes in the signature were involved in cellular processes associated with metastasis, such as cell adhesion, actin cytoskeleton rearrangement, and cell-cell interaction.

Although AR plays a functional role in most cases of CRPC, AR signalling is quite different in CRPC from that observed in androgen-dependent prostate cancer. In contrast to androgen-dependent prostate cancer where AR drives the G1/S cell-cycle transition via regulation of cyclin D1, p21, and p27 [15], in CRPC AR selectively upregulates M-phase cell-cycle genes. These include CDC20, UBE2C, and CDK1, which together function to inactivate the M-phase checkpoint and promote cell proliferation [16]. Capitalizing on the genomic repositioning of the AR in CRPC, David Neal and colleagues curated a signature of 16 AR-regulated genes that increased in CRPC patient tissue, was downregulated by castration, and reemerged after the transition to CRPC [17]. This gene signature could be used to make prognosis or monitor the progression of CRPC and, notably, was better able to identify CRPC than the larger AR expression signatures. Intriguingly, CRPC-specific AR-binding sites do not overlap with motifs for common AR cofactors such as FOXA1 but instead are enriched for STAT, MYC, and E2F motifs [17], suggesting that altered signalling in CRPC tissue reprograms the AR.

It has recently emerged that transcript levels of a few selected genes isolated in blood samples from prostate cancer patients can accurately identify and predict the severity of CRPC. The LPD1 expression signature—whose nine signature genes include HMBS, TMCC2, SNCA, SLC4A1, STOM, GABRARAPL2, TERF2IP, RIOK3, and TFDP1—was derived by analyzing mRNA expression data in whole-blood samples from men with metastatic CRPC compared to those with clinically indolent cancer [18]. The signature was associated with known prognostic markers of CRPC, such as elevated PSA and CTCs, and overall survival was significantly lower for men who tested positive for LPD1 than those who tested negative (9.2 months versus 21.6 months). Using a similar blood-based RNA expression profiling strategy, a six-gene signature consisting of ABL2, SEMA4D, ITGAL, C1QA, TIMP1, and CDKN1A could stratify men with low-risk and high-risk CRPC (7.8 months versus >34.9 months survival) [19]. Interestingly, many of the genes in the above signatures have a role in B cell and T cell function suggesting that poor prognosis could be related to diminished immune response.

The Genetic Landscape of Castration-Resistant Prostate Cancer

Characterization of the prostate cancer transcriptome and genome has identified chromosomal rearrangements and copy number changes that initiate progression to CRPC, most notably AR mutation and/or amplification, PTEN loss, and ETS gene family fusions [20]. Although the overall mutation rates are low in CRPC (~2.00 per megabase), genes that are recurrently mutated include TP53, BRCA2, AR, ZFHX3, RB1, PTEN, and APC (see Fig. 18.1) [21, 22]. Of these, AR mutations are very rare in

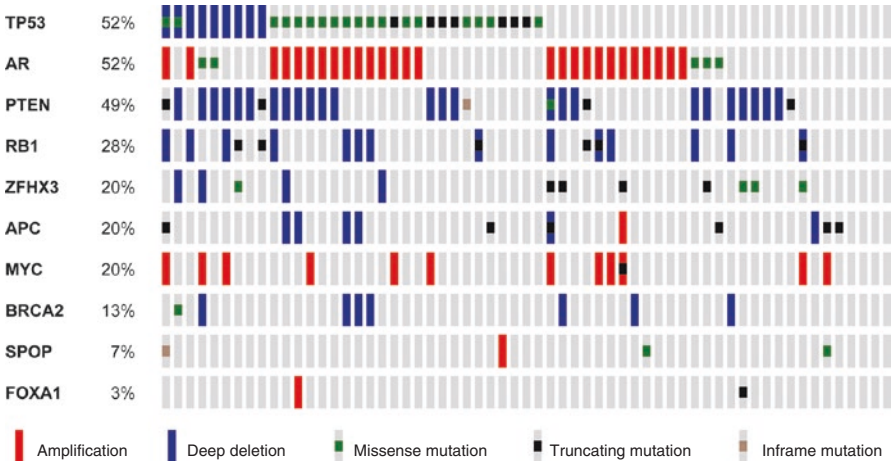

Fig. 18.1 Mutation and copy number changes in CRPC. Genome-wide genetic aberrations from 61 high-grade prostate cancer samples (represented by *gray squares*), including 50 metastatic CRPCs, were visualized using cBioPortal for Cancer Genomics [129, 130]. Genes are ranked by frequency of genomic alteration

early-stage untreated prostate cancer but are readily detected in CRPC; 10–30% of CRPCs harbor AR mutations, and 22–73% exhibit high-level amplification of the gene [23, 24].

Apart from the AR, CRPC driver mutations are clustered in key genes that confer enhanced proliferation and survival properties. The long arm of chromosome 10 (10q23), which contains the PTEN tumor-suppressor gene, is one of the most frequently deleted chromosomal regions in advanced prostate cancer; upward of 40% of CRPCs exhibit complete loss of PTEN via deletion or frameshift mutation [25]. This yields uninhibited activation of the AKT pathway, which is associated with cell survival, proliferation, and invasiveness. In addition to PTEN inactivation, loss of the retinoblastoma tumor-suppressor protein (RB) has been identified as a predominant compensatory mechanism for tumor maintenance under low-androgen condition. Relative to localized prostate cancer, RB expression is dramatically attenuated in CRPC, with both allelic deletion and methylation contributing to RB inactivation [26, 27]. These data are consistent with CRPCs clustering with a gene signature that is characterized by RB loss [28]. Mechanistically, loss of RB upregulates AR expression via the transcription factor E2F1 and increases recruitment of AR to the promoters of AR target genes associated with cell-cycle control. RB and/or PTEN loss in androgen-sensitive prostate cancer cells is sufficient to attenuate ADT and confer castration-resistant tumor growth, cementing them as key drivers of CRPC [28, 29].

Mutations in the DNA repair pathway occur with high frequency in CRPC and are largely associated with increased susceptibility to disease formation. The highest rate of mutation is located in the BRCA2 gene (12%), with mutations also identified in BRCA1 and ATM (8%) [25]. BRCA mutation carriers have an increased risk of developing prostate cancer, which presents with an aggressive, metastatic phenotype [30]. However, BRCA dysfunction itself is insufficient to promote carcinogenesis [31] but rather is believed to impair DNA repair thus facilitating genomic instability. This paves the way for secondary oncogenic events that lead to malignant conversion, such as TP53 deficiency or TMPRSS2-ERG fusion.

Although likely an early event in the genesis of prostate cancer, the expression of ETS gene fusions is maintained in many cases of advanced disease. In about one-third of CRPC patients, the androgen-regulated gene TMPRSS2 is fused with the ETS transcription factor family members, ERG, ETV1, or ETV4 [25]. These fusions, most commonly TMPRSS2-ERG, correlate with migratory cell phenotype, aggressive disease, and poorer prognosis [32]. In one particular study, all metastatic CRPC patient samples harbored ERG rearrangement by interstitial deletion, suggesting that it may be a requirement for progression to androgen independence [33]. However, this hypothesis was not supported by analysis of circulating tumor cells from patients with CRPC, which did not universally contain TMPRSS2-ERG [34]. This highlights the genetic basis of CRPC: there is not one defining “CRPC mutation” but a few distinct genomic alterations that can initiate disease progression.

Epigenetic Reprogramming in Castration-Resistant Prostate Cancer

Epigenetic alterations are also believed to represent important contributing factors in the genesis of CRPC. Genomic DNA from most CRPC is hypermethylated compared with benign prostate tissue [27], which functions to silence genes involved in hormone signalling, DNA repair, cell adhesion, cell-cycle control, and apoptosis. For example, glutathione S-transferase (GST), which protects cells from oxidative damage, is repressed in CRPC via DNA methylation of its CpG island-associated promoter. Methylated GST is detected in about 30% of men with CRPC and correlates with biochemical relapse and metastasis [35]. Interestingly, genes involved in the androgen biosynthesis pathway, such as CYP17A1 and HSD17B3, and the p53 signalling cascade, such as RB1 and TNFRSF10C, are particularly enriched for CpG methylation in CRPC [27]. These genes are also the target of copy number alterations and/or mutations [25, 26], suggesting that genetic aberrations and methylation work in concert to silence key tumor-suppressor pathways.

Histones are dynamic regulators of gene activity that undergo posttranslational modifications, including methylation and acetylation, to control chromatin accessibility. In particular, methylation of lysine 4 of histone H3 (H3K4) is an epigenetic mark correlated with an active transcriptional state. In CRPC the majority of genes that have H3K4 methylation near their promoter and/or enhancer also show AR binding [17, 36], highlighting the importance of the epigenome in the genomic positioning of the AR. For example, H3K4 is significantly methylated at the AR enhancer of the proto-oncogene UBE2C, which potentiates AR binding and UBE2C gene expression [16]. In addition to regulation of AR-binding dynamics, H3K4 methylation contributes to transcription of the AR gene itself. The second intron of the AR gene is associated with substantial levels of H3K4 methylation in cells adapted to androgen deprivation [37], consistent with this element functioning as an enhancer of AR gene expression and restored activity in CRPC. The establishment of unique H3K4 methylation patterns in CRPC is mediated by mutation of the H3K4 methyltransferase complex [22, 38] and/or altered activity of the AR cofactor lysine-specific demethylase 1 (LSD1) [37]. Ligand-bound AR recruits LSD1 to ARE-driven enhancers where it catalyzes the demethylation H3K4 to silence genes mediating androgen synthesis, DNA synthesis, and proliferation; however, castrate levels of androgen relive this LSD1-mediated repression [37].

Trimethylation of histone H3 on lysine 27 (H3K27) is also strongly associated with CRPC [39]. This epigenetic mark is mediated by the polycomb group protein EZH2, which acts in a large complex to silence genes involved in controlling cell identity. Typically, EZH2 expression is confined to stem/progenitor cells [40]; however, it is also found to be overexpressed in hormone-refractory, metastatic prostate cancer [41]. This could explain why CRPC cells exhibit a similar pattern of polycomb/EZH2 genomic occupancy and H3K27me3 marks as embryonic stem cells [42], indicating that developmental regulators are repressed by EZH2 in CRPC. Although the mechanism responsible for reactivation of EZH2 in CRPC is

poorly understood, transcriptional activation by ERG [43] and/or genomic loss of microRNA-101 [44], which targets EZH2, is likely responsible. In support of its role as an epigenetic driver of CRPC, a "polycomb repression signature" composed of 14 direct targets of polycomb/EZH2 correlates with prostate cancer progression, metastasis, and poor prognosis [42]. In particular, EZH2 has been shown to repress CDH1 (encoding E-cadherin) and DAB2IP, which trigger metastasis, as well as SLIT2, which promotes cell proliferation [45, 46].

Molecular Subtypes

The impressive, recent crescendo of whole-exome sequencing studies has made it possible to decipher molecular subtypes of CRPC [22, 26, 38]. The main division is based on the expression of ETS gene fusions: ETS fusion positive (35%) and ETS fusion negative (65%). These subtypes differ markedly in their gene expression and response to therapy; for example, ETS fusion-positive CRPC is associated with higher response rates to abiraterone acetate [47].

Of the ETS fusion-negative CRPCs, about 15% contain SPOP mutations, which anchor a distinct molecular subtype. In a study of 112 prostate tumors, more than 5000 somatic DNA mutations were identified with SPOP being the most frequently mutated gene in advanced-stage disease [38]. A subsequent in-depth analysis across multiple independent cohorts uncovered that all patients with SPOP mutations lacked the ETS family gene rearrangements and TP53 mutation. CHD1 deletion, which is overwhelmingly associated with ETS deletions [22], was harbored within the SPOP mutated population. Notably, tumors with SPOP mutations are enriched in PIK3CA mutations [38], suggesting they may be exquisitely sensitive to PI3K/AKT/mTOR inhibitors. In the future, expanded molecular subtyping of CRPC may illuminate molecular vulnerabilities and improve the stratification of patients in the neoadjuvant setting.

Mechanisms of Castration-Resistant Prostate Cancer

A number of adaptive mechanisms have been proposed which would allow CRPC cells to circumvent the restraint conferred by low levels of androgens (see Fig. 18.2): [1] amplification or overexpression of AR and its coactivators, which sensitizes cells to low levels of androgens; [2] AR mutation that decreases ligand specificity, thereby allowing AR signalling to be activated by nonandrogenic steroids; [3] activation of AR by nonsteroids such as growth factors and cytokines via deregulated kinase signalling pathways; and [4] complete bypass of dependence on the AR pathway. These mechanisms are not mutually exclusive but indeed work in concert during the development of CRPC, along with contribution from immune cells in the tumor microenvironment as well as cancer stem cells (CSCs).

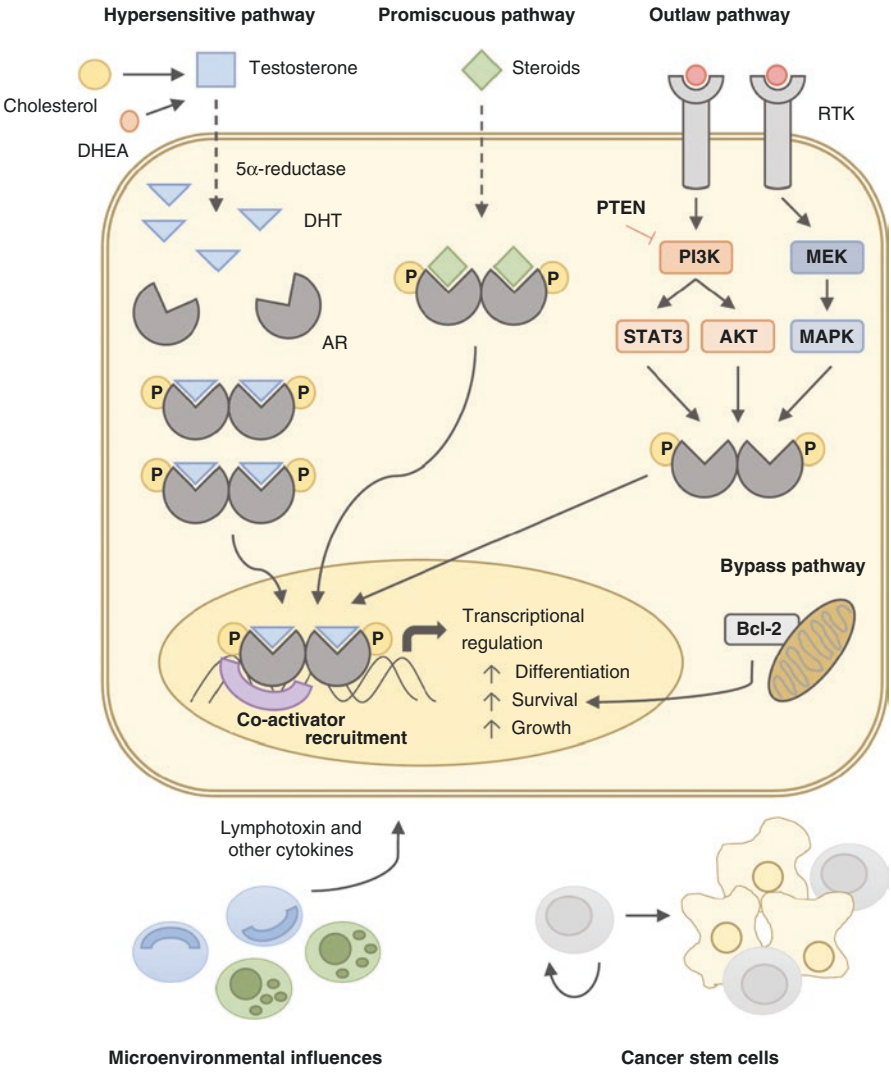

Fig. 18.2 Mechanisms of CRPC development. AR signalling can be activated via low levels of DHT (hypersensitive pathway) or nonandrogenic steroids (promiscuous pathway), while multiple signalling cascades, including PI3K and MAPK, stimulate and allow tumor cells to survive without androgens (outlaw pathway). In the absence of AR, survival can be enhanced through cell-intrinsic pathways, such as loss of PTEN or upregulation of anti-apoptotic Bcl-2 proteins (bypass pathway), as well as pro-growth signals from the microenvironment. Prostate cancer stem cells, which are not dependent on canonical androgen receptor signalling for survival, continually resupply the tumor cell population despite therapy. *AR* androgen receptor, *DHEA* dehydroepiandrosterone, *DHT* dihydrotestosterone, *RTK* receptor tyrosine kinase

Hypersensitive Pathway

One way in which CRPC cells circumvent the effects of androgen blockade is by increasing their sensitivity to very low levels of androgens. Prostate cancer cells that employ this mechanism are not, strictly speaking, androgen independent as they still depend on the activity of the AR signalling axis, but they have a lower threshold for androgens.

One potential mechanism to accomplish castration-resistant growth is through increased expression of the AR itself, leading to enhanced ligand-occupied receptor content. A gene-profiling study of isogenic pairs of androgen-sensitive and CRPC xenografts revealed that increased expression of AR is causally associated with castration resistance [12]. As previously discussed, overexpression of the AR can result from alterations in transcription factors, such E2F1 [28]; however, AR gene amplification is the most common mechanism for its overexpression in CRPC. Notably, AR amplification is significantly more prevalent in patients progressing on antiandrogen therapy than those receiving conventional chemotherapies, such a prednisone or docetaxel [48]. This suggests that AR aberrations are selected for during therapy and function to drive a resistance phenotype and CPRC progression.

Increased local production of androgens by prostate cancer cells themselves has been proposed as another mechanism for castration independence. Despite low-level serum androgens resulting from ADT, the intraprostatic concentration of DHT is usually reduced to a lesser extent than circulating testosterone (60–75% reduction) and is sufficient to maintain AR signalling [49]. These sustained levels of intratumoral DHT could result from elevated expression of enzymes converting adrenal androgens (e.g., dehydroepiandrosterone) [50] and cholesterol [51] to DHT, increased back conversion of the inactivated DHT metabolite androstanediol to DHT [52], or intratumoral de novo androgen synthesis by increased expression of enzymes involved in steroidogenesis, such as CYP17A1 [51, 53, 54]. A "back-door pathway" can serve as an alternative synthesis pathway utilizing progesterone as the primary steroidal precursor of DHT, bypassing testosterone as an intermediate altogether [55]. Therefore, the development of CRPC can be attributed to an incomplete blockade of androgen production with conventional ADT.

Promiscuous Pathway

Normally, the AR is activated only by testosterone and DHT; however, missense mutations in the ligand-binding domain can broaden this stringent specificity. As a result, CRPC cells can continue to activate the AR signalling axis and proliferate by using other circulating steroid hormones as substitute androgens.

The most common mutation of the AR in CRPC is a missense mutation in amino acid 877, which is detected in approximately 25% of CRPC patients [56]. This mutation results in the substitution of alanine for threonine at position 877 (T877A) located

in the ligand-binding domain. Molecular studies have demonstrated that hormones such as progestins, estrogens, and antiandrogens illicitly bind to this mutant AR and act as agonists [57]. In addition, a leucine-to-histidine substitution at amino acid 701 (L701H) enhances the ability of AR to bind adrenal corticosteroids, in particular cortisol and cortisone [58]. Recently, an F876L mutation in the AR has been linked to resistance to the clinically utilized second-generation antiandrogen drug enzalutamide [59]. This mutation promotes a switch from antagonist to agonist receptor function upon exposure to enzalutamide allowing for sustained proliferation during treatment.

Modulation of AR co-regulatory complexes has been shown to influence AR promiscuity by reprogramming the AR to new regions in the genome. Notably, many of the AR interacting proteins mutated in CRPC control chromatin and histone modification, including several members of the MLL complex (MLL2, MLL, ASH2L) as well as UTX and ASXL1 [22]. MLL2, which encodes a H3K4 methyltransferase to rearrange chromatin structure from a closed to open state, is most significantly mutated in nearly 10% of CRPC patients [22]. The resultant alteration in chromatin structure redistributes AR binding and promotes a new gene profile. Similarly, recurrent indel mutations in another AR collaborating factor, FOXA1, have a similar influence on AR chromatin accessibility. FOXA1 is mutated in about 3% of prostate cancers [22], which represses androgen signalling and promotes tumor growth. Finally, EZH2, which is overexpressed in metastatic CRPC [41], was found to bind and recruit AR to distinct genomic sites in CRPC [60]. Although the mechanism is not fully understood, the CRPC phenotype is likely mediated at least in part by cooperation between the AR and epigenetic modifiers.

Outlaw Pathway

Activation of AR signalling can occur independent of ligand binding. This can be accomplished through crosstalk with other signalling cascades, such as interleukin (IL)-6, growth factors (including insulin-like growth factor 1, keratinocyte growth factor, and epidermal growth factor (EGF)), human epidermal growth factor receptor 2 (HER2), and the proto-oncogene tyrosine-protein kinase Src. Indeed, IL-6 is elevated in the sera of patients with metastatic CRPC [61], and IL-6 signalling can activate the STAT3 and MAPK pathways to induce AR phosphorylation and activation [62]. Growth factors are also postulated to play a role in the regulation of AR transcriptional activity, particularly under androgen-depleted conditions. Engagement of the HER2 receptor by EGF activates the PI3K-AKT signalling pathway. Activated AKT directly phosphorylates AR at serine 213 and serine 791 to stimulate AR activity in the absence of androgens [63]. Similarly, EGF-dependent signalling activates Src kinase, and the subsequent phosphorylation of AR on tyrosine 534 is sufficient to facilitate androgen-independent growth [64].

Ligand-independent activation of AR signalling can also be achieved through alternative splicing of the AR. AR splice variants (ARVs) with a truncated, variable length ligand-binding domain are isoforms of AR that have been reported in prostate cancer cell lines, CRPC specimens, and metastatic lesions [65, 66]. To date, seven different

ARVs have been described with diverse activities ranging from constitutively active to dominate negative [67]. In particular, expression of the AR-V7 variant is increased upon antiandrogen therapy with abiraterone or enzalutamide [68]. This variant is constitutively active and its transcriptional activity is not regulated by androgens or antiandrogens. Notably, compared to the full-length AR, ARVs activate a distinct pro-proliferative transcriptional program that could confer castration resistance [68].

Bypass Pathway

The abovementioned mechanisms require the presence of the AR and its signalling cascade for the development of CRPC. However, it is also possible that complementary or alternative pathways can be invoked that are capable of bypassing the AR completely. AR activation stimulates androgen-dependent prostate cancer cells to proliferate, and depletion of androgens yields apoptosis. As such, an effective bypass of the AR signalling axis would upregulate parallel pathways that can provide a substitute survival signal, even in the absence of androgens and AR.

Blocking the apoptosis signal would be one such pathway for CRPC cell survival, with BCL2 being an obvious bypass candidate gene. BCL2 has anti-apoptotic function driven by its ability to inhibit caspase activity either by preventing the release of cytochrome c from the mitochondria and/or by sequestering apoptosis-activating factor (APAF1). It is not normally detected in the secretory epithelial cells of the prostate but is frequently overexpressed in CRPC [69]. In support of this mechanism, the emergence of BCL2 expression correlates with progression to CRPC in mouse models of prostate cancer [70].

In addition to BCL2, tumor-suppressor genes could have a similar bypass role in the development of CRPC. As previously discussed, PTEN is frequently inactivated in CRPC [25]. PTEN functions by antagonizing the PI3K-AKT-mTOR signalling axis, which functions as an alternative pathway to enhance cell proliferation and survival. As such, PTEN-null tumors are less dependent on AR signalling and, as such, are capable of proliferating under castrate conditions. The underlying mechanism for the increased cellular proliferation in the context of PTEN deficiency can be explained by unchecked AKT activation, resulting from downregulation of PHLPP, which encodes an enzyme that directly dephosphorylates AKT and protein kinase C [29]. Therefore, PTEN loss and the resultant AKT upregulation might provide alternative stimulatory signals to drive AR-independent cellular survival and growth to contribute to CRPC development.

Microenvironmental Influences

Despite the numerous cell-intrinsic pathways that endow CRPC cells with their remarkable propensity for growth and survival in androgen-depleted conditions, the interaction between the tumor and microenvironment plays an equally important

role in progression of the disease. Overall, the prostate tumor microenvironment is strongly immunosuppressive, creating a "fertile soil" for tumor growth and metastasis. There is a high degree of tolerance to prostate-specific antigens, which impedes antitumor immunity. For example, functionally suppressive CD4+ and CD8+ T regulatory cells and metabolically unresponsive T cells are found in primary prostate tumors [71] and prostate cancer islets [72]. This immunological tolerance may be linked to their expression of the T cell inhibitory checkpoint receptor programmed death-1 (PD-1), as there is a significantly increased frequency of PD-1+ prostate-infiltrating CD8+ T cells in patients with primary, hormone- and radiotherapy-naïve prostate cancer [73]. Interestingly, androgen ablation can mitigate this immunological tolerance and augment immune responses to CRPC tumors by allowing prostate-specific T cells to expand and develop effector function [74]. This is due, in part, to enhanced thymopoiesis following androgen deprivation; in turn, antigen-specific T cell effector and cytotoxic T cell functions are increased in response to prostate cancer-specific antigens [75–77]. In addition, apoptosis of prostate cancer cells following ADT has been shown to trigger an inflammatory response, leading to infiltration of regressing tumors with a myriad of immune cells, including T lymphocytes, B lymphocytes, natural killer cells, and myeloid cells [78]. Activation of IKK-β[beta] (inhibitor of nuclear factor KB kinase subunit β[beta]) in tumor-infiltrating B cells results in the production of lymphotoxin and other cytokines such as IL-6, IL-12, and TNF-α[alpha], which activate IKK-α[alpha] and STAT3 in prostate cancer cells to enable them to survive in the castrated state [78]. Notably, STAT3 is an anti-apoptotic, pro-tumorigenic transcription factor that when activated drives expression of genes central for proliferation, angiogenesis, and epithelial-to-mesenchymal transition [79].

Cancer Stem Cells

The acquisition of genetic or epigenetic alterations in prostate cancer cells or the surrounding microenvironment that promote survival in low-androgen conditions does not capture the entire complexity of CRPC progression. Both prostate cancer cell lines and patient tumors are heterogeneous with subclones of cells exhibiting varying degrees of androgen dependence even before ADT [80, 81]. Therefore, the outgrowth of pre-existing castration-resistant clones under the selective pressure of androgen deprivation likely occurs in parallel with adaptive mechanisms of resistance to drive CRPC progression.

Cancer stem cell theory proposes that cancer cell populations have a hierarchical developmental structure and a small fraction of cells, termed cancer stem cells (CSCs), can drive tumor growth and disease progression, perhaps through therapy resistance and metastasis [82]. Although not necessarily derived from normal tissue stem cells, CSCs share many similar characteristics with normal stem cells, including quiescence, expression of ATP-binding cassette transporters, common cell-surface markers and signal transduction cascades, and self-renewal capacity [83, 84].

These features could confer resistance to cancer therapy; hence, CSCs represent a plausible candidate to survive castrate conditions and reignite tumor growth.

CSCs have been identified in prostate cancer cell lines, xenografts, and patient tissue based on aldehyde dehydrogenase (ALDH) activity [85] and the combination of cell-surface markers such as CD44+, CD133+, and α[alpha]2β[beta]1hi [86]. The self-renewal capacity of CSCs in human prostate cancer has been successfully assessed by the formation of three-dimensional tumor spheroids in culture [80] as well as long-term tumor propagating capacity in mice [87]. Interestingly, all the identified subsets of putative prostate CSCs lack AR expression or have low AR activity [81, 86–88], which suggests that these cells might not be dependent on AR signalling for survival and growth. Indeed, the CSC population is expanded dramatically post-ADT both in mouse models and patient tumors [89, 90]. These cells have been shown to be capable of asymmetric cell division to regenerate a phenotypically mixed tumor, including AR- and PSA-positive cells [87]. Clearly, further studies are required to evaluate the biological characteristics and androgen dependence of prostate CSCs and their role in the genesis of CRPC.

Treatment of Castration-Resistant Prostate Cancer

An increased understanding of the molecular mechanisms that underlie CRPC has expanded the repertoire of therapeutic options (see Table 18.2). While docetaxel-based chemotherapy remains the cornerstone of CRPC treatment, a myriad of new drugs have entered the clinic that are well tolerated and significantly prolong survival in patients with CRPC. These include the taxane cabazitaxel, the CYP17 inhibitor abiraterone, the androgen receptor antagonist enzalutamide, and the vaccine sipuleucel-T. Clinical trials of targeted therapies directed against key biological mechanistic drivers of CRPC, such as metastases and cancer stem cells, are ongoing.

Table 18.2 Therapeutic agents for CRPC

Type of agent	Therapeutic agent	Mechanism of action	Clinical trial status	Therapeutic efficacy
Chemotherapy	Docetaxel	Stabilization of tubulin, induction of cell-cycle arrest, and inhibition of proliferation	FDA approved	Increase in OS (1.9–2.4 months)
	Cabazitaxel	Stabilization of tubulin, induction of cell-cycle arrest, and inhibition of proliferation	FDA approved for patients after failure of docetaxel	Increase in OS (2.4 months)

(continued)

Table 18.2 (continued)

Type of agent	Therapeutic agent	Mechanism of action	Clinical trial status	Therapeutic efficacy
AR-pathway targeting	Abiraterone acetate	Irreversible inhibition of CYP17 and subsequent androgen synthesis	FDA approved in pre- and post-docetaxel settings	Increase in OS (~4 months), radiographic progression-free survival, and time to PSA progression
	Enzalutamide (MDV3100)	AR antagonist preventing nuclear translocation and DNA binding	FDA approved in the pre- and post-docetaxel setting	Increase in OS (4.8 months), radiographic progression-free survival, and time to PSA progression
Immunotherapy	Sipuleucel-T (Provenge)	Enhancement of antigen-presenting cells to induce cytotoxic response against prostate cancer cells	FDA approved	Increase in OS but not progression-free survival
	PSA-TRICOM (PROSTVAC)	Poxviral-based PSA-targeting vaccine	Phase III in combination with GM-CSF or docetaxel	Results pending
	Ipilimumab	Monoclonal antibody that blocks CTLA4, a negative regulator of T cell activation	Phase III in combination with GVAX or PSA-TRICOM	Results pending
Bone targeting	Radium-223	Delivery of radiation to areas of high bone turnover	Phase III in comparison with placebo	Increase in OS (3.6 months) and decrease in time to first skeletal related event
CSC targeting	GDC-0449	Binds to and inhibits smoothened receptor to antagonize hedgehog signalling	Phase I/II in combination with hormone therapy	Results pending
	GSK2816126	Inhibition of EZH2	Phase I	Results pending
	JQ1	Inhibition of Myc	Phase I	Results pending

AR androgen receptor, *CSC* cancer stem cell, *CTLA-4* cytotoxic T lymphocyte associated protein 4, *FDA* US Food and Drug Administration, *GM-CSF* granulocyte macrophage colony-stimulating factor, *OS* overall survival, *PSA* prostate-specific antigen

Chemotherapy

Docetaxel-based chemotherapy is the current first-line standard-of-care treatment for patients with detectable metastatic CRPC, based largely on two pivotal trials TAX327 and SWOG 9916. In the TAX327 trial, patients treated with docetaxel plus prednisone (a corticosteroid that suppresses adrenal androgen production) demonstrated a statistically significant improvement in overall survival of 2.4 months compared with the de facto chemotherapy mitoxantrone plus prednisone [91]. A similar endpoint was achieved in the SWOG 9916 trial, which combined docetaxel with estramustine [92].

Most patients with metastatic CRPC experience disease progression during or following docetaxel therapy, and, until recently, no life-prolonging second-line treatment options were available. All this changed in 2010, when the FDA approved cabazitaxel for patients with metastatic CRPC previously treated with docetaxel. Cabazitaxel has the ability to overcome taxane resistance largely due to its low affinity for P-glycoprotein, a drug efflux pump that is overexpressed in taxane-resistant tumor cells [93]. The approval of cabazitaxel was based on data from the TROPIC study, which showed statistically significant and clinically relevant improvement in overall survival (15.1 months versus 12.7 months) in men treated with cabazitaxel plus prednisone compared with mitoxantrone plus prednisone [94].

AR-Pathway Targeting Therapy

Given that AR signalling remains active in patients with CRPC, targeting the androgen receptor axis continues to have an important role in the treatment of CRPC, with abiraterone acetate and enzalutamide being the most exciting developments. Abiraterone acetate is a highly selective irreversible inhibitor of CYP17, a critical enzyme for androgen biosynthesis in the adrenal gland and possibly also within prostate tumors [95]. In the phase III trial COU-AA-301, abiraterone acetate indicated superiority over placebo, demonstrating a 4-month gain in median overall survival from 12 to 16 months in the post-docetaxel setting [96]. Both groups also received prednisone because CYP17 inhibition has the potential to cause life-threatening adrenal insufficiency. In light of the positive results, in 2011, abiraterone acetate was approved as a second-line treatment for patients with CRPC.

A second study COU-AA-302 was designed to evaluate the effects of abiraterone acetate versus placebo in patients with asymptomatic CRPC without previous chemotherapy [97]. More deaths were observed in the prednisone arm alone than in the abiraterone acetate (34% versus 27%) prompting the recommendation that patients in the placebo arm switch to abiraterone acetate treatment. Radiographic progression-free survival was significantly better for patients who received abiraterone acetate, at a median of 16.5 months compared with 8.3 months for the placebo group. Based on the results from this trial, the use of abiraterone acetate with prednisone for treating chemotherapy-naïve CRPC was approved in 2012.

Enzalutamide (formally MDV3100) is a second-generation potent competitive inhibitor of the AR that impairs nuclear translocation and prevents DNA binding. In contrast to previous generation AR antagonists, such a bicalutamide, enzalutamide binds to the AR with greater relative affinity and has no agonistic activity at the wild-type receptor. It also induces shrinkage of CRPC tumor xenografts, whereas other conventional AR antagonists can only retard growth [98]. Enzalutamide was approved by the FDA in 2012 based on results from the AFFIRM study, which compared enzalutamide and placebo-treated patients that had progressed on docetaxel chemotherapy. Enzalutamide demonstrated a significant advantage over placebo in median overall survival of 4.8 months and all secondary endpoints, including radiographic progression-free survival and time to PSA progression [99]. The PREVAIL trial, which was set up to evaluate the benefit of enzalutamide in the pre-chemotherapy setting, revealed that enzalutamide not only delays the initiation of chemotherapy but also decreases the risk of radiographic progression and death [100]. Following this, in 2014, the FDA approved enzalutamide as a first-line therapy for use in chemotherapy naïve CRPC patients.

Immunotherapy

Although potent antiandrogen drugs improve CPRC patient survival, resistance is inevitable, leaving few other treatment options for men with this metastatic and lethal form of prostate cancer. The promise of improved survival with immunotherapy in prostate cancer is alluring; in 2014 there were over 2500 patients enrolled in global immunotherapy trials [101]. This is not surprising as prostate cancer is the only solid tumor type for which a vaccine is approved for the treatment of late-stage disease. In 2010 the FDA approved the autologous dendritic cell vaccine, sipuleucel-T (Provenge), for patients with metastatic CRPC [102, 103], and the poxviral vector-based vaccine, PSA-TRICOM (Prostvac-VF), is in late-stage clinical development [104]. Vaccines are a cornerstone of prostate cancer immunotherapies due to the well-characterized tumor associated antigens expressed uniquely by prostate tumor cells, including prostatic acid phosphatase (PAP—the target of Provenge) and PSA (the target of Prostvac-VF) [105]. These vaccines, therefore, are designed to facilitate the presentation of PAP or PSA antigenic peptides by dendritic cells to T cells in order to initiate antigen-specific killing of prostate cancer. Interestingly, although both vaccines have not shown improvements in improved time to radiographic or PSA progression in metastatic CRPC, they have resulted in significantly increased overall survival [102], leading to the approval of Provenge and Phase III clinical trials for Prostvac-VF. Importantly, retrospective analysis of the Provenge IMPACT trial showed that patients that most benefited from vaccination had baseline PSA values in the lowest quartile of those on study [106]. This lower burden of disease may allow for time for antigenic spread to occur as tumor cells are killed by vaccine-induced antigen-specific T cells, which has been documented in Provenge as well as Prostvac-VF-treated patients [107, 108], suggesting that Prostvac-VF may also prove most beneficial in patients with lower PSA.

The other major immunotherapeutic intervention for prostate cancer is the use of antibody-based therapies directed against T cell-inhibiting or "checkpoint" molecules like CTLA-4 and PD-1/PD-L1. These drugs enhance T cell antitumor responses by blocking inhibitory molecules like CTLA-4 or PD-1 on T cells from interacting with their ligands (CD80/86 or PD-L1 and 2, respectively), which are upregulated by tumors to evade T cell killing and/or by innate immune cells. While there is sound reason for the excitement over the durable responses after CTLA-4 blockade with ipilimumab and PD-1 pathway targeting agents in many cancers [109], neither have significantly improved survival in trials of CRPC patients [110–112]. However, ipilimumab treatment did show a trend to improved survival in metastatic CRPC, especially in patients with indolent disease features [111] and in an $n = 1$ report, a patient with metastatic CRPC showed a complete response after one dose of ipilimumab [113]. In addition, patients progressing after enzalutamide treatment show upregulation of PD-L1 on circulating immune cells, suggesting the presence of this immunotherapeutic target in this patient subset [114]. These more promising results suggest that choice of appropriate sequencing and combination therapies with checkpoint inhibitors may be the key to their success in improving CRPC outcomes.

At each stage of prostate cancer, from localized disease to advanced metastatic CRPC, there is strong rationale to integrate immunotherapies into the treatment landscape. Importantly, although immunotherapies were first tested in late-stage CRPC patients, results showing the most benefit from vaccines or ipilimumab in patients with low PSA or less clinically aggressive disease highlight the potential immunotherapies to alter prostate cancer progression much earlier. The many ongoing immunotherapy clinical trials now available to men with localized, castration-sensitive and nonmetastatic CRPC with checkpoint blockade or vaccination underscore this concept. In addition, the potential for synergy between standard-of-care radiation, androgen deprivation, and chemotherapy treatments with immunotherapies should be exploited and is not limited to one particular stage of prostate cancer. As such, vaccines and checkpoint inhibitors will undoubtedly play a major role in not only altering survival outcomes in prostate cancer patients but also how we study the mechanisms of prostate cancer progression.

Bone-Targeting Therapy

Patients with CRPC are particularly vulnerable to developing bone metastasis. This is associated with a significant risk of skeletal complications, such as pathologic fractures, debilitating bone pain, and spinal cord compression. Accordingly, a concerted effort has been made to identify therapeutic strategies that can prevent and/or treat prostate cancer spread to the bone. Denosumab is a human monoclonal antibody that targets the osteoblast-secreted receptor activator of nuclear factor KB ligand (RANKL) and prevents it binding to its receptor (RANK), leading to inhibition of bone loss [115]. It was the first bone-targeted agent able to delay bone

metastasis in patients with nonmetastatic CRPC by 4.2 months compared with placebo [116]. However, no difference in overall survival was found between denosumab and placebo groups.

Radiopharmaceuticals are bone-seeking agents that emit radiation or are conjugated to a radioactive emitter, enabling the preferential delivery of radiation to areas of high bone turnover. Strontium-29 and samarium-153 are FDA approved for palliation of pain caused by bone metastasis and are indicated in patients with multifocal bone metastasis [117]. Notably, radium-223 was the first radiopharmaceutical shown to improve overall survival in patients with symptomatic CRPC (14.9 months versus 11.3 months for placebo treated) [118]. Interestingly, radiopharmaceuticals are well known to increase antitumor immunity. Because of this, multiple trials have shown that combination of samarium-153 with PSA vaccines improves antigen-specific T cell responses [119] and progression-free survival [120], respectively.

Cancer Stem Cell-Targeting Therapy

The notion that CRPCs contain a rare and distinct subpopulation of CSCs that drive tumor regrowth following ADT and/or chemotherapy has gained increased acceptance in recent years [86, 90, 121]. This has led to the proliferation of novel targeted therapies aimed at key molecules and signalling pathways required to sustain CSCs. For example, GDC-0449 (Genentech) is a small-molecule inhibitor that binds to the smoothened receptor to antagonize the hedgehog signalling pathway. In preclinical studies GDC-0449 depleted the CSC population and reduced CRPC xenograft growth [122]. Similarly, inhibition of Myc, a transcription factor with a central function in CSC maintenance, was found to reduce the CSC population and suppress CRPC tumor growth and metastasis in mouse models [123]. While Myc-inhibitor design has been difficult due to the absence of a clear ligand-binding domain, BET inhibitors, such as JQ1, reduce Myc expression in prostate cancer models and have demonstrated astounding therapeutic efficacy in blocking CRPC tumor growth [124]. Finally, EZH2 represents a particularly alluring therapeutic target as it is overexpressed in prostate CSCs, which are addicted to it for growth and survival [125]. Pharmacological inhibition of EZH2 is associated with antitumor activity in mouse models of CRPC, mediated in part by eradication of the CSC population [126]. The EZH2 inhibitors GSK2816126 (GlaxoSmithKline) and E7438 (Epizyme) are currently being assessed in phase I clinical trials.

Ideal use of cancer stem cell-directed therapies will undoubtedly be in combination with other standard-of-care treatments such as antiandrogens, radiation, and chemotherapy. The rationale for combination therapy goes beyond the efficacy of each individual treatment and underscores the heterogeneity and plasticity of CRPC, which is comprised of a mixed population of AR-positive and AR-negative cells. As aforementioned, ADT has marked effects on enhancing the CSC population [89, 90]. Accordingly, targeting putative CSCs using an N-cadherin monoclonal antibody in combination with ADT markedly increased time to treatment failure [127].

In another study, eradicating the CSC population using a PI3K/mTOR inhibitor in combination with docetaxel had an enhanced antitumor effect relative to single-drug treatment [128]. These studies pave the way for designing rational combination therapies to optimize the clinical management and outcomes of patients with CRPC.

For the discussion of the highly aggressive variant of CRPC that presents with clinical features of small cell carcinoma (referred clinically to as neuroendocrine or anaplastic prostate cancer), see Chap. 19.

Acknowledgments A. Davies is a Prostate Cancer Foundation young investigator and receives additional support from the Canadian Institutes of Health Research. J. Bishop is supported by the Prostate Cancer Foundation, USA, and A. Zoubeidi by the Michael Smith Foundation for Health Research and Prostate Cancer Canada.

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Chapter 19
Neuroendocrine Prostate Cancer

Shaham Beg and Juan Miguel Mosquera

Contents

Abbreviations

AR	Androgen receptor
CRPC	Castration-resistant prostate cancer
NEPC	Neuroendocrine prostate cancer

S. Beg, M.D.
Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, New York, NY, USA

J.M. Mosquera, M.D., M.Sc. (✉)
Department of Pathology and Laboratory Medicine, New York-Presbyterian Hospital, Weill Cornell Medical College, New York, NY, USA

Englander Institute for Precision Medicine, New York-Presbyterian Hospital, Weill Cornell Medical College, New York, NY, USA
e-mail: jmm9018@med.cornell.edu

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*, Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_19

Introduction

Neuroendocrine prostate cancer (NEPC) is the clinical term used for advanced prostate cancer with clinical features of small cell neuroendocrine carcinoma. It is also referred to as anaplastic prostate cancer or small cell carcinoma of prostate, an aggressive clinical variant that can arise de novo in pure form or more commonly after hormonal therapy for usual prostatic adenocarcinoma. Incidence rate of this cancer subtype is estimated to be 0.35 cases per million per year, and more than 50% of the patients with NEPC have distant metastasis at the time disease presentation [1]. In the Surveillance, Epidemiology, and End Results (SEER) database on 3,830,355 patients diagnosed with prostatic adenocarcinoma from 1973 to 2008, approximately 10% of distant metastatic patients were diagnosed as NEPC [1]. This frequency can be much higher because of lack of diagnostic biopsy on metastatic sites and also due to increased use of androgen deprivation therapy (ADT) for treatment of advanced prostate cancer, which predisposes to neuroendocrine differentiation [2]. Moreover, age-adjusted incidence of NEPC still continues to rise as per recent statistics [3].

NEPC has poor prognosis. It is suspected in patients with treated prostatic cancer showing rapid progression of the disease with visceral metastasis, elevated neuroendocrine serum markers, AR expression loss, and no appreciable PSA elevation. Most patient have fatal outcome in very short duration.

Neuroendocrine Cells in the Normal Prostate

Normal adult prostate comprises of epithelial and stromal components. Epithelial components include secretory (luminal) cells, basal cells, and neuroendocrine (NE) cells. In addition, there are pluripotent stem cells, which cannot be seen on conventional histology section by light microscopy. NE cells are a normal component of prostate and are found in both glandular and duct epithelium, comprising less than 1% of benign prostatic cells. Periurethral and duct regions have the highest density of NE cells [4]. There are two types of NE cells, namely, open-type cells (in contact with lumen) and closed-type cells (not in contact with lumen). Open-type cells have long surface microvilli which connect with lumen and interact with luminal content, whereas closed-type cells rest on basal lamina and have dendritic like processes which extend to adjacent epithelial cells and receive stimuli from nerve endings and underlying stromal cells [5].

The precise function of NE cells in prostate is not fully understood, but they are thought to play a role in regulation, secretion, differentiation, and proliferation of secretory and basal cells. Ultrastructurally, NE cells of prostate contain large dense core secretory granules measuring 50–500 nm diameter, which are storage site for diverse neurosecretory peptides and hormones such as chromogranin A and B, neuron-specific enolase (NSE), serotonin, somatostatin, bombesin, histamine,

calcitonin, and other members of calcitonin gene family of peptides [6–10]. In clinical and research setting, NE cells are detected by using markers such as chromogranin, synaptophysin, NSE, and CD56 and showing negative expression of PSA, Ki-67, and androgen receptor (AR). Thus, they appear to be non-proliferative differentiated and androgen-independent cells.

Neuroendocrine Differentiation in Prostate Carcinoma

It is estimated that 5–10% of localized prostatic cancer show focal neuroendocrine differentiation (NED), and this differentiation proportionately increases with disease progression [11]. Acinar adenocarcinoma of prostate demonstrates NE cells by immunostains in highly variable number of cases, which depend upon number of slides studies and type of marker used [12]. Focal NED is still controversial with regard to its clinical meaning as different studies have shown conflicting data with regard to prognostic value of NED markers in surgically treated prostate cancer [13]. Acinar adenocarcinoma of prostate demonstrates NE cells by immunostains in highly variable number of cases, depending upon number of slides studies and type of marker used [12]. It is not recommended to routinely use immunostains to detect focal NED in typical early-stage prostatic adenocarcinoma [13, 14]. Focal NED cells may not be necessarily resembling normal NE cells but usually have same morphological appearance as that of usual adenocarcinoma [15]. Allelotyping of microdissected cells from prostatic carcinoma had shown NE cells and exocrine tumor cells from prostate to share identical allelic profiles and common origin [16]. This phenomenon along with other supported studies have led to the notion that adenocarcinoma cells have the capacity to transdifferentiate into NE cells and play role in tumor growth and progression. Support of a transdifferentiation model of NEPC and its clonal origin from prostate adenocarcinoma includes evidence that when prostate adenocarcinoma cells are exposed to various cytokines (IL6, IL8, heparin-binding EGF) or an androgen-depleted environment in cell culture, they are able to differentiate into neuroendocrine cells transiently and then revert back to their original phenotype when the inducer is removed [17]. Also, prostate adenocarcinoma cell lines (LNCaP) have also been shown to become “neuroendocrine like” when transfected with the gene that encodes the transcription factor (and oncogene) N-myc (MYCN), with upregulation of neuroendocrine markers and downregulation of androgen receptor and androgen-regulated genes occurring via direct binding of N-myc to promoters of synaptophysin (SYP), neuron-specific enolase (NSE), and AR [18]. As further support of a transdifferentiation model of NEPC and clonal origin from prostate adenocarcinoma, the frequency of the prostate cancer-specific ERG gene rearrangement is similar to that of prostate adenocarcinoma. Histologic evaluation of mixed tumors reveals that neuroendocrine and small cell areas and prostate adenocarcinoma can coexist and intermingle within the same tumor focus. Tumors that are ERG fusion-positive demonstrate rearrangement in both the neuroendocrine and adenocarcinoma foci (Fig. 19.1). Similarly, when ERG-positive gene

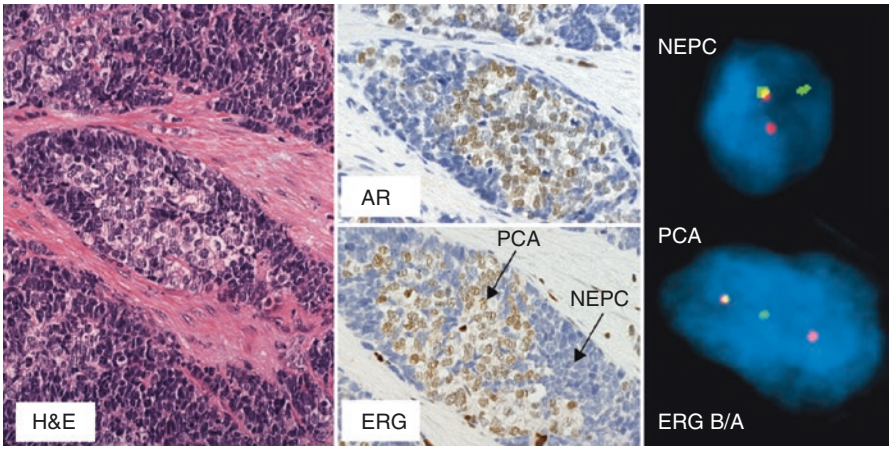

Fig. 19.1 Mixed small cell carcinoma and prostatic adenocarcinoma. H&E stain (*left*) shows a tumor with mixed small cell carcinoma and adenocarcinoma components. Immunohistochemistry (*center*) demonstrates that only adenocarcinoma cells are positive for AR and ERG, while ERG gene rearrangement by FISH (*right*) is present in both components (Reprinted with permission from Beltran H, Rickman DS, Park K, Chae SS, Sboner A, MacDonald TY, Wang Y, Sheikh KL, Terry S, Tagawa ST, et al.: Molecular characterization of neuroendocrine prostate cancer and identification of new drug targets. Cancer Discov 2011, 1:487–495)

fusion status of primary tumors is compared with that of local recurrences or metastases with neuroendocrine differentiation, rearrangement is present in both areas, i.e., acinar and neuroendocrine (Fig. 19.2).

De novo neuroendocrine prostate tumors are extremely rare cancers and composed of NE tumor cells without prior documentation of any known adenocarcinoma. Tumors that come under this category include carcinoid tumors of prostate, which have similar morphology to carcinoids at other anatomic sites, small cell carcinoma, and newly recognized entity large cell neuroendocrine carcinoma [19–21]. These tumors can present as pure form or admixed with variable component of prostatic adenocarcinoma.

The majority of studies on neuroendocrine tumors have shown that NE differentiation occur in hormone refractory prostate cancer after androgen deprivation study (ADT) [22–25]. It is considered to be the result of selective pressure caused by androgen deprivation as an escape route that enables adaptation in response to AR-targeted therapies [26–28]. Studies have shown that the NE component increases after a few months of introduction of ADT, and the diagnosis of castrate-resistant prostate carcinoma (CRPC) is made months or years after [22, 29, 30]. Androgen deprivation has been shown to activate epithelial mesenchymal transition (EMT) and neuroendocrine transdifferentiation, which are associated with resistance to treatment, tumor progression, distant metastasis, and aggressive behavior of the tumor [30]. Farach et al. recently showed that late stages of prostate cancer evolution involve neuronal transdifferentiation, which would enable cancer cells to acquire independence from the neural axis, critical in primary tumors [31]. It is estimated that at least a quarter of

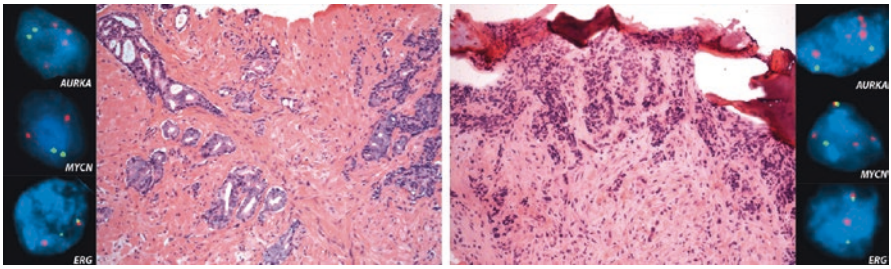

Fig. 19.2 ERG gene rearrangement and AURKA and MYCN amplification in prostate cancer. *Left panel* illustrates a needle biopsy from a patient with initial diagnosis of prostatic adenocarcinoma Gleason score 3 + 4 = 7 with amplification of AURKA (*upper inset*) but not MYCN (*middle inset*). Eight years after initial diagnosis and intermittent treatment, the patient developed pancytopenia and bone lytic lesions. The *right panel* shows a bone biopsy with metastatic small cell carcinoma (frozen tissue artifact present), consistent with spread from known prostatic primary. In addition to AURKA amplification (*upper inset*), clonal origin is confirmed by ERG rearrangement through translocation, also seen in his primary tumor (*lower insets*). The metastatic tumor demonstrates MYCN amplification (*middle inset*) (Reprinted from Neoplasia, Vol 15, Juan Miguel Mosquera et al., Concurrent AURKA and MYCN Gene Amplifications Are Harbingers of Lethal TreatmentRelated Neuroendocrine Prostate Cancer, p. 4, Copyright (2013), with permission from Elsevier)

patients with late-stage disease and aggressively treated will develop NEPC, and incidence will rise with introduction of more potent antiandrogen agents [32, 33].

Classification of Primary Neuroendocrine Prostate Tumors

A recent updated classification of primary neuroendocrine tumors of prostate has been published in the WHO tumor classification of the urinary system (Table 19.1) [12].

Adenocarcinoma with neuroendocrine differentiation is the usual prostate adenocarcinoma with scattered neuroendocrine cells indistinguishable from typical adenocarcinoma cells on routine histology section. NE cells can be identified by immunohistochemistry with neuroendocrine markers such as chromogranin, synaptophysin, NSE, and CD56. These NE cells are seen in 10–100% of typical adenocarcinoma cases depending upon number of slides studies and type of marker used [12]. These NE cells have also been seen in intraepithelial lesion [34, 35]. Both high-grade and high-stage prostatic adenocarcinoma show increased neuroendocrine differentiation [22, 23, 36]. The effect of this NE differentiation on patient outcome is controversial, and routine use of IHC markers for NE differentiation is not recommended in surgical pathology reporting [12–14].

Adenocarcinoma with Paneth cell-like neuroendocrine differentiation is characterized by presence of brightly eosinophilic cytoplasmic granules in cytologically bland neuroendocrine cells with typical nuclear features such as “salt and pepper”

Table 19.1 2016 WHO Classification of prostatic neuroendocrine tumors

• Adenocarcinoma with neuroendocrine differentiation
• Adenocarcinoma with Paneth cell-like neuroendocrine differentiation
• Well-differentiated neuroendocrine tumor (carcinoid tumor)
• Small cell neuroendocrine carcinoma
• Large cell neuroendocrine carcinoma

chromatin and absence of nucleoli. This Paneth cell-like change has been recognized for many years [37]. These granules are positive for NE markers by immunohistochemistry [38, 39]. Paneth cell-like cells may be present in well-formed glands of Gleason pattern 6 or can be found in cords and solid sheets typically defined as Gleason pattern 4 or 5 [37, 39]. It is recommended that this Paneth cell-like pattern should be excluded while determining Gleason grade pattern. In some cases, Paneth cell-like change can be seen in adjacent cells which lack cytoplasmic granules in tumor cells with deeply amphophilic cytoplasm [40]. The cells of this Paneth cell variant morphologically lack prominent nucleoli of usual adenocarcinoma cells and are diffusely positive for NE immunostains and should be excluded from Gleason grading. There is a high frequency of AURKA amplification in localized prostatic carcinoma with Paneth cell-like change, which clinical significance warrants further investigation [41].

Well-differentiated neuroendocrine tumors (carcinoid tumor) of prostate are tumors analogous to carcinoid tumors in other sites such as lung. Pure carcinoid tumors of prostate are extremely rare. These should be diagnosed only when they originate from prostatic parenchyma, are not associated with concomitant adenocarcinoma, and must be positive for NE markers and negative for PSA [14]. Usual adenocarcinoma of prostate can express NE markers and can have bland cytological appearance, but the diagnosis of carcinoid should only be rendered if such tumors are not close to usual adenocarcinoma and are negative for PSA; otherwise they should be regarded as prostate adenocarcinoma with neuroendocrine differentiation. Several carcinoid-like prostate tumors appear to be variants of Paneth cell-like NE differentiation with paucity or absence of eosinophilic granules, in which PSA may be negative [42]. If such diagnosis of pure prostatic carcinoid is rendered, then grading should be done using mitotic rate and Ki-67 proliferation index as done with these tumors at other sites. These tumors may present with locally advanced disease, including some with pelvic lymph node metastasis, but in general, they still have favorable outcome.

Small cell neuroendocrine carcinoma of prostate is high-grade primary prostate tumor with cell morphology similar to small cell carcinoma of the lung, which includes high nuclear to cytoplasmic ratio, nuclear molding, lack of prominent nucleoli, crush artifact, geographic necrosis, apoptosis, and high mitotic rate (Fig. 19.3a). Pure small cell carcinoma of prostate at initial diagnosis is seen in 50–60% of cases, and the rest of them have a component of usual prostatic adenocarcinoma [43]. Transition between acinar and small cell component is abrupt, so it is quite easy to appreciate on routine histology (Fig. 19.3b, c). Morphological variations of small cell carcinoma include intermediate cell type with slightly more

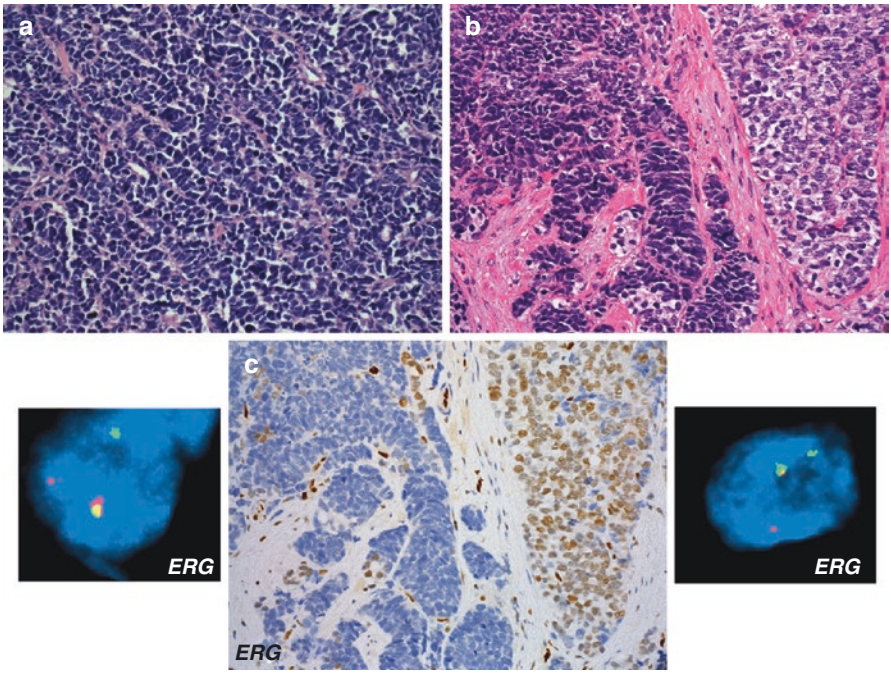

Fig. 19.3 Histologic features of small cell carcinoma. (**a**) Classic histomorphology of small cell carcinoma includes tumor cells with hyperchromatic nuclei, nuclear molding, and "crush" artifact. (**b**) Mixed small cell carcinoma—acinar adenocarcinoma. (**c**) Although ERG immunostain highlights nuclei only in the adenocarcinoma component, a FISH assay for ERG (*insets*) demonstrates break apart signal in both small cell carcinoma and acinar adenocarcinoma areas of this tumor

open chromatin and visible small nucleoli seen in 30–40% of the cases. Less common variation include presence of tumor giant cells and single file pattern [43]. Gleason grading is not done on the small cell component but in the acinar component, which shows Gleason score of >8 in 85% of the cases [43]. Classic morphology of small is quite distinct so routine use of immunostains is not necessary; approximately, 90% of the cases show immunopositivity for at least one NE marker [44, 45]. PSA and other prostate specific markers are focally positive in 17–25% of cases, usually cases in concomitant acinar component. Ki-67 labeling index is more than 50%. Positivity for p63 and HMWK is seen in 24 and 35% of cases, which are typically absent in usual adenocarcinoma [44]. TTF-1 is expressed in more than 50% of the cases, limiting its application in differentiating between primary small cell prostate carcinoma and metastasis from small cell carcinoma of the lung [43, 44, 46, 47]. Ancillary testing practical importance is the detection of prostate cancer-specific fusion between ETS family of genes and TMPRSS2, a common aberration found in small cell prostatic carcinoma (Fig. 19.3c) [48–50].

Being an aggressive tumor, approximately 60% of patients have with distant metastasis at time of presentation and less often paraneoplastic syndromes such as

those associated with ectopic adrenocorticotropic hormone, hypercalcemia, or inappropriate antidiuretic hormone production. Clinically localized small cell prostate cancer is treated aggressively with multimodal therapy including chemo- and radiation therapy, whereas metastatic small cell is treated with platinum-based combination chemotherapy [51–53]. A 2- and 5-year survival rate is 27.5% and 14.3%, respectively [54].

Large cell neuroendocrine carcinoma (LCNC) is a new entity added in prostate neuroendocrine tumors in the updated WHO 2016 classification. These are exceptionally rare high-grade tumors with cells in large nests with peripheral palisading, geographical necrosis, and cytologically resembling acinar high-grade adenocarcinoma (large cell size, abundant cytoplasm, prominent nucleoli, coarse clumpy chromatin) [55]. Neuroendocrine differentiation is supported by positivity of at least one NE marker (CD56, synaptophysin, or chromogranin) [14]. Ki-67 proliferation index is more than 50%. PSA and PSAP are either negative or focally positive in tumor areas. Pure or de novo large cell neuroendocrine tumors are exceptionally very rare tumors; the majority of cases reported are associated with prior prostate adenocarcinoma with long-standing history of hormonal therapy. For rendering diagnosis of this entity, both NE immunostains and morphological features should be considered. Most cases are associated with rapid progression, widespread dissemination, and eventually death due to metastatic disease [20, 55].

A recent proposed classification from a working committee to classify prostate cancer with neuroendocrine differentiation also includes CRPC with small cell carcinoma-like clinical presentation, which morphology is heterogeneous and includes pure or mixed small cell carcinoma, LCNC, and usual high-grade prostate adenocarcinoma with or without evidence of NE differentiation by immunohistochemistry [14, 56]. Patients with this aggressive variant of CRPC present with any combination of these manifestations: visceral metastases, lytic bone metastases, bulky lymphadenopathy, low PSA, elevated serum chromogranin, and history of hormonal therapy [33, 51, 56, 57].

Molecular Alterations in NE Differentiation in Prostate Carcinoma

In the past few years, significant amount of molecular data has emerged from genome, transcriptome, and epigenetic analysis of prostate cancer and has increased our understanding of the molecular basis of this disease. Several molecular aberrations have been implicated in this neuroendocrine development in prostate cancer progression including AR independence, AURKA amplification +/− MYCN amplification [18, 56, 58], REST downregulation or loss, TP53 loss, RB1 loss, PTEN loss, MYCL amplification, SMAD4 mutations, overexpression of stem cell transcription factor genes, upregulation of mitotic or proliferative genes, and upregulation of genes encoding NE markers [57, 59–63].

Androgen Receptor

Androgen receptors (AR) plays an important role in regulation of genes responsible for normal development and function of prostate, and specifically stromal AR controls prostatic epithelial cell proliferation, survival, and differentiation [64, 65]. Aberrations in AR gene such as amplification, point mutation, and splice variants which leads to increased activity are seen only in setting of metastatic and castration-resistant prostate cancer and not seen in clinically localized prostate cancer [66, 67]. The absence of AR gene lesions in localized tumors, and their emergence during treatment is hypothesized as a mechanism of resistance to drugs targeting androgen axis in advanced disease. Approximately 80% of castrate-resistant prostate carcinoma have been documented to bear an extra copy of AR gene, and in 30% cases, this is to the level of gene amplification, whereas this phenomenon is very rare in untreated prostate cancer [68]. Recently, it is shown that AR gene copy number change emerges during development of prostatic small cell carcinoma and is strongly associated with TMPRSS2-ERG rearrangement [69].

AR mutation are present in 10–30% in CRPC while being very rare in early stage untreated prostate cancer [68]. Majority of these mutations are gain of function mutation. These mutations most commonly affect ligand-binding specificity of AR leading to its activation [70]. Most common AR mutation seen in CRPC is T877A, other less common being L701H, V715M, V730M and H874Y.

AR splice variants are suspected to be major culprit behind development of resistance to ADT. Till now, more than 22 AR splice variants have been documented, but clinical relevance is seen in only 2 constitutively activated variants, namely, AR-V7 and ARV567es [71, 72]. ADT has been shown to cause inhibition of AR pathways, which in turn causes upregulation of AR-V7 and ARV567es [73, 74]. Expression level of these variants is known to be associated with poor patient outcome and also with CRPC [75].

Enzalutamide and abiraterone are potent AR-targeted therapies approved for the treatment of men with castration-resistant prostate cancer (CRPC) [76, 77]. Although the use of these agents improves the survival and quality of life of individuals with CRPC, most patients ultimately develop resistance to them [78]. Prostate adenocarcinomas may eventually completely escape androgen blockade and become truly hormone refractory (AR-independent), associated with the development of a predominantly neuroendocrine clinical phenotype (NEPC). In a recent study by Beltran et al., sequencing analysis of CRPC tumors that histologically were characterized as either adenocarcinomas or NEPC further supports divergent evolution of NECP from one or more CRPC cells rather than linear or independent clonal evolution, with decreased AR signaling and epithelial plasticity. In that study, genome-wide DNA methylation analysis also revealed marked epigenetic differences between NEPC and CRPC and also designated samples of CRPC with clinical features of AR independence as NEPC, suggesting that epigenetic modifiers may play a role in the induction and/or maintenance of this treatment-resistant state [79].

AURKA and MYCN

AURKA amplification has been reported in 65% of prostatic carcinoma (hormone naïve and treated) which subsequently developed NEPC after androgen deprivation therapy (ADT). Such gene amplification was present in 86% of distant metastases. Concurrent amplification of MYCN was present in 70% of primary tumors, 69% of CRPC, and 83% of metastases [56]. This is significant when compared to prevalence of these amplifications in only 5% of unselected prostatic carcinoma cases (Fig. 19.4). It has also been shown that transfection of AURKA and MYCN causes NE differentiation in normal benign prostate cell lines and use of AURKA inhibitors causes inhibition of MYCN transfected NEPC cell lines and shrinkage of NEPC xenograft [18, 56, 80]. AURKA amplification is also reported to be present in 45% of prostatic adenocarcinoma with

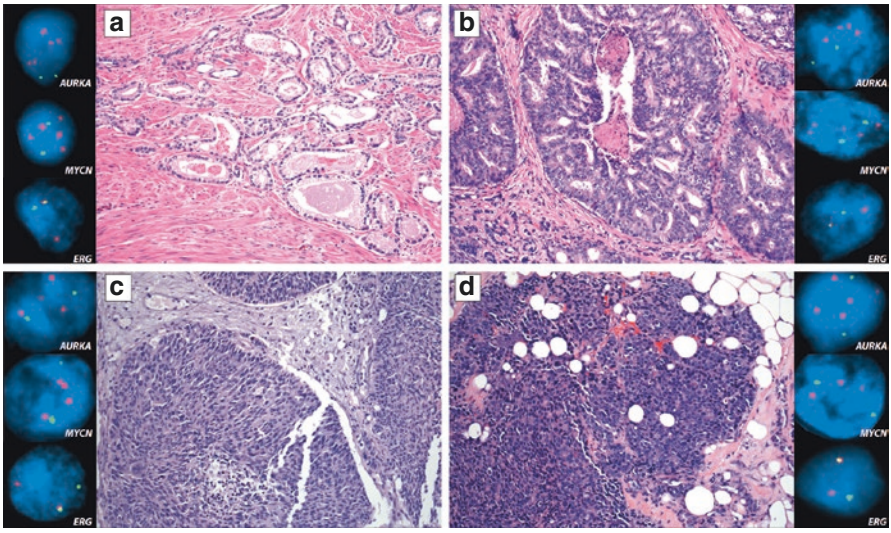

Fig. 19.4 AURKA and MYCN amplifications in primary prostatic adenocarcinoma may predict the subsequent development of neuroendocrine prostate cancer (NEPC). This four-image panel illustrates several specimens from a patient at different stages of disease progression to NEPC. (**a** and **b**) Images of hormone naïve prostate cancer with areas of Gleason score 3 + 3 = 6 (**a**) and 4 + 5 = 9 (**b**) at initial diagnosis. Concurrent AURKA (*upper inset*) and MYCN (*middle inset*) amplifications are present in both areas. (**c**) Subsequent local recurrence in the bladder demonstrates high-grade adenocarcinoma without neuroendocrine differentiation, exhibiting both AURKA and MYCN amplifications (*upper* and *middle insets*, respectively). (**d**) Five years after hormonal treatment, the patient presented with metastatic large cell neuroendocrine carcinoma in pelvic soft tissue. The tumor has both AURKA and MYCN amplifications (*upper* and *middle insets*, respectively). Clonal origin is confirmed by ERG rearrangement through translocation in all tumors (*lower inset*). (Reprinted from Neoplasia, Vol 15, Juan Miguel Mosquera et al., Concurrent AURKA and MYCN Gene Amplifications Are Harbingers of Lethal TreatmentRelated Neuroendocrine Prostate Cancer, p. 4, Copyright (2013), with permission from Elsevier)

Paneth cell-like neuroendocrine differentiation when compared to tumors without Paneth cell-like differentiation where it is present only in 7% cases [41]. These finding suggest that AURKA and MYCN play significant role in development of NEPC and can be exploited as therapeutic target. AURKA inhibitors are currently in stage of clinical trials, and a ray of hope is seen for NEPC patients in whom targeting hormone pathway is no longer effective [81]. In addition, recent data demonstrate that N-Myc overexpression in preclinical models drives aggressive prostate cancer that mimic NEPC at the molecular level and sensitizes cells to AURKA and EZH2 inhibition [82].

REST downregulation: Repressor element-1 silencing transcription factor (REST) also known as neuron restrictive silencing factor (NRSF) is a transcriptional repressor of neuronal-specific genes and has been shown to play crucial role in during embryogenesis and neural development [83–85]. It is proposed to be one of the key mediators for NE differentiation caused by androgen depletion [86]. Recently, it has been shown that REST downregulation is essential for NE differentiation of prostate adenocarcinoma induced by hypoxia through activation of autophagy [87]. REST is identified to be crucial regulator for CRPC to acquire EMT-like and stem cell phenotype and may serve as a potential therapeutic target for CRPC [88].

TP53 Loss

Aberration on TP53 gene has been seen in approximately 53% of metastatic CRPC [72]. NE cells of small cell neuroendocrine carcinoma are highly proliferative, compared to NE cells of benign prostate and those present in usual prostate adenocarcinoma, and lead to early metastasis [89]. It is postulated that TP53 mutation leads to inactivation of IL8–CXCR2–p53 signaling pathway, resulting in the loss of important growth inhibitory mechanism and the hyper-proliferation of NE cells in prostatic small cell carcinoma [90]. It is reported that TP53 mutation also leads to Aurora kinase A expression, which has a critical role in rapid proliferation and aggressive behavior of small cell prostatic carcinoma [58].

RB1 Loss

Rb protein loss has been seen in almost 90% of prostate small cell carcinoma cases with RB1 allelic loss in 85% of cases, whereas this Rb protein loss is rare in high-grade acinar tumors, suggesting that it is a critical event in the development of small cell carcinomas, prostate cancer progression, and metastasis [91, 92]. Rb loss along with hypoxia also leads to aberrant expression of hypoxia-regulated genetic programs that causes invasiveness and enhanced neuroendocrine differentiation in prostate cancer [93].

PTEN Loss

PTEN is one of the commonest inactivated tumor suppressor genes in human malignancies. PTEN aberrations such as deletion and mutation are seen in approximately 50% of primary prostate cancers with even higher incidence in advanced prostate cancers [94–98]. PTEN deletion is more frequent event in prostate cancer compared to point mutation [99]. PTEN loss mediates prostate tumor growth and metastasis via AKT activation, which may contribute to neuroendocrine differentiation [100, 101]. PTEN loss is associated with worst survival outcome in ADT-treated CRPC as well as those treated with radical prostatectomy [102, 103].

Recently, using next-generation RNA sequencing, an NEPC-specific RNA splicing signature has been identified, which is controlled by serine/arginine repetitive matrix 4 (SRRM4), which drives the progression of NEPC. This has been proposed to be a novel potential therapeutic target for NEPC [104]. IL6 is also hypothesized to induce neuroendocrine differentiation in prostate cancer cells via peroxisome proliferator-activated receptor γ and adipocyte differentiation-related protein (a major component of adiposome) which could be exploited as novel drug targets for CRPC [105].

Recurrent MYCL amplification, SMAD4 mutation along with other rare molecular aberrations have also been linked to neuroendocrine differentiation of prostate cancer [60, 62].

Clinical Presentation and Therapy

Primary or de novo NEPC (most commonly with small cell histomorphology) is rare (<1%) and tends to occur at younger age, and most patients present with overt metastases. There are no well-defined risk factors. Patients that do present with localized disease usually have few symptoms. Distant spread is often to visceral organs (such as liver and brain) or lytic bone lesions, unlike acinar prostatic adenocarcinoma that tends to metastasize to bone and produce sclerotic lesions. Presenting symptoms of primary NEPC may include constitutional symptoms, hydronephrosis, bone pain, abdominal pain, hematochezia, or hematuria. Occasionally, patients may have paraneoplastic syndrome due to ectopic production of hormones such as adrenocorticotropic hormone and antidiuretic hormone.

Differential diagnoses of pure NEPC include small cell carcinoma from other primary sites such as the lung, specifically in the setting of widespread metastatic disease. Accurate diagnosis can be challenging in this clinical scenario. Histologically, tumors from other sites may have similar morphological appearance as well as similar immunostaining profile (negative for AR and positive for NE markers). Detection of ERG gene rearrangement by FISH is helpful in such scenarios as this rearrangement is positive in more than 50% of prostatic NEPC whereas universally negative in small cell carcinoma from other sites such as the lung and bladder [48, 106].

Much more commonly, NEPC arises in the setting of androgen deprivation therapy of prostate adenocarcinoma. Resistance eventually develops to antihormonal drugs, and some hormone refractory tumors progress toward NEPC [22]. Clinically, suspicion of NEPC should be made in a patient with advanced prostate cancer showing rapid progression, especially development of visceral metastases, without appropriate rise of serum PSA level. Serum NE markers such as chromogranin A and NSE are frequently elevated in advanced prostate cancer, but extremely high values support the diagnosis of NEPC. Circulating tumor cell analysis has recently been shown to enable detection and characterization of NEPC, but it still remains as a research tool [107, 108]. Metastatic tumor biopsy is the gold standard for diagnosing NEPC.

Patients with treatment-related neuroendocrine prostate cancer will likely not respond to hormonal agents but may initially respond to platinum-based chemotherapy. Therefore, treatment of NEPC is somewhat similar to that of small cell carcinoma of lung. Radiotherapy is occasionally added for local control or palliation of symptoms. Currently, there has also been growing concern for toxicities of continuous ADT (CADT). Treatment of NEPC is still an area of active research. Currently phase II clinical trial of PARP inhibitor olaparib with or without cediranib in men with metastatic castration-resistant prostate cancer-inducing NEPC is still ongoing (https://clinicaltrials.gov/ct2/show/NCT02893917).

Conclusion

In summary, NEPC is a highly aggressive form of prostate cancer that most commonly arises in the setting of hormonal treatment and very rarely de novo. Most of these tumors progress rapidly and develop visceral metastases and lytic bone lesions in the setting of low PSA. Due to widespread use of hormone therapy in treatment of prostate cancer, incidence of NEPC is anticipated to rise. Biopsy is the gold standard for diagnosing NEPC. Chemotherapy is the mainstay of treatment as used in small cell neuroendocrine carcinoma of other sites. More targeted approaches are being developed based on an emerging molecular understanding of this aggressive form of prostate cancer.

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Part IV
Key Molecular Pathways in Prostate Cancer Development and Progression

Chapter 20
The Role of Androgen Receptor in Prostate Cancer

Adeline Berger and David S. Rickman

Contents

Androgen Receptor (AR): Description and Function

AR Gene and Protein Description

The androgen receptor (AR) is encoded by a 186,588 base-pair, 8-exon gene located on chromosome X (Xq11–12) and belongs to the steroid hormone nuclear receptor superfamily. AR expression is cell-dependent and regulated by androgen [1]. The AR gene encodes for a 919 amino acid nuclear receptor (~110 kDa) that acts as a

A. Berger, Ph.D.
Department of Pathology and Laboratory Medicine, Weill Cornell Medicine, New York, NY 10065, USA
e-mail: asb2018@med.cornell.edu

D.S. Rickman, Ph.D. (✉)
Department of Pathology and Laboratory Medicine, Weill Cornell Medicine, New York, NY 10065, USA

Meyer Cancer Center, Weill Cornell Medicine, New York, NY 10065, USA

Englander Institute for Precision Medicine, New York-Presbyterian Hospital, Weill Cornell Medicine, New York, NY 10065, USA
e-mail: dsr2005@med.cornell.edu

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*, Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_20

hormone-dependent DNA-binding transcription factor. This modular protein is composed of four domains [2]: the N-terminal domain (NTD), the DNA-binding domain (DBD), the hinge region, and the C-terminal domain (CTD). The NTD contains residues involved in the recruitment of transcriptional co-regulators and is required for the activation of transcriptional activity. The autonomous transcriptional activation function (AF1) of the NTD is ligand-independent and required for maximal activity of AR [3]. The DBD is composed of two zinc-finger motifs involved in the recognition of specific DNA sequences, located in AR target genes [4, 5], known as androgen response elements (AREs: canonical consensus sequence = 5′-AGAACA-3′ in direct repeats or inverted repeats separated by a spacer of three base pairs for classical and selective AREs, respectively [6]). Noncanonical elements have also been described but are bound by AR with a lower affinity [7]. The DBD is separated from the CTD by the hinge region, a flexible region that includes the nuclear localization signal sequence (NLS). The CTD contains the ligand-binding domain (LBD), which is formed by 12 α[alpha]-helix arranged in a globular structure surrounding the hydrophobic ligand-binding cavity, and a second transcriptional activation function (AF2). The AF2 is ligand-dependent and has a synergistic effect with AF1 that results in full AR transactivation [8]. Despite a high similarity of sequence between members of the hormone nuclear receptor superfamily, some key residues in the AR AF2 core domain are essential for AR functionality and make it distinct from the other hormone nuclear receptors [9]. The CTD also contains the nuclear export signal (NES), which is used to export AR to the cytoplasm upon ligand withdrawal.

The Role of AR in Normal Development

AR is weakly expressed in numerous tissues and cells yet highly expressed in the adipocytes, liver, and prostate (http://biogps.org/#goto=genereport&id=367), directly linking its function to male dimorphism. Following androgen activation, AR participates widely in the male reproductive tract including gonadal development and maintenance [10]. During embryogenesis, AR signaling regulates the reproductive tract patterning by determining the Wolffian duct differentiation and inducing the development of the male reproductive tissues [11, 12]. In adults, AR remains essential for seminiferous tubule and prostate function in terms of epithelial and stromal compartment maintenance [13] and maintenance of paracrine factor-mediated secretory functions of the epithelial cells [14]. AR signaling is also involved in other physiological processes linked to sexual dimorphism, such as muscle development, lipid accumulation, and bone homeostasis [13].

AR Transcriptional Regulation

The activation of the AR signaling pathway depends on ligand binding to AR. In the absence of ligand, AR is localized in the cytoplasm and forms a complex with HSP90/HSP70-based multiprotein chaperone machinery, protecting it from

degradation [15]. Barring 10% that is produced in the adrenal cortex, the vast majority of circulating androgen is testosterone produced by the testes. Circulating testosterone enters prostate epithelial cells and is rapidly converted to the potent AR ligand 5α[alpha]-dihydrotestosterone (5α[alpha]-DHT) by the enzyme 5α[alpha]-reductase to DHT (dihydrotestosterone or androstanolone) [16]. DHT binds the LBD of AR with a twofold higher affinity and a fivefold decreased dissociation rate compared to its precursor [17]. Binding of DHT to the LBD of AR induces conformational changes resulting in AR dissociation from the HSP90/HSP70 complex and the exposition of the AF2 domain and the NLS. AR is then shuttled to the nucleus via the microtubule network [18, 19]. The AR NLS interaction with importin-α[alpha] and importin-β[beta] mediates the translocation of AR across the nuclear membrane [20]. Upon entry into the nucleus, AR homodimerizes and binds to AREs associated with AR target genes. These sequences are found in both proximal promoters and distal enhancers, up to several thousand base pairs upstream or downstream of the transcription start site [21]. The AR cistrome has been defined by multiple research groups in cell lines, such as LNCaP [22–24] and VCaP cells [25], in transgenic mouse models [26], and in human clinical samples of prostate cancer tissues [27] including castrate-resistant prostate cancer [28]. These studies clearly demonstrated that the AR cistrome is reprogrammed in human prostate tumorigenesis [27, 28]. AR binding to AREs leads to recruitment of co-regulators (replacement of transcriptional corepressors with coactivators), general transcription factors, and RNA polymerase II to induce transcription activation. Two well-described AR target genes and indicators of androgen signaling pathway activation are prostate-specific antigen (PSA) and transmembrane protease, serine 2 (TMPRSS2). A noncanonical pathway of androgen signaling, independent of androgen binding and involving cofactors or crosstalk with other intracellular signaling pathways, has also been characterized.

AR Signaling Regulation

AR Co-regulators and Cofactors

AR activity is modulated through an interaction with specific cofactors. Depriest et al. recently generated a database referencing 274 AR-associated co-regulator genes [29]. According to Heemers and Tindall [30], AR-interacting proteins can be classified into three main groups: (1) general transcription factors, comprising the classical transcriptional machinery, (2) co-regulators that shift the balance toward expression or repression of the transcriptional activity, and (3) specific transcription factors.

The first class includes TFIID/B/F proteins, required for the recruitment of RNA polymerase II, and TFIIE/H. Of these, AR directly interacts with TFIIF, TFIIH, and polymerase II through its RPB2 subunit.

The second class of cofactors is composed of more than 160 proteins [30] including components of the chromatin remodeling complex (e.g., ARIP4 [31], BAF57 [32], the SWI3-related gene product SRG3 [33], and SRCAP [34]) and histone modifiers (e.g., members of the p160 SRC gene family [35, 36]). P300 [37] and CREB-binding

protein (CBP) also directly acetylate AR through their acetyltransferase activity. This acetylation allows for the recruitment of other coactivators to serve as molecular bridges between AR and the transcriptional machinery [37]. Histone modifiers either promote (e.g., demethylases such as LSD1 and JMJD2C [38, 39]) or repress (e.g., deacetylases such as SIRT1 [40]) AR-mediated transcriptional activity. AR also interacts with ubiquitination/proteasome and SUMOylation pathway components, proteins involved in splicing and RNA metabolism, DNA repair proteins, chaperones, cell cycle proteins, signal integrators, and apoptosis regulators [30, 41].

The third category of AR-interacting proteins [30] regulates AR signaling by defining the temporal, spatial, and functional binding pattern of AR [42]. This class of cofactors acts by modifying the interaction of AR with DNA [43], titrating other co-regulators [44], or recruiting AR on non- or partial AREs [42]. For example, DNA motifs recognized by the three transcription factors FoxA1, GATA2, and Oct1 are enriched at AR half-site motifs [42]. FoxA1 is a known pioneering factor that is essential for maximal prostatic gene activation [45] by facilitating AR binding on FoxA1-dependent AR binding sites. More recently, FoxA1 has been shown to regulate AR function by masking AR binding sites, which become functional upon FoxA1 depletion [23]. While FoxA1-AR interaction is not affected by ligand binding, AR interacts with GATA2 and Oct1 in a hormone-dependent manner. These collaborating partners have distinct functional roles in androgen-dependent gene transcription and cell proliferation [42].

These observations highlight the balance between coactivators and corepressors of AR and their importance on its activity. AR cofactors are differentially expressed in prostate cancer and have the potential to drive disease progression.

AR Protein Posttranslational Modifications and Signaling Crosstalk

Although androgen binding is the primary means for AR activation, protein posttranslational modifications also influence AR activity. AR, at the protein level, can be altered by up to five well-described modifications (phosphorylation, acetylation (discussed above), SUMOylation, methylation, and ubiquitination) on a subset of 23 different amino acids [46].

AR phosphorylation occurs at serine 16, 81, 256, 308, and 424 in the presence of androgen, but the impact of phosphorylation at any one of these sites in terms of AR activity remains unclear. For example, stress-induced JNK1 phosphorylation on serine 650 regulates nuclear export of AR, antagonizing AR-mediated transcription [47]. AR phosphorylation at specific tyrosines results from specific growth factor signaling. Growth factors such as IGF-I (insulin-like growth factor-I), KGF (keratinocyte growth factor), and EGF (epidermal growth factor) are able to activate androgen signaling through AR phosphorylation, leading to an increase of PSA level. This activation is inhibited by the AR antagonist Casodex, highlighting the specificity of the mechanism for AR [48]. EGF, for example, induces the activity of Src and Ack1 kinases, which, in turn, phosphorylate AR at tyrosines 534 and 267, respectively. Phosphorylation at these sites increases AR transcriptional activity by

enhancing its nuclear translocation and DNA binding. EGF can also modify AR activity by inducing IL-6 upregulation in prostate cancer cells.

AR SUMOylation, occurring at regulatory amino acid SUMO acceptor motifs at lysines 386 and 520, results in an inhibition of both the ligand-activated and the basal ligand-independent activity of AR [49]. AR is also regulated by mono- and polyubiquitination, which impact the stability and turnover of the protein or its activity, depending on the topology of the polyubiquitin chains [28, 50, 51]. The position of the lysine residue used for ubiquitin chain branching dictates the fate of the substrate. For example, the polyubiquitination of AR mediated by MDM2 induces AR degradation by the 26S proteasome [51], while the one driven by RNF6 promotes AR transcriptional activity [50].

AR Involvement in Primary Prostate Cancer

After skin cancer, prostate cancer is the most common and the third most lethal form of cancer in men in the United States, with 161,360 estimated new cases in 2017 [52]. Over 50 years ago, Huggins' work [53, 54] demonstrated a direct relation between androgen and prostate cancer, reporting a regression of prostate cancer after orchiectomy. High levels of AR in prostate cancer luminal epithelial cells are associated with a high tumor grade, deregulation of cell-cycle genes [55–57], inhibition of apoptosis [58], increased angiogenesis [59], and crosstalk with PI3K-AKT-PTEN, RAF, Wnt, and DNA repair signaling pathways [60]. AR has also been implicated in the development of chromosomal rearrangements, such as the TMPRSS2-ERG gene fusion, detected in around 50% of prostate cancer patients [61–63]. Clinically, androgen signaling is monitored using the prostate-specific antigen (PSA) level, encoded by the AR target gene *KLK3* [64]. The level of circulating PSA is measured to track prostate cancer progression and disease recurrence in the context of androgen deprivation therapy (ADT) [64–66], which is the first line of treatment for advanced prostate cancers. Current ADT approaches are aimed at chemically lowering circulating testosterone levels by the administration of luteinizing hormone-releasing hormone (LHRH) analogs. Since these approaches target only 90% of androgen production (not from the adrenal glands), they are often used in combination with the classic antiandrogen compounds (e.g., flutamide, bicalutamide, and nilutamide). Despite encouraging initial response following ADT, relapse occurs for almost all cases within several months and leads to a more aggressive form of prostate cancer defined as castration-resistant prostate cancer (CRPC).

Mechanisms of AR Reactivation Associated with CRPC

There are several mechanisms of resistance associated with the onset of metastatic CRPC (mCRPC) tumors, among which include AR-related alterations (e.g., *AR* gene amplification or mutations [60, 67, 68], alternative splicing of *AR* mRNA [69–72], and posttranslational modifications of AR protein (Fig. 20.1)), crosstalk with other

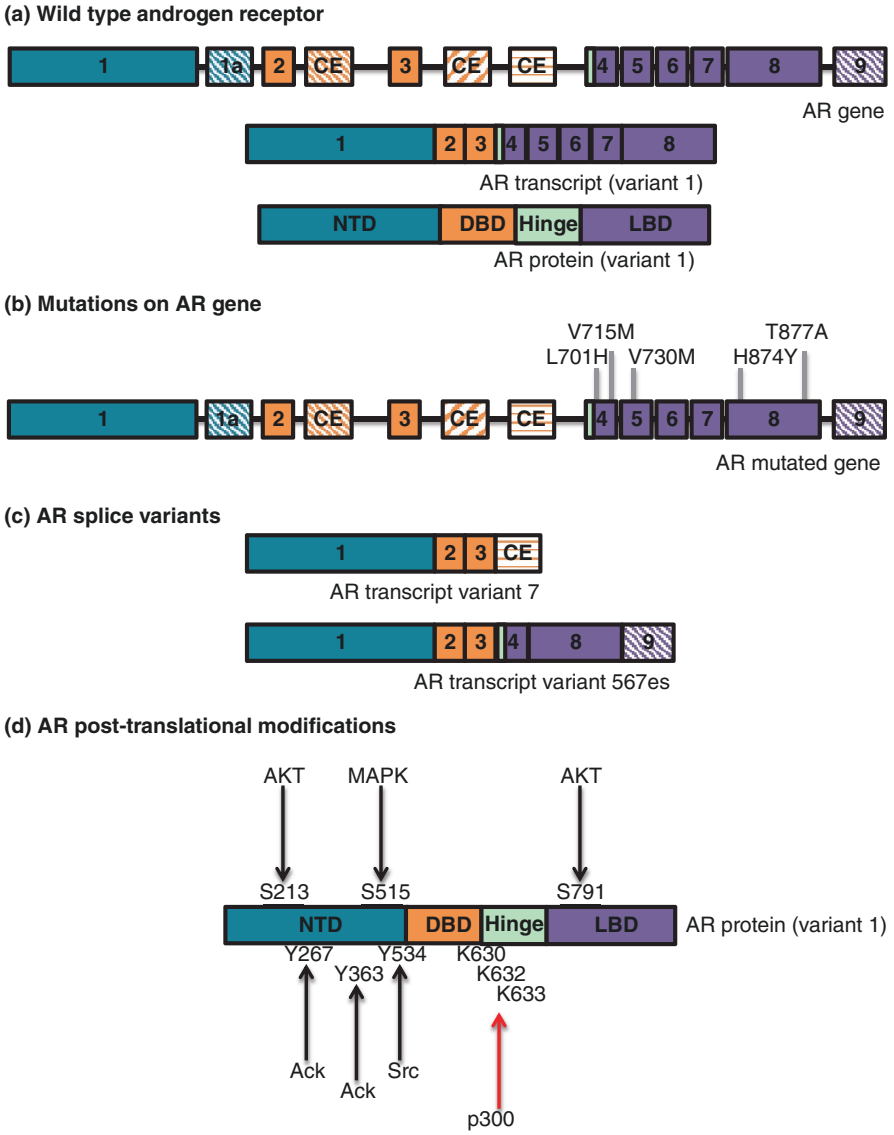

Fig. 20.1 AR modifications associated with castrate-resistant prostate cancer development. (**a**) Wild-type AR gene, RNA, and protein. (**b**–**d**) Alterations to the AR gene DNA, mRNA, or protein-associated androgen deprivation therapy resistance. These alterations result in constitutive activation of AR, due to mutation in the LBD (**b**) or splicing variant (**c**). (**d**) The most common posttranslational modifications of AR that enhance its transcriptional activity and that are driven by one of the AR cofactors (e.g., p300) or crosstalk with other signaling pathways (e.g., AKT, MAPK, Ack, and Src). *Black arrows* represent phosphorylation, while the *red arrow* represents acetylation. CE = cryptic exon, NTD = N-terminal domain, DBD = DNA-binding domain, hinge = hinge domain, LBD = ligand-binding domain

cancer-promoting signaling pathways, genomic alterations involving cofactors/co-regulators and other AR signaling proteins, and intraprostatic generation of androgen [73, 74]. These findings have led to the development of second-generation antiandrogens, which are improved AR antagonists (e.g., enzalutamide) or inhibitors targeting the biosynthesis of AR (e.g., abiraterone acetate). Enzalutamide (MDV3100) is a targeted AR inhibitor that competitively binds to the LBD of the androgen receptor and inhibits androgen-receptor translocation to the cell nucleus, recruitment of AR cofactors, and AR binding to DNA [75, 76]. The 17α[alpha]-hydroxylase/C17,20-lyase (CYP17) inhibitor abiraterone acetate acts as an antagonist to AR and inhibits 3β[beta]-hydroxysteroid dehydrogenase blocking androgen synthesis in the adrenal glands, testes, and within the prostate tumor [77, 78]. Despite improved response rates and overall survival with these molecules [79], almost all metastatic CRPC patients develop resistance to these agents as well. Recent genomic sequencing studies of large cohorts with resistance to these molecules have recently been reported and show further AR signaling reactivation alterations [60]. Below, we discuss the mechanisms, linked to AR or AR signaling, known to be involved in the resistance of mCRPC to second-generation ADT.

AR Gene Amplification

Early studies using both targeted or genome-wide approaches of hormone-naïve versus hormone-refractory primary prostate cancers led to the finding of an acquired increased copy number (up to 60 copies per cell) at chromosome Xq11–13 including the genomic loci of AR in roughly 30% of recurrent tumors [80–82]. Other studies have also reported an AR amplification in more than 50% of circulating tumor cells (CTC) from metastasized CRPC [83, 84]. This observation is consistent with the frequency of AR amplification found in recent genome sequencing studies [60, 85]. AR amplification drives its overexpression and increases the likelihood of androgen-AR interaction, thus reactivating the AR signaling pathway.

AR and AR-Associated Gene Mutations

AR mutations in the context of CRPC were first described roughly 20 years ago [67, 68] and have since been characterized in around 20% of CRPC [86]. Numerous AR mutations have been described in prostate cancer, approximately 45% of which are somatic single-base substitution occurring in the LBD [87]. Several mutations in this region affect the ligand specificity of AR, allowing its activation by non-androgenic steroids or antiandrogens [88]. Recent genomic sequencing analyses of metastatic prostate cancers have shed a considerable amount of light regarding mutations to AR and AR signaling genes such as NCOR1, NCOR2, FOXA1, and NKX3.1 [85, 86, 89–91]. The most recent study, based on a large sequenced CRPC patient cohort treated with the most up-to-date standard-of-care antiandrogen

therapy (abiraterone or enzalutamide) or through a cohort of prospective clinical trials (n = 150), found that upward of 70% of cases harbored AR pathway aberrations [60]. The majority (63%) of alterations impacted AR directly, through amplifications and mutations including hotspot mutations that confer agonism to AR antagonists such as flutamide (T878A) and bicalutamide (W742C) [92]. This agonism to enzalutamide has also been described with the F876L mutation [93] as well as to glucocorticoids in case of L702H mutation. In addition to AR mutations itself, Robinson et al. also observed alterations in AR pathway members such as NCOR1, NCOR2, and FOXA1 [51] and AR-associated genes such as deletion of ZBTB16 [94, 95] and SPOP mutations [89, 96].

AR Splice Variants

More than 20 different AR variants have been described in preclinical or clinical CRPC samples ([60, 69–72], reviewed by Lu C. and Luo J. [97] and more recently by Wadosky and Koochekpour [98]). The AR variants are generated from multiple alternative splicing events (e.g., aberrant splicing, inclusion of an alternative exon, or insertion of cryptic exons) of the *AR* mRNA. Structural alterations in the *AR* gene resulting in AR variant expression have also been described [99, 100]. Insertions of cryptic exons downstream of the sequences encoding the DBD or deletions of the exons encoding the LBD result in a truncated AR protein devoid of the functional LBD. AR splice variants that lack the LBD (encoded by exons 5–6-7–8) generate constitutively active forms of AR [70, 71]. The activity of these AR variants is no longer regulated by androgens. They are thus resistant to antiandrogen therapies and constitutively activate the AR signaling pathway [71, 72]. Moreover, while AR is translocated to the nucleus via microtubule transport, AR-V7 (the most characterized AR variant lacking the LBD) exploits another way to its translocation that is still under investigation [101]. AR-V7 and AR-v567es are the most commonly detected AR variants in prostate cancer and thus, the most studied to date (Fig. 20.1). A genome-wide occupancy study using ChIP-seq found that AR variants bind DNA as dimers and display a binding preference for the same canonical high-affinity AREs that are engaged by AR-FL, albeit with lower affinity [102]. While initially described as heterodimers with AR full length (AR-FL), the variants have since been implicated in homodimerization and driving AR signaling independently of AR-FL [102–105]. Based on nuclear AR expression using N- and C-terminal-specific AR antibodies, Zhang et al. found an increase in the prevalence of AR variants in CRPC clinical samples compared to primary prostate cancer [106]. Another study of 13 CRPC bone metastasis samples found that the level of AR variant protein constituted 32% (range 0–95%) of the AR full length. Meanwhile, the RNA level was relatively weak compared to the full length, suggesting that AR variants could be posttranscriptionally stabilized in CRPC [107]. AR-FL, AR-V1, and AR-V7 transcripts were detected in most of the nonmalignant primary tumors and metastatic samples examined, while the AR-V567es transcript was detected in only 7 (23%) CRPC bone

metastases. The expression of these variants is also associated with a poor prognosis of patients, most likely due to their constitutive activation [107]. AR variants drive androgen-independent cell proliferation in a manner that is resistant to antiandrogens, including enzalutamide [108], and are widely expressed in the context of metastatic CRPC (SU2C cohort) and to a lower extent in pre-abiraterone/enzalutamide primary prostate cancer (TCGA cohort [60]). While controversial [109], AR-V7 expression has been associated with abiraterone and enzalutamide resistance [110–113]. More recently AR-V9 has also been associated with abiraterone resistance [114]. AR variants are hypothesized to induce epithelial-to-mesenchymal transition and stem cell phenotypes [115], but a further validation of this notion is needed.

AR Signaling and Crosstalk with Other Signaling Pathways Associated with CRPC

Many signaling pathways interacting with AR have been observed as altered or dysregulated in prostate cancer cells. For example, (1) the loss of PTEN and subsequent activation of PI3K/AKT are critical event in human prostate cancer [116, 117], (2) increased expression of EGFR correlates with the evolution of prostate cancer [118], (3) elevated circulating IL-6 and IL-8 levels have been observed and associated with advanced prostate cancer cases [119–123], and (4) members of SRC family have been described as increased in prostate cancer, even at higher levels in CRPC [124, 125].

Some of them have been shown to enhance AR signaling in the context of CRPC that arises either as a feedback following androgen withdrawal and/or compensation from growth factors and other signaling ligands. The expressions of several peptide growth factors, such as EGF/TGFα[alpha] and IGF-1, have been shown to be increased during progression to CRPC [118, 123, 126] and either induce AR transcriptional activity irrespective of androgen stimulation or sensitize AR to low concentrations of androgens (Fig. 20.2) [48, 127, 128]. More recently, another growth factor, CXCL12, has been characterized as androgen-independent AR activator in prostate cells [129]. Interleukins are also able to induce androgen-independent AR activity. IL-6 (interleukin-6), a multifunctional cytokine produced by prostate cells, binds to its specific receptor and induces a signaling cascade including JAK, STAT3, and p300. The N-terminal domain of AR directly interacts with STAT3, after IL-6 induction through phosphorylation of mitogen-activated protein kinase (MAPK) pathway [130]. This interaction leads to the activation of the AR NTD. IL-8 is also able to increase AR expression and promote its activity in an androgen-independent manner [131]. In addition, several protein kinases (e.g., MAPK, Akt/PKB, PKA, and PKC) and non-receptor tyrosine kinases (ERBB2/HER-2/neu, Src, FAK, and Etk/BMX) modulate AR activity by direct phosphorylation of serine/threonine or tyrosine residues, respectively, on AR or one of its cofactors (e.g., TIF2 and SRC1) [127, 128, 132]. The ERBB2/HER-2/neu tyrosine kinase modulates AR signaling [133], through MAP kinase pathway [134] or AKT pathway [135] or when associated with ERBB3 through

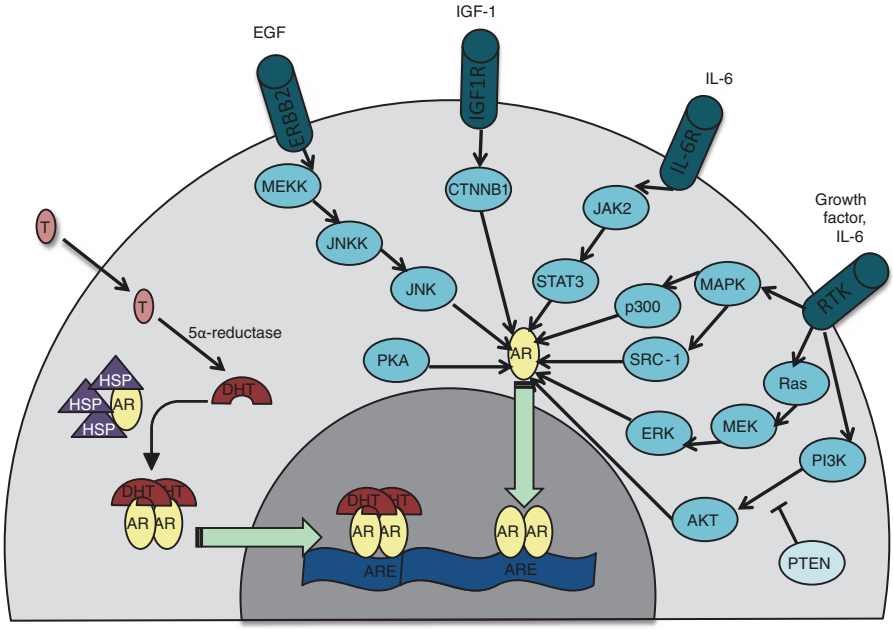

Fig. 20.2 Androgen-dependent and independent AR activation. AR can induce androgen-signaling pathway upon androgen binding (on the *left*) or activation through interaction with other signaling pathways (on the *right*)

a mechanism that remains to be elucidated [136]. As mentioned above, some specific AR polyubiquitinations serve as negative regulators of AR by enhancing its degradation [51]. This degradation is inhibited by HER2/ERBB3/PI3-kinase pathway in the context of hormone-refractory prostate cancer, providing a mechanism of enhanced AR stability as an additional mechanism of resistance [136]. PKA (protein kinase A), whose activity is dependent on the cellular level of cAMP, activates AR in the absence of androgen (Fig. 20.2) [137, 138]. There is a well-described and dynamic interplay between PI3K/AKT/mTOR and AR signaling axes during prostate cancer progression as well as a mechanism of ADT resistance. In the presence of androgen, AKT phosphorylates AR on Ser 213 and Ser 791, inducing a modification in AR signaling [139–141]. However, activation of the PI3K/AKT/mTOR pathway resulting from PTEN loss is associated with androgen insensitivity and the development of CRPC [142]. To address the mechanism underlying this finding, two independent groups found that the loss of PTEN in prostates results in a decrease in transcription of AR target genes through derepression of negative regulators of AR activity, EGR1, and c-Jun [143, 144]. In addition, loss of AR signaling either through genetic or pharmacological manipulation with enzalutamide leads to a reduction of FKBP5, an AR target gene. Low FKBP5 levels lower the AKT phosphatase and negative regulator PHLPP protein levels. In addition, mTOR inhibition in the background of PTEN loss leads to an increase in AR levels through upregulation of HER3, which increases

AR stability. Altogether, these data show how PI3K/AKT/mTOR pathway activity in the context of CRPC alters the need of the restricted levels of circulating androgens.

Moreover, an increase of AR acetylation enhances the binding of p300 on AR, reducing N-CoR/HDAC/Smad3 corepressor binding. This effect leads to a modulation of the transcriptional activation on AR-responsive genes, resulting in an aberrant cell growth in prostate cancer stable cells [37].

The activity of the glucocorticoid receptor (GR) has also been described as a potential mechanism of resistance to enzalutamide and ARN-509 in a preclinical model and has been confirmed in patient samples [145]. Glucocorticoids administrated at a low dose inhibit adrenocorticotropic hormone (ACTH) production by the pituitary and initially result in reduced androgen levels. However, a high expression of GR by the prostate cancer will result in GR activation in tumor cells. In this context, a more efficient strategy could be to combine AR and GR inhibition. This is currently being explored in an early phase clinical trial combining enzalutamide and mifepristone (NCT02012296).

Next Generation of AR-Targeted Therapies

Even in the face of potent second-line AR antagonists (enzalutamide) or CYP17 inhibitors (abiraterone), metastatic CRPC tumors continue to evolve different mechanisms to reactivate AR signaling, which has spurned the development of further agents targeting the AR signaling axis. New inhibitors have emerged that efficiently block both AR full length and variant action by targeting the N-terminal domain of AR [146, 147] or inducing degradation of AR mRNA [148] or protein [149–151]. None of them have been fully tested on patients and FDA-approved. One of the two most promising compounds is EPI-506 (binds the NTD of AR), which is currently in phase I/II clinical trial (NCT02606123). While the patients will not be selected based on AR-V7 status, responses to treatment will be stratified based on AR-V7 expression in CTCs. The second promising compound is the FDA-approved niclosamide, which promotes AR-V7 degradation and potentially restores the sensitivity of the tumors to second-generation ADT [152–154]. Two clinical trials are currently ongoing to assess the efficiency of niclosamide in combination with enzalutamide (NCT02532114) or abiraterone (NCT02807805).

The concept of bipolar androgen therapy is also emerging. This is based on the observation that the growth of AR-positive human CRPC cell lines is inhibited by supraphysiologic levels of androgens [155–157]. This has recently been the subject of a pilot clinical trial [158]. These newer strategies may also prove beneficial in combination with inhibitors targeting key crosstalk pathways (e.g., PI3K/AKT/mTOR or ERBB2/Her2/neu) or microtubule-targeting agents (e.g., taxanes). One consequence of taxane treatment is the inhibition of AR nuclear trafficking [18, 19, 159, 160]. This mechanism predicts synergy between effective AR-targeted therapy and taxanes, clinically validated by the unprecedented survival results for men with advanced, hormone-naïve prostate cancer in CHAARTED and STAMPEDE clinical trials (Sweeney ASCO 2014; James ASCO 2015 [161–163]).

Conclusion

Androgen signaling is a cellular pathway activated upon androgen binding to its specific receptor AR, leading to the transcriptional activation of androgen-responsive genes. Regulation of this pathway occurs through the action of numerous co-regulators of AR and is influenced by crosstalk from other signaling pathways in the cell and the microenvironment. Activation of AR is essential for the male dimorphism and also determinant for the development and maintenance of the prostate. Androgen signaling is also essential for the maintenance and progression of prostate cancer, making chemical castration, in the case of non-organ confined disease, the first line of intervention. While the involvement of androgen receptor in prostate cancer progression is established, the therapeutic strategy targeting androgen signaling-driven prostate cancer still needs to be improved for the incurable forms of the disease.

Despite continued hormonal therapy, including the most robust and potent second-line antiandrogens (e.g., enzalutamide) or CYP17 inhibitors (e.g., abiraterone), metastatic CRPC tumors evolve complex and ever-adapting mechanisms to reactivate AR signaling or other mechanisms that render the tumor cells indifferent to AR signaling. Recent evidence shows that neuroendocrine prostate cancer can arise in later stages of prostate cancer progression from a pre-existing adenocarcinoma during the course of treatment resistance to AR-directed therapies [164]. This is as an adaptive resistance mechanism.

The complexity and variability of mechanisms of resistance that have been described to date emphasize the growing need for better model systems that recapitulate clinically relevant mechanisms and a precision strategy adapted to each patient. The synergistic effect observed in recent combinatory treatments also highlights the important question of the timing, order, and/or combination of drugs in future strategies.

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Chapter 21
PI3K/Akt/mTOR/PTEN and ERK/MAPK Pathways

Tamara L. Lotan

Contents

PTEN/PI3K/AKT/mTOR

Overview of PTEN/PI3K/AKT/mTOR Signaling in Prostate Cancer

Phosphoinositides are membrane glycerophospholipids which make up ~15% of the phospholipid content in most cells. PIP3 (phosphatidylinositol [3,4,5]-trisphosphate) is the most potent signaling intermediate and is rapidly and transiently produced on the cell membrane downstream of receptor tyrosine kinase activation, following membrane recruitment of phosphoinositide-3-kinase (PI3K) and subsequent phosphorylation of the more common phosphoinositide, PIP2 (phosphatidylinositol [4,5]-trisphosphate). PTEN (phosphatase and tensin homologue on chromosome 10) is a lipid phosphatase that removes the 3-phosphate group from PIP3 at the plasma membrane, functionally antagonizing the oncogenic PI3K signaling pathway [1]. Once formed, PIP3 can recruit PH domain containing signaling intermediates to the cell membrane, such as the protein kinase AKT, which may

T.L. Lotan, M.D.
Johns Hopkins School of Medicine,
1550 Orleans, CRB2 Rm 343, Baltimore, MD 21231, USA
e-mail: tlotan1@jhmi.edu

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*,
Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_21

then be phosphorylated and activated. AKT signaling is one of the most commonly upregulated oncogenic pathways in human cancer and results in downstream activation of a number of additional pathways, including mTOR. mTOR kinase is simultaneously upstream and downstream of AKT function because mTORC1 is indirectly activated by PI3K/AKT (via inactivation of TSC1/2) and mTORC2 phosphorylates AKT, fully activating it [2]. PI3K/AKT/mTOR signaling results in diverse cellular outcomes relevant to tumor initiation and progression, including promotion of proliferation, inhibition of apoptosis and senescence, as well as effects on cell adhesion and migration.

Among genes altered in the PI3K/AKT/mTOR pathway in prostate cancer, PTEN deletion is by far the most common and well studied (see below). Other aberrations in PI3K/AKT/mTOR pathway components have been described, but these generally occur in a small minority (1–5%) of cases. PIK3CA (the gene encoding for the p110α catalytic subunit of PI3K) amplifications (20% of cases) and gain-of-function point mutations (<5% of cases) occur, often, but not always, mutually exclusively with PTEN loss [3–7]. PIK3CB alterations are even less common in human prostate tumors [3–7], though mouse models suggest that PIK3CB may be essential for AKT signaling in the context of PTEN loss [8]. Interestingly, AKT1/2 alterations are almost never seen in human prostate tumors, though deletions involving the AKT phosphatase PHLPP1 [9–11] may occur in a minority of cases. Rare loss of other phosphatases that may compensate for PTEN loss in murine models, such as INPP4B, have also been described [12, 13]. Mutations in GSK3B, a kinase functioning downstream of PI3K, are also rarely reported [3–7]. Of note, alterations involving mTOR signaling are relatively rare in prostate tumors relative to other epithelial malignancies [3–7].

Mechanisms of PTEN Inactivation in Prostate Cancer

PTEN genomic deletion was first identified as a molecular aberration common in prostate cancer nearly 15 years ago [14, 15], and recent sequencing efforts have demonstrated that PTEN is the most commonly lost tumor suppressor gene in primary disease [3–7]. Depending on the cohort examined and the methodology used, the reported rate of PTEN gene deletions in prostate cancer varies widely because it is highly correlated with increased Gleason grade and tumor stage. In early studies using microsatellite analysis, loss of heterozygosity (LOH) at the PTEN locus was reported in 10–55% of primary tumors from surgical cohorts [14, 16–20]. In studies using fluorescence in situ hybridization (FISH), loss of at least one PTEN allele has been reported in as few as 17% of patients with tumors incidentally discovered on transurethral resection (TURP); however, PTEN allelic loss is present in 17–68% of primary tumors from various surgical cohorts [21–30]. Despite the variations in reported rates of genomic PTEN loss, a nearly universal finding is that loss of one PTEN allele is significantly more frequent than loss of both PTEN alleles in surgical cohorts. Consistent with the strong correlation with tumor stage, PTEN loss is more common in prostate cancer metastases than in primary tumors, with rates of loss reported near

50% in most studies [5, 20, 28, 31, 32]. Recent data from a castrate resistant prostate cancer (CRPC) cohort showed deep deletions in ~30% of patients [7].

Though most commonly inactivated by biallelic deletion in prostate cancer, PTEN may be inactivated by alternative genetic and epigenetic mechanisms. PTEN may also be lost by chromosomal rearrangements which have been reported in primary prostate cancer; however, the relative frequency of these events remains unclear [33]. The frequency with which PTEN is inactivated by mutations also remains unclear in prostate cancer. Although early Sanger sequencing studies reported a high rate of mutations and methylation in the PTEN promoter region, it is likely that some of these studies were confounded by the existence of a PTEN pseudogene (PTENP1) that harbors a high rate of such changes [34, 35]. More recent exon sequencing studies have shown mutation rates hovering around 5% in primary tumors, of which many have hemizygous deletions involving the second allele [3–7]. Of interest, the majority of mutations are truncating mutations, with relatively few missense mutations. However, larger whole genome sequencing studies will be required to fully assess PTEN mutation frequency.

The advent of highly specific mouse monoclonal antibodies for detection of PTEN protein has enabled the development and validation of immunohistochemistry (IHC) assays to assess prostate cancer for PTEN protein loss [36, 37]. In addition to allowing larger-scale studies of association of PTEN loss with clinical outcome (see below), these assays have shed important light on mechanisms of PTEN inactivation in prostate cancer. First, PTEN protein loss is commonly focal or heterogeneous in primary prostate cancer, occurring in some but not all tumor cells within a given nodule (Fig. 21.1). This suggests that PTEN loss is a relatively late (at least compared to ERG gene rearrangement [29, 38–40] event during the evolution of a primary tumor and is consistent with the relatively low rates of PTEN loss observed in isolated prostatic intra-epithelial neoplasia (PIN), widely believed to represent a precursor to invasive prostate cancer [41, 42]. In addition, comparison of PTEN IHC to PTEN FISH has revealed that non-deletion events to inactivate PTEN may be relatively common in invasive tumors. In all of the studies that have carefully compared IHC and FISH, ~30% of cases with PTEN protein loss fail to show underlying PTEN homozygous deletion [22, 28, 36] (Fig. 21.2). Though this may be in part explained by the relative frequency of smaller insertion/deletion events (indels) that are not detectable by FISH, mutations involving the PTEN promoter, epigenetic modifications, or other alternative mechanisms of PTEN inactivation may be more common than previously believed. Indeed, the relative frequency of PTEN promoter methylation in prostate cancer appears to be low [35, 43], and when it occurs, the functional consequences of this finding are unclear. The PTEN promoter is shared with that of a p53-target gene (KILLIN) which may be a tumor suppressor in its own right [44]. Recent studies have elucidated the role of microRNAs and pseudogene deletion in the regulation of PTEN protein levels [45, 46]. Further, the first whole genome sequencing study for prostate cancer identified chromosomal translocations in MAGI-2, a membrane-associated guanylate kinase known to bind and stabilize PTEN protein [3]. While the rate of alterations in MAGI-2 in prostate cancer remains unclear, the small number of cases reported did not show coexisting PTEN deletions, suggesting that these genetic changes may be

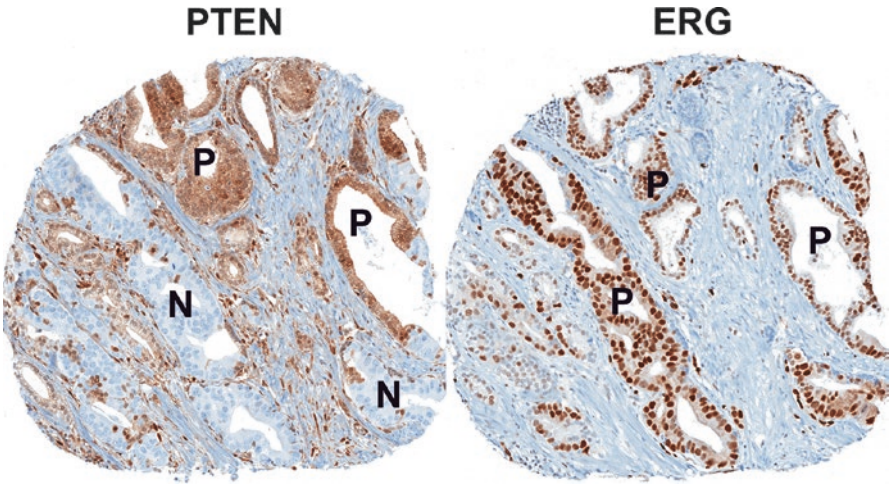

Fig. 21.1 PTEN loss most likely occurs after ERG rearrangement. PTEN and ERG immunohistochemistry (IHC) in a representative prostate tumor sample, demonstrating heterogeneous PTEN loss in tumor glands with some positive (P) areas and some negative (N) areas on a homogeneous ERG background

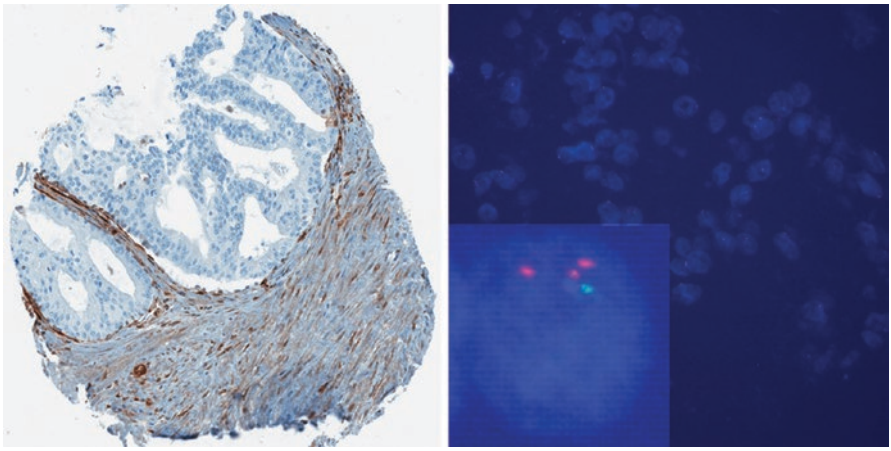

Fig. 21.2 Prostate tumor case with discordant PTEN fluorescence in situ hybridization (FISH, *right panel*) and immunohistochemistry (IHC, *left panel*) result suggests frequent occurrence of PTEN protein loss in context of one apparently intact allele of PTEN by FISH. PTEN FISH depicted here uses a four-color probe across PTEN (*orange*), two flanking genes on 10q (*green* and *aqua*) and a centromeric probe (*red*). This suggests the presence of a mutation, indel, or epigenetic event silences the other allele in some cases leading to absence of protein expression in tumor glands compared to stroma which serves as internal positive control

functionally redundant for tumor cells. Overall, these data strongly suggest that PTEN inactivation in prostate cancer occurs through a number of mechanisms, many of which have yet to be described.

Association of PTEN Loss with Clinical Outcome in Prostate Cancer

The wide range in reported frequency of PTEN genomic loss in prostate cancer likely reflects the close association of PTEN loss with high-risk pathologic features and an association with development of CRPC. Innumerable studies have found a significant correlation between PTEN loss and high-risk pathologic variables at radical prostatectomy, most importantly with increased pathologic stage and Gleason grade [36]. Accordingly, PTEN loss in Gleason score 6 needle biopsies is independently associated with an increased risk of upgrading to Gleason score 7 or higher at radical prostatectomy [47]. Despite this close association with pathologic variables, PTEN loss is also independently correlated with decreased time to biochemical recurrence after radical prostatectomy in multiple studies [23–25, 30, 43]. Though the association with biochemical recurrence is most significant for homozygous gene deletions, many studies have documented an association with hemizygous deletions as well. This may indicate that either: (1) PTEN is a haploinsufficient gene or, (2) in cases of hemizygous loss, the second allele is commonly inactivated by additional mechanisms which are not detected. Interestingly, there is limited evidence for PTEN haploinsufficiency in mouse prostatic tumorigenesis [48–50]. PTEN loss is also associated with an increased risk of death from prostate cancer, though fewer studies have investigated this since large cohorts are required to examine prostate-cancer-specific mortality (PCSM). Using FISH or immunohistochemistry, PTEN loss was strongly associated with PCSM in a cohort of conservatively managed prostate cancer patients diagnosed on transurethral resection of the prostate (TURP) [51]. In surgical cohorts, PTEN loss by immunohistochemistry is associated with increased risk of metastasis in high-risk radical prostatectomies [36, 52], as well as increased risk of CRPC and PCSM in a smaller biopsy cohort [53].

Association of PTEN Loss with Androgen Receptor (AR) Signaling, Response to Androgen Deprivation Therapy (ADT), and Development of CRPC

Mouse models have suggested that AR signaling and PI3K/AKT signaling may be subject to reciprocal inhibition in the prostate [10, 11, 54]. Thus, PTEN inactivation may promote castration-resistant tumor growth through suppression of androgen receptor (AR) levels and AR transcription factor activity with

accompanying inhibition of AR-regulated negative feedback of PI3K/AKT signaling. Gene expression data from human tumors has largely supported this hypothesis [10, 11, 54], and murine prostate tumor models with PTEN loss appear to be more likely to develop androgen-independent lesions than many models without PTEN loss [10, 55, 56]. Of interest, ERG may modify the association of PTEN loss with suppressed androgen signaling [54]. The association of PTEN loss with development of CRPC in humans has also been studied. PTEN loss is relatively enriched in CRPC compared to primary, untreated prostate tumors, suggesting potential selection for the alteration upon androgen inhibition [3, 5, 28, 36]. There is limited evidence that PTEN loss may portend resistance to androgen deprivation therapies (ADT), including some of the newer drugs such as abiraterone [57]; however, additional studies are required to test whether PTEN loss may serve as a predictive biomarker in the setting of ADT.

Association of PTEN Loss with ERG Rearrangement and Other Molecular Alterations in Prostate Cancer

PTEN deletion is 2–5 times more common among prostate tumors with ERG gene rearrangements compared to those without ERG rearrangements [4, 25, 28, 33, 38, 43, 58–61]. Given that ERG expression is most commonly homogeneous within a given primary tumor nodule and PTEN loss is frequently heterogeneous on this background, PTEN loss almost certainly occurs subsequent to ERG gene rearrangement in most cases [29, 38, 40]. This fact led several groups to hypothesize that there may be a synergistic effect of ERG expression and PTEN loss on prostate cancer progression, and data from mouse models has supported this possibility [54, 58, 59]. However, the data from human studies have been mixed. While some studies have suggested that ERG-rearranged PTEN-deleted tumors may have an increased risk of biochemical recurrence compared to PTEN-deleted tumors lacking ERG rearrangement [25, 62, 63], the largest FISH-based study showed no interaction of PTEN and ERG status with respect to risk of biochemical recurrence [30]. Only one study has examined the interaction of PTEN and ERG and their association with prostate-cancer-specific mortality in a cohort of 308 patients managed conservatively [60]. Interestingly, PTEN deletion detected by FISH was associated with increased risk of prostate cancer mortality among ERG-rearrangement-negative but not among ERG-rearrangement-positive tumors. However, in a subsequent study of 652 patients (including the original 308 patients), the authors failed to validate this interaction between PTEN deletion and ERG status with respect to prostate cancer death [51].

Though numerous additional genomic changes have been shown to be associated with PTEN loss, the strong association of PTEN loss with ERG gene rearrangement is a confounder in many studies. Genome-wide copy number

evaluation has suggested that PTEN loss is strongly associated with a higher burden of overall copy number alterations (CNA) in prostate cancer [64]. These data may be consistent with in vitro experiments from other systems suggesting that PTEN has critical nuclear functions, including regulation of DNA double-strand break repair and sensitivity to genotoxic stress [65, 66]. However, overall burden of CNA is itself among the most highly prognostic genomic classifiers in prostate cancer [67]; thus, it remains formally possible that PTEN deletion is simply more common with a generalized higher CNA burden, which is the central driver of prognosis.

Targeting Tumors with PTEN Loss and/or PI3K Activation in Prostate Cancer

Numerous PI3K/AKT/mTOR inhibitors have been developed over the past decade. However, despite widespread activation of PI3K/AKT/mTOR signaling in epithelial tumors, single-agent therapy with targeted inhibitors for this pathway has largely failed in most clinical trials. In prostate cancer, this may be consistent with preclinical models showing reciprocal inhibition between AR signaling and PI3K signaling [10, 11]. Indeed, clinical trials testing the utility of PI3K/AKT/mTOR inhibition in the context of AR axis signaling suppression are currently underway (e.g., phase 1/2 single-agent ARN-509, phase 1/2 abiraterone + mTOR/PI3K inhibitor BEZ235, phase 2 Akt-inhibitor GDC-0068 or PI3K/mTOR inhibitor GDC-0980 with abiraterone vs. abiraterone alone, phase 2 ARN-509 + PI3K/mTOR inhibitor everolimus). The hope is that the results from these trials will be similar to the breast cancer BOLERO-2 trial, where a combination of hormonal therapy and mTOR inhibition was quite promising [68].

However, to fully evaluate these precision medicine trials, it will be critical to examine the efficacy of each therapy in the context of PTEN status, since tumors with PTEN loss would be predicted to derive the largest benefit from combination therapy. This will require the development of clinical-grade assays to reliably assess PTEN status, at the DNA and/or protein level, using formalin-fixed paraffin embedded (FFPE) tumor tissue available from prostate or metastatic biopsy and/or radical prostatectomy. Because PTEN loss is frequently focal in primary tumors and may be missed by assays requiring nucleic acid purification, in situ assays such as IHC or FISH will likely be most useful to screen for PTEN loss and to fit into the standard workflow of most pathology laboratories [36]. Though many such assays have been validated for use in research environments, none to date have been validated to the level required for FDA clearance of a companion biomarker assay. Ultimately, surmounting the hurdles of pre-analytic, analytic, and post-analytic validations of PTEN loss assays will be critical to advancing the promise of precision medicine from the bench to the bedside [69].

RAF/MEK/ERK

Overview of RAF/MAPK/ERK Pathway Signaling in Prostate Cancer

In contrast to the central role played by PTEN/PI3K/AKT/mTOR signaling in prostate cancer, less is known about the role the mitogen-activated protein kinase (MAPK) pathways. ERK is one of best characterized MAPK family members and the one most commonly activated by upstream growth factor stimulation via activation of RAS/RAF/MEK signaling [70]. Once activated by receptor tyrosine kinase signaling, RAS, a GTPase, is recruited to activate the protein kinase activity of RAF, which can phosphorylate and activate MEK, with subsequent activation of ERK. ERK phosphorylation induces nuclear translocation, with concomitant phosphorylation and regulation of multiple transcription factors impacting cellular behaviors relevant to tumorigenesis. The central role of this pathway in other solid tumors is evidenced by the frequency of activating KRAS and BRAF mutations in pancreatic adenocarcinomas, papillary thyroid carcinomas, and melanomas [70]. High rates of RAS/RAF activation reported for primary (~40%) and metastatic prostate tumors (~90%), primarily based on copy number alterations in a number of genes in the pathway (KRAS, PTPN11, NRAS, BRAF, RAF1, SPRY1, SPRY2) [32]. However documented activating mutations in these genes occur in only 1–2% of prostate cancers in keeping with the relatively low rate of mutations overall in prostate cancer [3–7]. Though activating gene fusions involving KRAS [71] and BRAF [72] have been reported as one mechanism of ERK activation in prostate cancer, these are also quite rare (1–2% overall). Of interest, these rare RAF kinase fusions result in constitutive RAF activity that is sensitive to RAF inhibitors [72].

In Vitro and Preclinical Studies

Despite a lack of concrete evidence that RAS/RAF/MAPK/ERK are genetic drivers in human prostate cancer, older in vitro signaling studies in prostate cancer cell lines have supported a role for MAPK signaling in the development of androgen independence [73–76]. BRAF activation (via expression of BRAF V600E) is sufficient to drive prostatic tumorigenesis in the mouse [77], though KRAS activation (G12D) did not initiate tumor development [78]. In addition, there is ample evidence of cross-talk between the PI3K and MAPK signaling pathways in many systems, and the prostate is no exception. Accordingly, in mouse models, co-activation of PI3K signaling (via conditional ablation of PTEN) and MAPK signaling (via expression of oncogenic KRAS or BRAF mutants) results in highly penetrant metastatic prostate cancer [78–80].

Conclusions

As the genomic landscape of prostate cancer has become increasingly clear, the molecular mechanisms by which oncogenic intracellular signaling circuits are dysregulated in prostate cancer are slowly coming to light. Loss of the PTEN tumor suppressor by genomic deletions is a key mechanism underlying upregulation of PI3K/AKT/mTOR signaling in prostate tumors. In addition, there may be sizable populations of patients with rarer activating mutations and amplifications of other components of PI3K/AKT signaling. Though less is known about the role of MAPK signaling in prostate tumor initiation and progression, cross-talk between the PI3K and MAPK pathways is clearly significant and inhibiting both pathways will undoubtedly be critical to avoiding the development of therapeutic resistance [81]. With increasing numbers of drugs available to target these pathways, the current challenge of molecular pathology is to analytically and clinically validate assays to query aberrations and signaling activity along these circuits in order to guide our ever-increasing armamentarium of targeted therapies to the patients who will benefit most.

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Chapter 22
The Cell Cycle and Androgen Signaling Interactions in Prostate Cancer

Simone de Brot and Nigel P. Mongan

Contents

The Cell Cycle and Prostate Cancer

In this chapter, we will review the cell cycle in normal and malignant prostate cells. In particular, we will outline how androgen signaling and cell cycle are mechanistically inter-dependent and implicated in prostate cancer (PCa). Huggins and Hodges pioneering 1941 study established the essential role for androgen signaling in prostate carcinoma [1]. In the intervening decades, the molecular basis of this androgen dependence has been extensively studied. The molecular functions of the androgen receptor (AR) as a ligand-dependent transcription factor have been described elsewhere in this book (part IV, Chap. 20). Androgens and the AR are now known to play essential roles in driving cell cycle progression of androgen-dependent cells [2, 3]. Conversely, androgen deprivation induces cell cycle arrest in those PCa cells

S. de Brot, D.V.M., Dip. E.C.V.P.
Faculty of Medicine and Health Sciences, School of Veterinary Medicine and Health Science, University of Nottingham, Nottingham, UK

N.P. Mongan, B.Sc (Hons), Ph.D, F.R.CPath (✉)
Faculty of Medicine and Health Sciences, School of Veterinary Medicine and Health Science, University of Nottingham, Nottingham, UK

Department of Pharmacology, Weill Cornell Medicine, New York, NY 10065, USA
e-mail: nigel.mongan@nottingham.ac.uk; npm2001@med.cornell.edu

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*, Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_22

which depend on androgens. Here, we will describe the role of androgen signaling in driving cell cycle progression and will summarize the current knowledge of how critical cell cycle proteins including cyclins and cyclin-dependent kinases (CDKs) interact with the AR, modulate transcriptional activity in response to androgens, and contribute to PCa pathogenesis.

Normal Cell Cycle

The normal mammalian cell cycle (Fig. 22.1) consists of transitions through several temporally distinct phases monitored by intracellular checkpoints [4]. These states include the initiation and completion of DNA replication (synthesis or S-phase) and

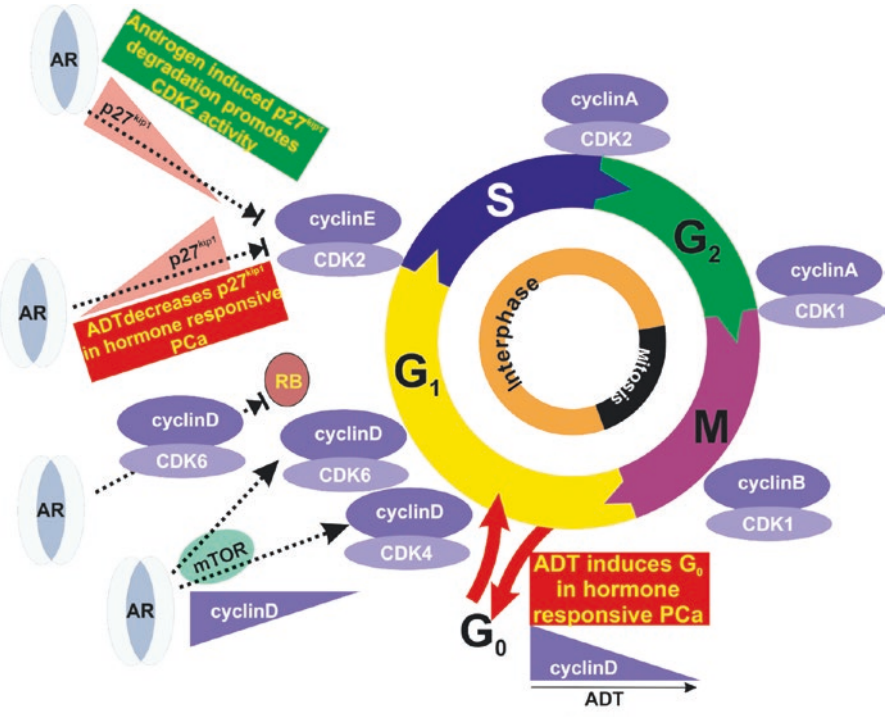

Fig. 22.1. The normal mammalian cell cycle consists of transitions through several temporally distinct phases monitored by intracellular checkpoints. These states include the initiation and completion of DNA replication (synthesis or S-phase) and of cell division or mitosis (mitosis or M-phase). Between these phases are gaps (G1 and G2 phases). In response to mitogenic signals such as androgens, quiescent cells (G0 phase) enter the cell cycle, and genes encoding D-type cyclins (D1, D2, and D3) are induced. As the cells progress through G1 phase, the cyclins assemble with their catalytic partners, CDK4 and CDK6, to drive cell cycle progression. During androgen deprivation therapy (ADT)-induced cell cycle arrest, cyclin D levels decrease and p27kip1 increases. Conversely, androgens acting via the androgen receptor promote cyclin-CDK complex activity and decreased p27kip1 to drive cell cycle progression

of cell division or mitosis (mitosis or M-phase). Between these phases are gaps (G1 and G2 phases). In mammalian cells, the restriction point in late G1 denotes a point at which the cell commits itself irrevocably to another round of DNA replication [4]. Passage through the restriction point and the different cell cycle phases are controlled by cyclin-dependent protein kinases (CDKs) that are regulated by cyclins [5].

In response to mitogenic signals, quiescent cells (G0 phase) enter the cell cycle, and genes encoding D-type cyclins (D1, D2, and D3) are induced. As the cells progress through G1 phase, the cyclins assemble with their catalytic partners, CDK4 and CDK6 [6]. Assembled cyclin D-CDK complexes then enter the cell nucleus where they phosphorylate the retinoblastoma protein (Rb), contributing to its inactivation in mid-G1 phase [6]. Cyclin E-CDK2 subsequently becomes active and completes this process by phosphorylating Rb on additional sites [6]. Cyclin A-dependent and B-dependent CDKs activated later during the cell division cycle maintain Rb in a hyperphosphorylated inactive form until cells exit mitosis [6]. Rb hyperphosphorylation disrupts its association with the E2F transcription factor, allowing the transcription of genes that mediate S-phase entry [6–8].

CDKs activities are constrained by two families of CDK inhibitors (CKIs). The first class includes the INK4 proteins (inhibitors of CDK4) p16INK4a, p15INK4b, p18INK4c, and p19INK4d that specifically inhibit the catalytic subunits of CDK4 and CDK6 [6]. The INK4 proteins can be contrasted with the more broadly acting CDK inhibitors of the Cip/Kip family that include p21CIP1, p27KIP1, and p57KIP2 [5, 6]. The gene for p21CIP1 (CIP1) can be induced by the tumor suppressor protein p53 [5], whereas p27KIP1 and p57KIP2 are more directly involved in restriction point control [5]. Expression of p21CIP1 (CIP1) is androgen regulated [9, 10], providing a mechanism for androgen-mediated fine-tuning of cell cycle progression in non-malignant cells.

Although cell cycle transitions depend on the underlying CDK cycle, superimposed checkpoint controls help ensure that certain processes are completed before others begin [5]. The tumor suppressor protein p53 is an archetypal checkpoint regulator [5, 11]. p53 is promoted by p14 alternative reading frame (ARF) (p14ARF) which prevents the degradation of p53 by its inhibitor MDM2 (mouse double minute 2 homolog) [11–13]. As we will see later, p14ARF may have a pro-oncogenic role in PCa.

Cell Cycle and Cancer

Cell cycle deregulation is a common feature of human cancer [5, 14, 15]. The three basic cell cycle defects are (1) unscheduled cell proliferation, (2) genomic instability resulting in increased DNA mutations and chromosomal aberrations, and (3) chromosomal instability resulting in changes in chromosome number [14]. These cell cycle defects are largely mediated, directly or indirectly, by misregulation of cyclin-dependent kinases (CDKs) [14]. The cyclin/CDK/retinoblastoma (Rb) axis is a critical modulator of cell cycle entry, and cyclins and CDKs are often

overexpressed in many human cancers [16, 17]. Accordingly, the INK4 and CIP/KIP CDK inhibitors are frequently mutated, deleted, or silenced in tumors, leading to their loss of function in cancer cells [17].

Cell cycle deregulation with subsequent oncogenic proliferation occurs through two principal means in tumors: (1) loss of the CDK inhibitor p16INK4a and (2) loss of the tumor suppressor protein Rb [18]. Functional inactivation of the p16INK4a/Rb axis is common in many cancer types [19]. Loss of P16INK4a prevents the suppression of CDK4 or CDK6 activity, whereas loss of Rb deregulates downstream signaling in the cell cycle [18, 20, 21]. The p16 encoding gene (CDKN2A) is mutated or silenced in many tumor types [22–24]. Rb is believed to be directly or indirectly inactivated in nearly all human cancers [20, 21, 25]. Its inactivation occurs either by direct mutation/deletion or indirectly through altered expression/activity of upstream regulators [25]. These regulators include cyclin D1, CDK4, and loss of p16INK4a, among others [25]. Another mechanism of deregulating the CDK4/CDK46-Rb axis is the direct oncogenic activation of CDK4 or CDK6 activity. Overexpression of CDK4 and CDK6 is observed in several malignancies [14, 18, 26, 27]. Of the various cyclins, amplification or overexpression of cyclin D1 is most frequently associated with human malignancies [15, 18, 28, 29]. In most cancer types, cyclin D1 overexpression results from induction by oncogenic signals rather than amplification of the cyclin D1 (CCND1) gene [28]. Interestingly, E-type cyclins are often overexpressed in human tumors [18, 30–34], allowing cancer cells to bypass the need for CDK4 or CDK6 activity to initiate the S-phase. Furthermore, deregulation of CDK2 also occurs frequently in certain types of cancer [18, 34], contributing to the further phosphorylation of Rb and the initiation of DNA replication. The CDK inhibitor p27KIP1 is inactivated in many cancers through impaired synthesis, accelerated degradation, and mislocalization, although it is rarely mutated or deleted [18, 35]. Interestingly, in contrast to the genetic deregulation of the CDKs that coordinate the S-phase, there is limited evidence to show that CDK1 activity is dysregulated by direct genetic alteration in tumorigenesis [18, 36]. Disruption of p53 signaling or of DNA damage checkpoints indirectly leads to the deregulation of CDK1, and high cyclin B1 expression is generally associated with a more aggressive cancer phenotype [18, 36, 37]. However, the requirement that CDK1 activity must be attenuated to exit mitosis and the lethal aspects of uncoordinated CDK1 activity are likely to limit its potential as a direct oncogenic driver. Inactivation of the tumor suppressor gene p53 contributes to the carcinogenesis and/or progression of a substantial fraction of all human cancers [38, 39].

Cell Cycle Regulators and Prostate Cancer

Numerous proteins involved in cell cycle regulation are often mutated in PCa (Fig. 22.2, Table 22.1) [12, 39–84]. The diversity of currently identified somatic genetic abnormalities associated with PCa suggests that there is not a single dominant molecular pathway required for prostate carcinogenesis, but rather the complex

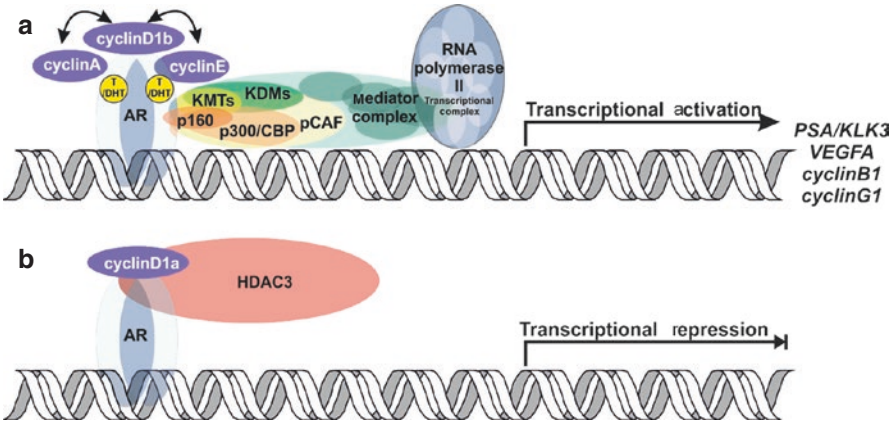

Fig. 22.2. (**a**) Androgen (testosterone/dihydrotestosterone)-activated androgen receptor (AR) interacts with multiple enzymatically diverse epigenetic coregulators including lysine acetyltransferases (KATs, p300/CBP, and pCAF), methyltransferase (KMTs), demethylases (KDMs), and the multimeric mediator complex to stabilize the recruitment of RNA polymerase II to androgen-activated loci. Cyclin A, cyclin E, and cyclin D1b interact with AR and promote androgen signaling. (**b**) In contrast, the cyclin D1a isoform interacts with AR but facilitates the recruitment of a histone deacetylase (HDAC)-3 containing transcriptional repressor complex which functions to repress AR-mediated transactivation

Table 22.1 Cell cycle proteins commonly mutated in prostate cancer

Factor	Study size (*n*)	Change observed	Ref.
p16INK4a	*n* = 5 cell lines (Met) *n* = 18	(*n* = 1) Two SSCPs: one missense mutation (codon 84) and one polymorphism ((C/G) in base 494) (*n* = 18) No DNA mutations (*n* = 1) One SSCP ((C/G) in base 494)	[40]
	n = 104	Overexpression in 95%; loss of nuclear expression in 13%; no prognostic significance	[41]
	n = 88	Nuclear overexpression in 43% and loss of expression in the remaining 57%; overexpression is associated with tumor recurrence and a poor clinical course	[42]
	n = 72 *n* = 49 PCa *n* = 23 Met	Overexpression in 83%, in correlation with more rapid treatment failure and higher histologic grade; loss of expression more commonly in metastatic specimens	[43]
	n = 111 *n* = 69 low-grade *n* = 42 high-grade	Overexpression in 25% low-grade PCa and 43% high-grade PCa, respectively	[44]
	n = 612	Reduced expression is associated with a higher risk of distant metastases in patients undergoing androgen-deprivation therapy	[45]
	n = 70	Expression has no prognostic significance	[46]
	n = 137	Overexpression in 86%	[47]

(continued)

Table 22.1 (continued)

Factor	Study size (*n*)	Change observed	Ref.
Rb	*n* = 40	60% with LOH of the Rb gene	[48]
	n = 41	37% with loss of a single Rb1 allele	[49]
	n = 26	35% with LOH of the Rb locus; 33% of specimens with LOH show low or no Rb protein	[50]
	n = 116 *n* = 112 PCa *n* = 4 Met	3/68 PCa with Rb gene alterations; 17% with reduced Rb mRNA expression, in correlation with increasing tumor stage and grade	[51]
	n = 81 *n* = 33 AD therapy *n* = 48 untreated	Reduced expression in 36% of antiandrogen treated and in 13% of untreated PCa; low Rb correlates with higher tumor grade	[52]
	n = 85 *n* = 63 after ADT *n* = 22 before ADT	Loss of the Rb gene is four times more frequent after AD therapy than before therapy (22% versus 6%)	[53]
	n = 72 *n* = 49 PCa *n* = 23 Met	19% with loss of expression; no correlation with p16INK4a expression; loss of expression more commonly in metastatic disease (55%)	[43]
	n = 156 CRPC *n* = 22 human xenografts	Rb loss is overrepresented in CRPC; Rb loss is associated with hormone therapy failure and poor outcome	[54]
	n = 218	Downregulated Rb gene expression in 5% of primary but 37% of metastatic samples	[55]
CyclinD1	*n* = 140	78% with no or low expression	[56]
	n = 66	70% with no or low expression	[57]
	n = 156	Overexpression is rare and only seen in the most poorly differentiated and advanced-stage lesions	[58]
	n = 36	Expression in 36%; the majority with high cytoplasmic and no nuclear expression; nuclear expression correlates with grade	[59]
	n = 119	Nuclear expression in 57% (primary tumors) and 62% (metastases); cytoplasmic expression in 61% (primary tumors) and 50% (metastases); no correlation of expression with different Gleason patterns; in the metastases, high-level cytoplasmic cyclin D1 expression predicted poor outcome	[60]
	n = 85	Expression in 75%; high-grade Gleason score group with a higher (40%) mean expression compared to the low-grade Gleason score group (27%)	[61]
	n = 30	Expression in 100% with intense expression in 47% (24/30 with both nuclear and cytoplasmic expression); no correlation between intensity of expression and histologic tumor grades	[62]

Table 22.1 (continued)

Factor	Study size (*n*)	Change observed	Ref.
P27KIP1	*n* = 50 *n* = 45 PCa *n* = 5 Met	Reduced or no (68%) expression; reduced expression correlates with increased tumor grade; the loss of p27KIP1 is associated with an increase in the proliferative index	[63]
	n = 138	Low expression is associated with a higher Gleason score and poor prognosis	[64]
	n = 130	Abundant p27KIP1 mRNA but either high or low to undetectable levels of p27KIP1 protein. Low protein expression is associated with a more aggressive tumor type	[65]
	n = 96	Reduced expression is associated with tumor grade, with an increased probability of recurrence and decreased survival	[66]
	n = 113	Reduced expression and correlating with higher Gleason grade	[67]
	n = 86	Absent or low expression is an adverse prognostic factor	[68]
	n = 83 *n* = 73 PCa *n* = 10 Met	Reduced expression in primary tumors and metastatic lymph nodes (Met) with lowest expression in Met; no association between p27KIP1 expression and Gleason score or clinical stage	[69]
	n = 95	Loss of expression is associated with disease recurrence after radical prostatectomy	[70]
	n = 92	Low (<50%) expression is associated with a poor outcome	[71]
	n = 86	Loss of expression is associated with disease recurrence after radical prostatectomy	[72]
	n = 70	Expression has no prognostic significance	[46]
	n = 3701	Expression negative in 18.6%, weak in 33.5%, moderate in 28.4%, and strong in 19.5%; loss of expression associated with low-grade tumors; no correlation with clinical outcome	[73]
p53	*n* = 137	6% with high, 11% with low, and 83% with no expression; high expression associated with high histologic grade, high cell proliferation rate, and poor prognosis	[74]
	n = 150	Nuclear accumulation of (mutated) p53 in 13%, in correlation with disease stage	[39]
	n = 92	Nuclear accumulation of (mutated) p53 is associated with tumor stage, grade, and androgen sensitivity	[75]
	n = 139	Expression in 15%, independent of tumor stage and Gleason score, and with no effect on prognosis	[76]
	n = 61 *n* = 26 CRPC *n* = 27 PCa *n* = 8 Met	Elevated expression in 94% of hormone refractory specimens, 50% of metastatic tumors, and 22% of primary tumors; clear progression of increased p53 alteration from primary to hormone refractory disease	[77]

(continued)

Table 22.1 (continued)

Factor	Study size (*n*)	Change observed	Ref.
	n = 69 *n* = 36 PCa *n* = 17 Met (lymph node) *n* = 15 Met (bone)	Nuclear expression in 28% of the primary tumors, 59% of the lymph node metastases, and 43% of the bone metastases; increased expression is correlated with increased histologic grade and with the presence of metastatic disease	[78]
	n = 109	Expression in 21%, in correlation with tumor progression	[79]
	n = 109 *n* = 62 PCa *n* = 47 Met	PCa: 10/55 with loss of the p53 gene, overexpression in 2/38; metastatic tissue (Met): Overexpression in 62%; no correlation between expression and progression or outcome	[49]
	n = 129	Abnormal expression in 18%, associated with incidence of metastases and poor prognosis	[80]
	n = 37	Loss of functional p53 in 46%, associated with progression	[81]
p14ARF	*n* = 9	Elevated p14ARF mRNA in 22%	[82]
	n = 16	3/16 with loss of expression, correlating with homozygous deletion or promoter hypermethylation	[83]
	n = 32	3% with methylation of p14ARF gene	[12]
	n = 111 *n* = 69 low-grade PCa *n* = 42 high-grade PCa	Expression in 100% of both low-grade and high-grade PCa	[44]

AD androgen deprivation, *CRPC* castrate-resistant prostate cancer, *LOH* loss of heterozygosity, *Met* metastatic, *PCa* prostate cancer, *Rb* retinoblastoma protein, *SSCP* single-strand conformation polymorphism

effects of mutations that disrupt cell cycle control pathways [84]. Cell cycle proteins commonly mutated during PCa progression (summarized in Table 22.1) include the negative cell cycle regulators Rb, p14ARF, p16INK4a, p53, and p27KIP1 [12, 39, 48, 63, 75, 84]. Mutations within these proteins result in defective cell cycle checkpoint control, leading to further chromosomal instability and inactivation of the tumor protective cellular senescence [84].

p14^{ARF}/p16^{INK4a} (CDKN2A) and PCa

As mentioned previously, loss of the negative cell cycle regulators p16INK4a and Rb is common during the tumorigenesis in many human cancers [18]. Loss of p16INK4a is however only infrequently observed in PCa, whereas inactivation of Rb is common [12, 21, 48, 84, 85]. Unlike other primary tumors, p16INK4a inactivation, through deletions, mutations, or promoter methylation, seems to be an infrequent event in primary PCa [21, 40, 46]. Surprisingly, p16INK4a seems to be overexpressed rather

than lost in a large proportion of prostate carcinomas [21, 41–44, 46, 47], and increased p16INK4a levels have been associated with poor prognosis [21, 40, 42, 43, 45]. More frequent alterations of p16INK4a in metastatic disease suggest that this may be a later event during PCa progression [43, 45]. Loss of the p53 stabilizer and tumor suppressor p14ARF is frequently found in various cancers [12]. The roles of p14ARF in cancer remains however poorly understood [86]. In PCa, however, p14ARF is reported to be upregulated, and its increased level is associated with advanced and metastatic stages [44, 82, 83, 87, 88]. These observations suggest that p14ARF can act in a p53-independent manner to promote the progression of some tumors [88]. Interestingly, it could recently be shown that p14ARF can bind to the androgen receptor (AR) and function as an AR corepressor in both an androgen-dependent and androgen-independent manner [87]. This direct interaction of p14ARF with the AR may contribute to the development and progression of PCa [87].

Rb and PCa

The primary function of Rb is to repress expression of proteins required for cell cycle progression and thereby induce cell cycle arrest. This is achieved primarily by blocking the action of the E2F transcriptional activator ([89] and references therein). Inactivation of the tumor suppressor Rb is common in PCa and appears to be an early event in prostatic tumorigenesis [21, 43, 48–55, 84, 90, 91]. Strikingly, recent observations indicate that Rb has specialized roles in controlling androgen receptor (AR) expression in PCa and primarily protects against progression to castration resistance [54, 92, 93]. Hence, Rb is frequently lost or functionally inactivated in castrate-resistant PCa [21, 52, 54, 92], suggesting that loss of Rb is associated with transition to the incurable, castration-resistant disease state [21, 54, 92]. Indeed, loss of Rb function represents a critical event in sensitizing PCa cells to androgens. Rb loss relieves repression on E2F activation of cyclins required for G1 and enables E2F activation of AR expression [94], which in turn promotes G1 transition [95]. Furthermore, Rb is required for AR-regulated repression of the EZH2 (enhancer of zeste homolog 2) polycomb protein [96]. Thus, loss of Rb contributes to the increased EZH2 levels found in aggressive PCa [97]. Therefore, inactivation of Rb function (1) sensitizes PCa cells to androgens, (2) drives PCa progression by a convergence of multiple mechanisms, and (3) enables E2F transcriptional activation of the AR. Collectively, loss of Rb primes PCa cells to have sufficient AR to drive transcriptional activity and mediate mitogenic effects of androgen [94].

Cyclin D and PCa

Amplification or overexpression of cyclin D is one of the most commonly observed alterations in human malignancies [28]. However, in PCa both low [56–58] and elevated cyclin D1 expression has been reported [59–62]. Hence, the importance of

elevated cyclin D1 expression in prostatic tumor development or progression is unclear [21]. Interestingly, modest elevations of cyclin D1 in the presence of androgen inhibit, rather than enhance, cellular proliferation [28, 98, 99]. This unexpected capacity of cyclin D1 to attenuate cell cycle progression is specific to AR-positive PCa cells and indicates a functional relationship between cyclin D1 and AR [98]. Consistent with this, cyclin D1 can bind AR and has been shown to act as a critical regulator of androgen-dependent transcription and cell cycle progression [28, 62, 93, 98, 100]. Modest increases in cyclin D1 levels are sufficient to suppress both AR activity and androgen-dependent proliferation in AR-positive PCa cells [98, 101, 102] (Fig. 22.3). These AR regulating functions of cyclin D1 are disrupted in PCa [98]. Furthermore, cyclin D levels decrease following androgen deprivation therapy (ADT) and contribute to cell

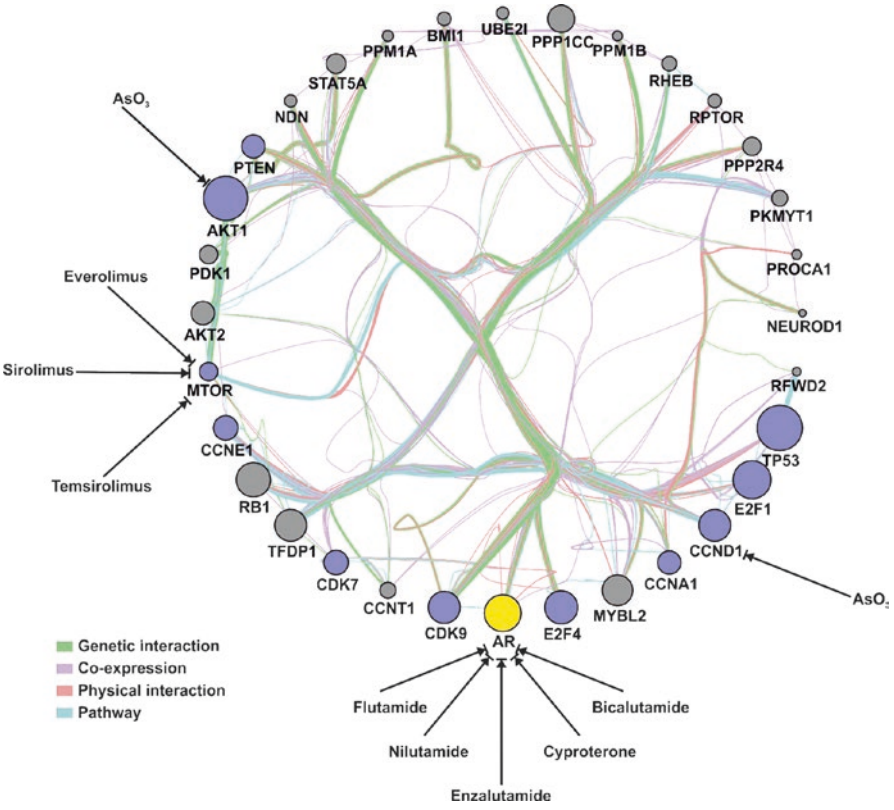

Fig. 22.3 The GeneMANIA Cytoscape plugin [101] was used to illustrate multiple functional interactions with the AR (indicated in *yellow*) and components of the cell cycle regulatory apparatus. Cell cycle proteins indicated in *blue* interact with AR [102]. Current pharmacotherapies which will influence the cell cycle include the antiandrogens (flutamide, nilutamide, enzalutamide, bicalutamide, and cyproterone acetate). The mTOR complex is targeted by everolimus, sirolimus, and temsirolimus. Both AKT and cyclin D1 are targeted by arsenic trioxide which is approved for use in acute promyelocytic leukemia

cycle arrest in hormone-dependent PCa [3, 103]. Restoration of functional androgen signaling restores cyclin D translation in an mTOR (mammalian target of rapamycin)-dependent mechanism [103]. As we will discuss later, the exact role for different cyclin D1 isoforms in the transition to androgen independence is still being studied [21].

p27^{KIP1}/CDKN1B and PCa

The CDK inhibitor p27KIP1 is commonly inactivated in several cancer types, and this loss is generally associated with poor prognoses [18, 35, 67, 104]. Loss of p27KIP1 has been shown to occur through miRNA-mediated inhibition of translation in PCa [35]. However, the prognostic relevance of p27KIP1 loss in PCa remains unclear [35, 40, 46, 64–73]. While quiescent normal prostate epithelia express high levels of nuclear p27KIP1 [35, 63, 73], p27KIP1 expression is reduced in malignant prostate tissue [63, 68–72]. Expression of p27KIP1 increases in hormone-dependent PCa cells in response to ADT [3]. Conversely, androgens can upregulate [105] or downregulate [106] p27KIP1 levels depending upon cellular context.

p53 and PCa

Inactivation of the p53 tumor suppressor gene is implicated in a large number of human cancers [38, 39]. Likewise, p53 mutations are believed to play a role in the progression of at least a subset of aggressive prostate cancers [39, 107, 108]. Although the frequency of p53 mutations in early PCa is low, mutated p53 alleles are found in 20–25% of advanced cancers [39, 75, 76, 84, 109]. Furthermore, functional loss of p53 is associated with advanced metastatic stage, loss of differentiation, and transition from androgen-dependent to androgen-independent growth [39, 74, 75, 77–81, 109]. Consistent with this, it is notable that expression of the AR gene is negatively regulated by p53 in nonmalignant prostate epithelial cells [110]. Therefore, loss of normal p53 function can contribute to increased AR expression. Indeed, p53 gain of function mutations contribute to androgen independence in experimental cell lines [111]. Furthermore, p53 status influences response to radiation therapy [112, 113]. For this reason, the development of novel therapies which restore p53 function is an area of active research [114].

PTEN/AKT/mTOR Signaling and PCa

As outlined in Chap. 21, the phosphatase and tensin homolog deleted on chromosome ten (PTEN) gene is a well credentialed tumor suppressor gene [115] implicated in advanced stage, higher-grade PCa [116, 117]. Targeted deletion of PTEN in

mouse models of PCa has revealed important roles for PTEN, p53, mTOR signaling, and p27 in metastases [118–122]. An important function of PTEN phosphatase activity is to inhibit PI3K/AKT pathway. Thus, loss of PTEN leads to an increase phosphoinositide 3-kinase (PI3K) activity, increased cellular PIP3 levels, and activation of downstream effectors including the AKT and mTOR protein kinases [123]. AKT influences multiple pro-oncogenic pathways including angiogenesis [124, 125] and the transition to hormone refractory PCa [126]. Indeed, there is evidence from mouse models that while reduced androgens and AR signaling enhance AKT activity [127], AKT can in turn function to upregulate AR and androgen signaling [128]. Similarly, mTOR signaling is implicated in prostate carcinogenesis [129, 130] and progression [131]. Preclinical and clinical studies of the combinatorial effects of AKT and mTOR pharmaco-inhibitors have shown significant promise in inhibiting proliferation of hormone refractory PCa cells [132–134].

Cell Cycle Regulators and Androgen Signaling Form Reciprocal Regulatory Circuits in PCa

The expression of important cell cycle regulators is altered in PCa (Fig. 22.4) [135]. In this section, we describe the mutual functional interactions between critical cell cycle regulators and androgen signaling, and we will relate these to mechanisms related to PCa carcinogenesis and progression.

Functional Interactions of AR and Cyclin D1

Complex mechanistic links between androgen signaling and cell cycle regulators have emerged and are well established for cyclin D1. The predominant cyclin D1 isoform, cyclin D1a, inhibits the transcriptional activity of AR [136], through

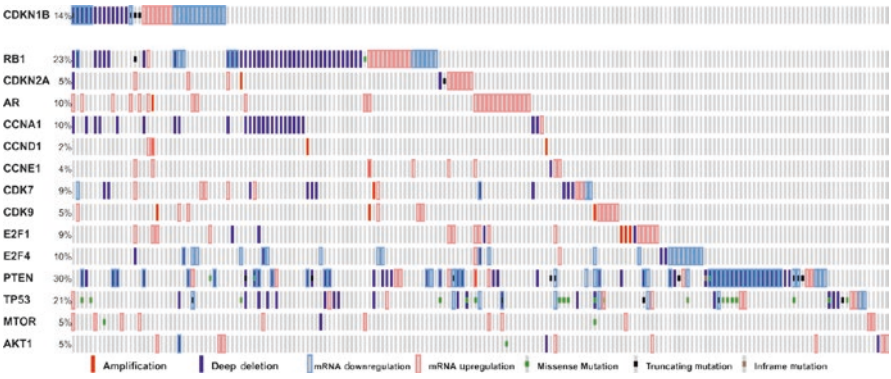

Fig. 22.4 The cBio portal [135] was used to characterize genetic changes in expression or mutation in key cell cycle proteins in the cancer genome atlas PRAD dataset ($N = 257$)

epigenetic mechanisms independent of its role in cell cycle regulation [3, 136–139]. Cyclin D1 directly interacts with AR [137, 138] and regulates AR recruitment to androgen-responsive loci [140]. The AR-cyclin D1 interaction does not require the cyclin D1 LxxLL motif commonly exploited in coactivator recruitment by agonist-activated nuclear receptor ligand-binding domains (LBD) [141]. Consistent with this, cyclin D1 has been shown to be recruited by N-terminal [100] and hinge [138] AR domains. Cyclin D1 appears to attenuate androgen signaling by impairing recruitment of the p160, p300, ARA70, and p/CAF coactivators [100, 138], enabling histone deactylase-3 (HDAC3) recruitment [142] and modulating AR-chromatin interactions [140]. As cyclin D1 inhibits androgen-induced transcription, cyclin D1 also reduces androgen-induced PCa cell proliferation [100]. While cyclin D1a is the most common splice form, cyclin D1b is an alternative splice form linked with the CCND1 G/A870 polymorphism associated with PCa [143, 144]. Cyclin D1b can interact with AR like cyclin D1a [139]. However, cyclin D1b fails to inhibit androgen-induced transcription and proliferation [139, 145]. Thus, whereas cyclin D1a mRNA expression is often reduced in PCa [55] and rarely increased [146], expression of cyclin D1b is frequently increased in PCa [145] and enhances androgen-induced proliferation [139]. More recently, evidence has emerged supporting a reciprocal cyclin D1-AR regulatory loop in specific cell contexts. The recruitment of the AR and DAX1 repressor to the CCND1 promoter cooperates to reduce cyclin D1 protein levels in MCF-7 breast cancer cells [147]. While the relevance of this AR-DAX1 mechanism to PCa is unclear, DAX1 is expressed in PCa [147]. Thus, while AR can increase cyclin D1 protein levels via the mTOR pathway in PCa cells [103], it is possible that AR-DAX1 inhibition of CCND1 expression overrides this and contributes to reduced cyclin D1 mRNA expression [55] and by extension relieves cyclin D1 inhibition of AR function in PCa.

Functional Interactions of AR and Cyclin A1 and Cyclin E1

While the functional interactions of cyclin D and androgen signaling are well established, there is evidence of mechanistic convergence of androgen signaling and additional cell cycle regulators. Functional interactions have also been described for AR and cyclin A1. Evidence from mouse models suggests cyclin A1 functions predominantly in the G2/M transition in meiotic cell division of male germ cells [148] and in leukemic stem cell division [149] and transition from G1 to S-phase in somatic cell division [150]. Cyclin A1 has long been implicated in leukemia [151, 152] and has more recently been implicated in solid tumors, including PCa [153–157]. Epigenetic silencing of CCNA1 expression by DNA promoter hypermethylation suggests cyclin A1 may act as tumor suppressor in head and neck squamous cell carcinoma (HNSCC) [156] and oral [157] and cervical [158] cancers. Cyclin A1 is regulated by p53 [159] and in turn appears to function in a positive feedback loop to promote p53 action to enhance genomic stability [160]. Thus, loss of cyclin A1 can disrupt normal p53 function. However, cyclin A1 also possesses pro-oncogenic functions in other solid tumor contexts, most notably PCa. Expression of

cyclin A1 is significantly higher in poorer differentiated PCa tumors as compared to well- and moderately differentiated tumors [155]. Ectopic expression of cyclin A1 in PCa cells enhances both apoptosis resistance [154] and pro-angiogenic signaling by increasing VEGF (vascular endothelial growth factor) levels [155], but only in the presence of functional AR. This cyclin A1-mediated increase in VEGF expression involves both Rb and androgens [155]. Over-expression of cyclin A1 in mouse PCa xenografts promotes invasion, metastases, and tumor vascularization [153]. Cyclin A1 interacts directly with AR, and in the presence of androgen, the AR-cyclin A1 complex is believed to be recruited to the VEGF promoter [153] together with epigenetic coregulators such as KDM1A [161]. Thus, cyclin A1 appears to function as an AR coactivator to promote angiogenesis. Interestingly, there is evidence that mRNA and expression of cyclin A1 is itself androgen regulated in androgen-responsive prostate cells [162]. This regulation of cyclin A1 involves the NCOA3/SRC3/ACTR/AIB1 AR coactivator [163, 164] which is itself implicated in PCa harboring SPOP mutations [165]. Furthermore, AR phosphorylation (at Ser213) as a consequence of elevated PIM1 kinase levels enhances androgen-induced expression of cyclin A1 [166]. Thus, cyclin A1 and the AR-NCOA3-KDM1A coregulator complex form a mutual regulatory circuit which amplifies androgen responsiveness in prostate cells.

There is also evidence linking androgen signaling with cyclin E1 and cyclin B1 function. Cyclin E1 also appears to possess both kinase-dependent and kinase-independent functions in cell cycle regulation [167, 168]. In addition to this role in the cell cycle, cyclin E interacts with AR N-terminal domain and enhances AR transactivation activity [169]. In contrast, AR can promote [170] and repress [171] expression of cyclin B1 in PCa and associated stromal cells, respectively. The AR, acting via an androgen response element in the cyclin b1 promoter, represses expression by facilitating the displacement of E2F1 by an E2F4 complex involving the silencing mediator of retinoid and thyroid hormone (SMRT) corepressor and HDAC3 [171]. In PCa cells, the AR acting in concert with the Jagged1 notch ligand via Akt promotes cyclin B1 promoter activation and expression [170]. Therefore, by selective mechanistic interactions, androgens and the AR promote proliferation of PCa cells and inhibit proliferation of PCa-associated stromal cells.

Functional Interactions of AR and Cyclin-Dependent Kinases

Androgen signaling is also regulated by CDK-mediated AR phosphorylation. AR phosphorylation (at serine 515) by CDK1 [172] coincides with enhanced AR stability and activity [173] and correlates with decreased time to biochemical recurrence [172]. CDK6 interacts with the AR and enhances androgen-regulated transcription independent of both cyclin D1 and the intrinsic kinase activity of CDK6. However, the ability of CDK6 to enhance androgen signaling was itself reduced by cyclin D1 and p16INK4A [174]. CDK5 phosphorylation of AR (at serine 81 and serine 308) promotes AR protein stability [175, 176] and in turn enhances AR-driven

transcription and proliferation. This pro-proliferative effect of CDK5 depends upon AKT activation [175]. Thus, CDK5 is central to the integration of androgen and AKT signaling in the cell cycle. However, phosphorylation of AR-serine308 by cyclinD3-CDK11p58 attenuates AR action [177, 178]. Collectively, these findings indicate that specific combinations of AR phosphorylation can influence androgen signaling and program the AR to prefer subsets of androgen-regulated promoters for transcriptional activation [175].

Conclusions

The ability of hormone refractory PCa to evade ADT has long been a major challenge in treating men with advanced disease. Indeed, the mean duration of response is commonly <24 months. Abiraterone, enzalutamide, and taxane-based chemotherapies have delivered some survival benefits in hormone refractory PCa; however, these have been modest [179, 180]. Indeed, one potential consequence of these androgen blocking treatments is the increasing incidence of neuroendocrine PCa which has a poor prognosis [181]. With our increasing understanding of how androgen signaling influences cell cycle progression and how cell regulators can in turn promote androgen signaling, the potential may soon exist to develop novel approaches to dissociate androgen signaling and cell proliferation. However, several key questions remain unanswered. For example, does the distinct transcriptional network regulated by androgens and AR in hormone-dependent and refractory PCa cells [182] change cell cycle regulation, or does alteration in cell cycle regulator expression during tumor progression enforce changes in the transcriptional networks regulated by AR in hormone-dependent and refractory tumors? With a greater understanding of how the AR regulates the cell cycle and how specific cyclins and CDKs in turn influence AR function, novel therapies targeting the AR complex transcriptional activity may emerge which can prevent, delay, or reverse the transition to hormone refractory disease and blocks the emergence of neuroendocrine disease.

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Chapter 23
DNA Damage Repair

Jacqueline Fontugne

Contents

Introduction

DNA is vulnerable to various damages, ranging from single- or double-strand breaks to nucleotide mismatches or inter-strand cross-links. These damages may be the result of DNA-damaging agents (e.g., UV rays, radiation, chemotherapy) or endogenous physiological errors during DNA replication. To maintain genomic integrity, normal cells are equipped with multiple repair mechanisms that counterpart the wide variety of DNA damages [1]. In fact, the repair machinery holds a complex assembly of proteins destined to either detect or fix DNA errors or to induce cell death if the damage is considered too severe. Specific repair mechanisms target distinct damage types, summarized in Fig. 23.1. Importantly, DNA damages occur genome-wide, but those occurring in cancer-related genes may lead to tumorigenesis [2]. Unlike some tumor types, such as breast or colon cancer, prostate cancer is not commonly described as driven by genomic alterations involving DNA damage response (DDR) genes. However, several recent findings demonstrate that DDR pathways may play an important role in prostate cancer tumorigenesis and progression.

J. Fontugne, M.D.
Department of Pathology and Laboratory Medicine,
Englander Institute for Precision Medicine, Weill Cornell Medical College and New York-Presbyterian, New York, NY, USA
e-mail: fontugnej@gmail.com

© Springer International Publishing AG 2018

B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*,
Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_23

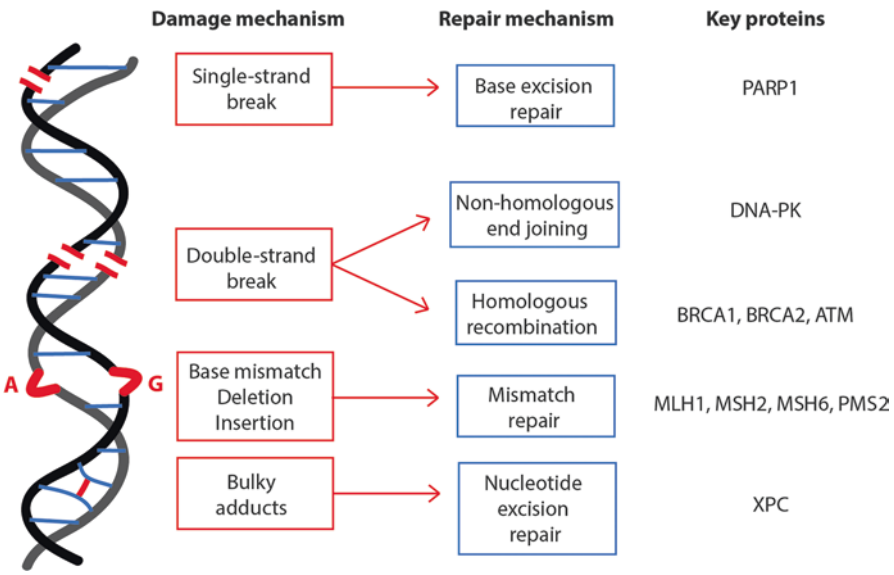

Fig. 23.1 Schematic representation of major DNA damage mechanisms and repair processes. DNA is constantly subject to various damages. In normal cells, each damage mechanism is counteracted by a specific repair process. Key DNA damage repair proteins mentioned in this chapter, specific to each repair mechanism, are listed

DNA Damage Response Alteration in Prostate Cancer

Alterations in DNA Damage Response Genes in Prostate Cancer

Although next-generation sequencing studies have revealed recurrent genomic alterations in prostate cancer, alterations in DNA damage repair genes do not seem predominantly involved. However, DDR genes have been reported as altered in a small subset of primary prostate cancers, and more recent studies of advanced or castrate-resistant prostate cancer have revealed more frequent mutations in DDR pathways, suggesting that they increase as a function of disease progression [3]. The main DNA damage response-related genes or pathways that have been found recurrently altered in prostate cancer are p53, BRCA2, and mismatch repair genes.

p53

The tumor suppressor gene p53 is linked to DNA damage response, although not in direct repair. One of the functions of p53 protein is to block cell cycle progression at the G1-S interphase in the event of DNA damage, allowing time for the DNA damage repair processes to intervene or to initiate cell apoptosis. Therefore, p53 plays a role in DDR through cell cycle arrest, preventing the cell from duplicating a

damaged DNA strand, thus maintaining genomic integrity [4, 5]. p53 function is altered in numerous cancers, mainly through missense mutations of the p53 gene. In fact, p53 seems to be the most frequently mutated gene across human cancers [6, 7]. In prostate cancer, p53 is mutated in a range of 2.5–40% of tumors, depending on the study sample size, tumor stage, and methods of detection [8, 9]. Alteration of p53 may be more frequent through copy number alteration rather that mutation in prostate cancer [10]. Additionally, p53 has been suggested to be a predictor of tumor recurrence and metastasis [8, 11, 12], and p53 loss seems to increase as disease progresses [13].

BRCA Genes

BRCA proteins, BRCA1 and BRCA2, play a critical role in DNA damage response, specifically in repair of double-strand breaks through homologous recombination [14]. Loss-of-function germline mutations in BRCA genes have been well described in tumorigenesis of hereditary breast and ovarian cancer syndromes [15–17]. Furthermore, epidemiologic studies have reported families of hereditary breast cancer which were aggregated with prostate cancer, either in male relatives of women [18] or men with breast cancer [19]. It was subsequently determined that carriers of germline mutations in the cancer susceptibility gene BRCA2 are at a significantly higher risk of developing prostate cancer and at a younger age [20–22].

DNA sequencing studies of sporadic primary prostate cancers have not revealed recurrent alterations in BRCA genes thus far [23, 24]. However, Beltran et al. interrogated advanced metastatic prostate cancer cases—hormone naïve or castrate resistant—for genomic aberrations, through targeted next-generation sequencing. Mutation and copy number analysis identified recurrent BRCA2 loss in 3/25 (12%) of metastatic castrate-resistant prostate cancer cases. This novel finding, also supported by similar recent findings by Robinson et al. [3], suggests that somatic alterations in DDR genes such as BRCA2 may play a key role in prostate cancer resistance and progression. In terms of inherited mutations, the recent multicenter study by Pritchard et al. illustrates that germline DNA-repair gene mutations among men with metastatic prostate cancer are significantly higher (11.8%) than the incidence in men with localized disease. Mutations were found in 16 genes including BRCA2, ATM, CHEK2, BRCA1, RAD51D, and PALB2 [25].

Mismatch Repair System

Among various DNA damage response processes, the mismatch repair system detects and repairs base mismatches generated during DNA replication. These mismatches can occur through an erroneous insertion or deletion of a base (forming insertion/deletion loops) or by mis-incorporation of a base with another. This system requires six main proteins, which function as heterodimers: MLH1, MSH2, MSH3, MSH6, PMS1, and PMS2 (Fig. 23.2). In combination, these proteins

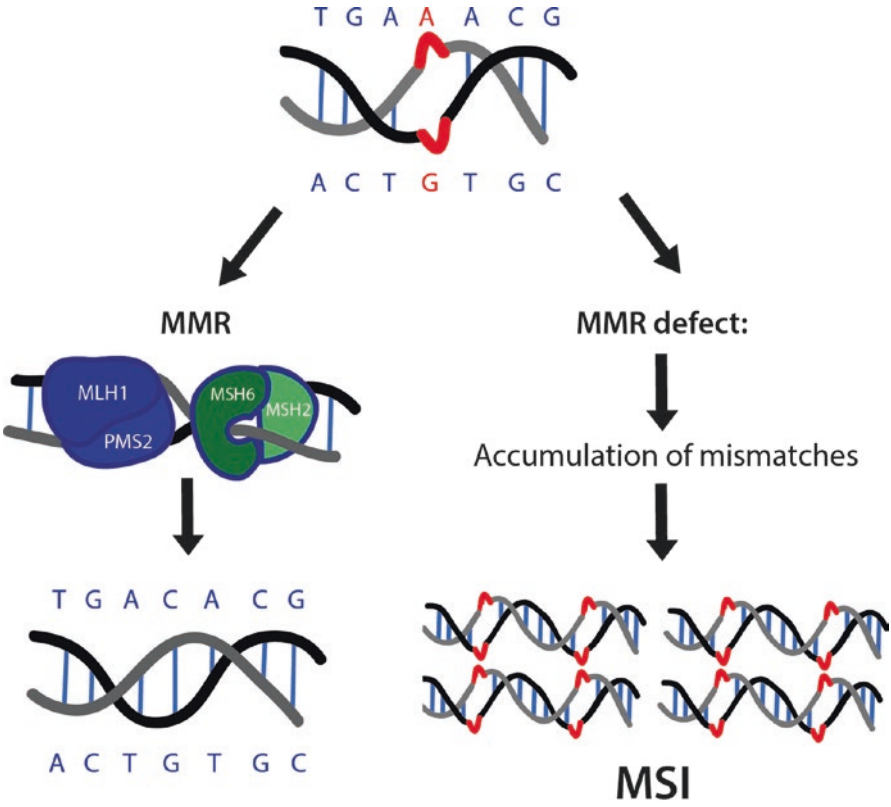

Fig. 23.2 Schematic representation of the base mismatch repair system and consequence of its defect. In a normal cell, a base mismatch is detected then repaired by MMR proteins such as MLH1, PMS2, MSH2, and MSH6. In a cell lacking MMR, mismatch errors accumulate, in particular in microsatellite repetitive sequences, leading to microsatellite instability. *MMR* mismatch repair, *MSI* microsatellite instability

detect and excise the nucleotide strand containing a mismatch or single-base loop, subsequently followed by synthesis of the excised nucleotides and ligation [26].

Defects in MMR can lead to the accumulation of mutations genome-wide, described in tumors as a "mutator phenotype," which harbor an increased rate of mutations compared to the same tumor type with an efficient MMR system [27]. The accumulation of mismatches occurs more frequently in particular repetitive sequences named microsatellites, resulting in microsatellite instability (MSI), which can be detected and used as a biomarker of MMR deficiency [28]. In prostate cancer, sequencing studies have reported somatic mutations involving MMR genes, hypermutated phenotype in a subset of tumors, as well as MSI [29], although rare. In fact, multiple studies have reported isolated MSH6-mutated cases that harbored five- to tenfold more somatic mutations than the average mutation rate per tumor, suggesting mutator phenotypes [10, 24, 30].

The MMR system was discovered and described in patients with hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome, an autosomal dominant disease characterized by a germline mutation in one of the MMR genes, most commonly MLH1, MSH2, MHS6, or PMS2 [28, 31]. These patients are at a high risk of colon and endometrial cancer but also, although less frequently, gastric, ovarian, urinary, and skin cancers [32, 33]. Regarding prostate cancer, recent studies analyzing close to 200 Lynch syndrome families each determined a cumulative lifetime risk of prostate cancer of two- to fivefold higher than the general population [34, 35]. Therefore, it is suggested that prostate cancer may be added to the Lynch syndrome spectrum.

Other DDR Gene Alterations

Sequencing techniques identified additional mutations in genes involved in a number of DNA damage repair processes but mostly in isolated cases. For example, Taylor et al. found mutations in BLM, involved in double-strand break repair, and in XPC, linked to nucleotide excision repair [10]. Similarly, Grasso et al. identified mutations in PRKDC (which encodes DNA-PKcs, DNA-dependent protein kinase catalytic subunit), related to double-strand break repair. Additionally, Beltran et al. described recurrent ATM mutations in castration-resistant prostate cancer [9]. ATM is an additional key protein in double-strand break repair [36].

A recent sequencing study in the context of a precision medicine trial identified a hemizygous deletion in the DNA damage repair gene FANCA, in both primary and metastatic tumor tissues in one patient, who also showed a mutator phenotype [37]. FANCA encodes for a protein of the Fanconi anemia family, involved in the repair of DNA cross-links [38]. There was also putative partial loss of function of the second allele through a missense variant. These findings explained the extreme clinical response of this patient's cancer to platinum, a chemotherapy that creates DNA cross-links.

The Role of Prostate Cancer Recurrent Alterations in DNA Damage Repair

As previously described, direct alterations to DNA damage response genes are rather infrequent in prostate cancer and have only been reported in a subset of tumors. However, the concept that other key pathways in prostate cancer may interact with the DDR machinery is emerging.

Genomic Instability in Prostate Cancer

Genomic instability, represented by chromosomal breaks, rearrangements, and fusions, is a key feature of prostate cancer genomics and an indication of some defect in DNA integrity maintenance. In fact, approximately half of prostate cancers

harbor chromosomal rearrangements creating a fusion between the 5′ untranslated region of TMPRSS2, an androgen-regulated gene, and various members of the ETS transcription factor family genes, most frequently ERG [39, 40]. The TMPRSS2-ERG fusion defines a molecular subclass of prostate cancer and leads to the overexpression of a truncated ERG protein [41–43].

Furthermore, through whole genome sequencing of seven primary prostate cancers, Berger et al. identified a "complex pattern of balanced genomic rearrangements," beyond the TMPRSS2-ERG fusion [23]. This pattern of balanced rearrangements is characterized by multiple DNA breaks and inter-chromosomal religations, without genomic material loss.

This finding was further supported by Baca et al., who performed next-generation sequencing of 57 whole genomes—55 primary prostate cancers and 2 prostate cancers with neuroendocrine differentiation [13]. Complex chains of multiple rearrangements involving several chromosomes were identified and termed "chromoplexy." A subset of cases showed even more dramatic rearrangements subsequent to chromatin shattering, resembling chromothripsis, described in other tumor types [44, 45]. Importantly, these rearrangements seem to disrupt and dysregulate several driver cancer-related genes.

Although the implication of genomic instability in prostate cancer initiation and progression is not yet fully elucidated, it conceivably illustrates some extent of DNA damage repair impairment.

The Role of TMPRSS-ERG Fusion in DNA Damage Repair

It is well known that the TMPRSS2-ERG fusion leads to the aberrant overexpression of its product, a truncated ERG protein, which has a role in tumor initiation and progression, in particular if associated with other genomic alterations, such as the loss of the tumor suppressor PTEN [46–50]. However, the full interactions and functions of ERG are still to be determined. In order to further understand the biological role of ERG, Brenner et al. utilized immunoprecipitation techniques to identify proteins that directly interact with ERG [51]. Interestingly, ERG physically interacts with two DNA damage repair proteins involved in double-strand break repairs: poly-(ADP-ribose) polymerase (PARP1), an enzyme involved in homologous recombination, and DNA-dependent protein kinase catalytic subunit (DNA-PKcs), required for nonhomologous end joining [52, 53]. These interactions were shown in prostate cancer cell lines, as well as in human prostate cancer tissues overexpressing ERG, i.e., harboring the TMPRSS2-ERG fusion. Of note, PARP1 and DNA-PKcs seemed to function as modulators of ERG transcriptional activity. Additionally, aberrant overexpression of ERG seems to increase DNA susceptibility to double-strand breaks, which is potentiated by inhibition of PARP1. Directly linking ERG to a DNA damage response actor PARP1 is a crucial finding, given the importance of the ETS gene fusion axis in prostate cancer genomics.

The Role of the Androgen Receptor Pathway in DNA Damage Repair

Unexpectedly, recent findings demonstrate that signaling pathways linked to steroid hormones, such as estrogen and androgen, modulate DNA damage repair in hormone-dependent tumor types, like breast and prostate cancer, respectively. In breast cancer, radiation therapy (RT), which induces DNA damage through double-strand breaks, is widely utilized. Interestingly, clinical observations show an enhanced response to RT when adjuvant therapy with tamoxifen, an estrogen signaling suppressor, is administered in patients with estrogen receptor (ER)-positive tumors [54]. Additionally, although the underlying biology behind this improved response is not fully defined, ER does interact with important actors of DNA repair, such as DNA-PK, which modulates ER signaling by phosphorylation of estrogen receptor-α[alpha] [55].

Prostate cancer strongly depends on the androgen receptor (AR) signaling pathway at all stages of disease. In fact, the AR pathway holds a crucial role in tumor initiation, maintenance, and progression [56–58]. Consequently, therapies targeting the AR axis are a major therapeutic alternative at all stages of disease progression [59, 60]. Similarly to breast cancer, RT is widely utilized as a primary treatment option, specifically in localized prostate cancer [61, 62], and phase III randomized trials demonstrate that RT combined to antiandrogen therapy (ADT) improves overall survival compared to ADT alone, in locally advanced prostate cancer [63, 64]. These clinical observations suggest that AR signaling may impact sensitivity to DNA damage, hence to radiation therapy. Recently, Goodwin et al. investigated the potential underlying mechanisms of crosstalk between AR signaling and DNA damage repair [65]. They determined through in vitro studies that AR activity is induced by DNA damage. AR increases DNA double-strand break resolution through direct upregulation of expression and activity of DNA damage proteins, such as DNA-PKcs, previously mentioned. This discovered link between AR signaling and DNA damage repair may be a critical foundation for the development of novel therapeutics, such as DNA-PKcs inhibitors, currently in phase I trials [66].

Additionally, recent in vitro work demonstrated that PARP1 modulates AR activity and reversely, PARP1 inhibition reduced expression of AR-dependent target genes [67].

The Role of c-Myc in DNA Damage Repair

The oncoprotein c-Myc is a transcription factor that plays a role in cell differentiation, proliferation, and apoptosis [68] and is known to be overexpressed in a number of prostate cancers [69]. Interestingly, c-Myc has been linked to DNA damage response through interaction with ATM signaling, involved in double-strand break repair [70, 71].

Therapeutic Relevance

Prostate cancer is the leading cause of cancer-related deaths in men in the United States [72]. Surgical or medical castration is the main therapeutic choice, but androgen deprivation resistance and disease progression are inevitable, possibly resulting in castration-resistant prostate cancer and metastasis. Recent efforts have been concentrated on developing new highly potent drugs targeting the androgen axis (e.g., abiraterone that blocks androgen production) [73]. However, there is currently no cure for advanced prostate cancer, and identifying new therapeutic targets is an unmet need. Although direct sporadic alterations to DNA damage repair genes seem to be somewhat rare drivers in prostate cancer, key pathways in prostate cancer directly cooperate with DNA damage repair processes, as previously described. Therefore, identifying prostate cancer patients harboring a deficiency in DDR mechanisms is crucial, due to the fact that these alterations are potential drug targets. As an example, in the aforementioned example of a precision medicine clinical trial patient, identification of a DDR gene FANCA alteration explained a dramatic response to a cross-linking agent, platinum [37].

Tumors harboring a defect in double-strand break repair, specifically homologous recombination (e.g., deficiency of BRCA genes or ATM), are sensitive to PARP1 inhibitors, such as olaparib [74, 75]. Biologically, the combination of PARP1 inhibition and BRCA defects synergistically allows for DNA damages to persist in tumor cells, leading to cell death; this concept is referred to as "synthetic lethality." Olaparib showed a significant antitumor activity in prostate cancer patients with known germline BRCA mutations [76, 77]. Additionally, there are multiple ongoing trials testing PARP1 inhibitors in metastatic prostate cancer [78]. Other clinical observations demonstrate that therapeutic agents that target the DDR pathway, such as radium-223 dichloride, which induces double-strand breaks, improved survival in patients with castration-resistant prostate cancer and bone metastasis, consistent with the postulate that a subset of advanced tumors become reliant to DDR signaling [79, 80]. Basically, PARP1 inhibition is a promising therapeutic option in tumors with defects in DDR genes like BRCA. However, these defects are infrequent in prostate cancer; therefore identifying other determinants of PARP1 inhibition sensitivity in prostate cancer is of interest.

Directly inhibiting transcription factors such as ERG may be difficult, but targeting cofactors, such as PARP1, is a feasible alternative. Because PARP1 and ERG directly physically interact, PARP1 inhibition may rationally be a more accessible and effective target in tumors harboring ERG rearrangement. Promising in vivo work interrogated response to olaparib in mouse xenograft models and in prostate cancer cell lines and established that ERG-rearranged tumors or cells were preferably sensitive to the PARP inhibitor, compared to ERG-negative tumors [51]. This study was further supported by similar findings in Ewing sarcoma, another tumor type that also carries ERG rearrangements [81].

Additionally, it has been shown that radiosensitization of prostate cancer triggers PARP1 hyperactivation [82], and inhibition of PARP1 potentiates sensitivity

to radiation therapy. Thus, a PARP1 inhibitor in association with radiation is an appealing combination. This finding may be particularly relevant in tumors overexpressing ERG, since the ERG/PARP1 interaction may provide radiation resistance, potentially reversible through PARP1 inhibition [83]. Similarly, recent work determined that radiation of prostate cancer cells combined with a PARP1 inhibitor was most effective in cells harboring combined ERG fusion and PTEN loss [84].

Another potential DNA damage drug target is DNA-PKcs, which interacts with the androgen receptor pathway and ERG, as detailed above. Because the AR pathway is crucial in prostate tumorigenesis and progression, identifying new drugs interfering with this axis is of interest, and phase I trials of DNA-PKcs inhibitors are ongoing.

Conclusions and Perspectives

Multiple human cancers are initiated and driven by direct genomic alterations to DNA damage repair genes, which lead to the accumulation of deleterious DNA errors, potentially in cancer-related genes. In prostate cancer, germline or sporadic genomic alterations in DNA damage genes are limited to a subset of prostate cancers, although sporadic alterations seem to increase as a function of disease progression. However, clinical observations support the hypothesis that prostate cancer may rely on DNA damage pathways. In fact, multiple lines of evidence demonstrate that complex cross talks exist between DDR pathways and known key pathways in prostate cancer development and progression. Importantly, these findings may lead to future therapeutics, as DNA damage repair actors are potential drug targets. Therefore, identifying specific defects in DDR pathways through precision medicine, as well as their link to known prostate cancer pathways, represents a unique opportunity for the development of synergistic therapies.

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Chapter 24
Tyrosine Kinase Receptor Signaling in Prostate Cancer

Heather Johnson, Lingwu Chen, Kefeng Xiao, and Jenny L. Persson

Contents

H. Johnson, Ph.D.
Olympia Diagnostics, Inc., Sunnyvale, CA, USA

Institute of Basic Medical Sciences, Beijing, China

L. Chen, M.D., Ph.D.
Department of Urology, First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China

K. Xiao, M.D., Ph.D.
Department of Urology, The Second Clinical Medical College of Jinan University (Shenzhen People's Hospital), Shenzhen Urology Minimally Invasive Engineering Center, Shenzhen, China

J.L. Persson, Ph.D. (✉)
Department of Translational Medicine, Clinical Research Centre, Lund University, Malmö, Sweden

Department of Molecular Biology, Umeå University, Umeå, Sweden
e-mail: jenny_l.persson@med.lu.se

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*, Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_24

Introduction

Prostate cancer (PCa) is the most common type of cancer in men and one of the leading causes of cancer-related death in Western world [1, 2]. Elevated levels of the male hormones, androgens, are known to contribute to development of PCa. As the growth of PCa at initial stage is dependent on hormones, hormone-deprivation therapies are therefore used as standard treatment to induce tumor regression in PCa patients [3]. Despite hormone-deprivation treatment, most of treated PCa will resume the growth and become hormone-refractory, also termed castration-resistant PCa (CRPC) [3, 4]. CRPC is no longer responsive to most of the available therapies and is highly invasive with metastatic potentials to disseminate to distant organs including the lung, bone, and brain [5]. Thus, CRPC represents a major clinical challenge.

Androgens including testosterone, dehydroepiandrosterone, and dihydrotestosterone (DHT) are produced by testes, adrenal glands, and prostate itself [3, 4]. Under castration-resistant state, despite the absence of or minimal levels of androgens, PCa cells are capable of growing rapidly and gain survival and invasive advantages [5, 6]. This suggests that PCa cells may use alternative mechanisms without consuming large quantities of androgens, thereby bypass androgen-dependent pathways.

Emerging evidence has suggested that various types of growth factors may replace androgen effects to stimulate growth and survival of PCa cells under castration-resistant conditions. Epidermal growth factor (EGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and insulin-like growth factor-1 (IGF-I) are the major growth factors that are involved in growth and invasion of PCa cells [7–9]. The effect of these growth factors is mediated by a superfamily of transmembrane receptors also termed receptor tyrosine kinases (RTKs). At least 58 members of RTKs have been identified so far, and these include EGFR, FGFR, PDGFR, VEGFR, and IGFR, which all share a conserved structure [10–15].

One of the major features of PCa is its heterogeneity nature. A prostate tumor often contains a mixture of heterogeneous populations including cancer cells and endothelial cells of the blood and lymphatic vessels, stromal cells, fibroblasts, smooth muscle cells, neuromuscular tissues, infiltrating immune cells, and the tumor-specific extracellular matrixes (ECM) [3, 4]. It is now clear that abundant growth factors are not only secreted by the cancer cells but are also produced by the tumor-specific stromal cells, fibroblasts, ECM, and other cell types. The growth factor receptors (RTKs) play an essential role in facilitating proliferation, survival, and migration in response to the stimulation by various types of growth factors [16]. It is of importance to gain deeper understanding of the cellular mechanisms underlying the interplay between PCa cells and PCa-associated microenvironment during progression of CRPC and to specifically gain deeper knowledge about the role of RTKs in proliferation, survival, and migration of PCa cells and PCa-associated cells during development of CRPC.

The regulation of growth factors and their receptors is mediated through autocrine- or paracrine-dependent manners [10, 11]. In PCa, abnormal levels of the growth factors are frequently observed in serum and in PCa tissues from PCa patients [9]. Similar to their ligands, alterations in expression and activity of RTKs also occur in various types of cancers including cancers of the lung, colon, breast, pancreas, and prostate [17–19].

During the past years, several new classes of inhibitors to RTKs have been developed and shown promising effects to target metastatic diseases in lung, breast, and colorectal cancer [18]. Two EGFR inhibitors, cetuximab and panitumumab, have been approved by FDA and are currently used for treatment of patients with lung cancers [18]. These inhibitors induce apoptosis in cancer cells by blocking multiple growth and survival signaling which are EGFR-dependent. The effects of cetuximab and panitumumab on castration-resistant PCa remain to be further investigated in preclinical models and in patient-based clinical trials.

In this chapter, we will review series of important studies on investigation of clinical importance of RTKs including EGFR, FGFR, PDGFR, VEGFR, and IGFR in CRPC. We will discuss the cellular mechanisms by which alterations in RTKs allow PCa cells to bypass androgen-dependent pathways and take advantages of tumor-associated microenvironment [32, 33]. Moreover, we will highlight the ongoing development of targeted therapies by using cancer immunotherapy and small molecule-based targeted therapy for treatment of CRPC and metastatic PCa.

EGFR Signaling in Development of Castration-Resistant and Metastatic PCa

EGF family of growth factors consists of EGF, transforming growth factor-α[alpha] (TGF-α[alpha]), amphiregulin, epigen, betacellulin, heparin-binding EGF and epiregulin tumor necrosis factor-α[alpha] (TNF-α[alpha]), vascular endothelial growth factor (VEGF), and RANKL [17, 19]. FGF interacts with its four receptors including EGFR (known as ErB-1 or Her 1), Her 2/neu (ErbB-2), Her 3 (ErbB-3), and Her 4 (ErbB-4), all belong to the ERBB family of RTKs [17, 20, 21]. Upon binding to its ligands EGF or TGF, EGFR becomes active by forming into homodimers. The homodimers of EGFR phosphorylate and interact with its downstream factors which regulate the fundamental cellular events including proliferation, survival, and migration [21]. Alteration in EGFR often triggers the oncogenic events such as malignant transformation and growth of cancers of the lung and colon and brain tumors [17]. Expression of EGFR is low in normal prostate tissues [21]. However, EGFR is highly expressed in primary PCa tissues and in PCa bone marrow metastases [19, 22–25], suggesting a role of EGFR in development of PCa. The correlations between EGFR expression and clinical characteristics and patient outcome have been investigated in cohorts of PCa patients. In these studies, elevated expression of EGFR was associated with high Gleason scores and the increased incidence

of disease recurrence, as well as rapid progression to CRPC [23]. Further, elevated level of EGFR also correlated with increased expression of its ligand TGF-α[alpha] in CRPC, indicating the presence of an autocrine ligand-receptor loop between EGFR and TGF-α[alpha] in CRPC cells [22, 24, 25]. Since excess levels of EGF and EGFR are produced by both PCa cells and tumor-specific stromal/fibroblasts, it is likely that EGFR signaling in cancer cells is activated by its ligands from both cancer cells and tumor-specific stromal/fibroblasts through paracrine and autocrine loops, leading to the growth and survival of PCa cells in the absence of androgens [19, 21, 22].

The precise mechanisms underlying EGFR activation by the tumor-associated microenvironment are largely unknown. It has been shown that the growth of androgen-sensitive PCa cell line, LNCaP cells, was increased in the presence of osteoblastic cells [25]. Further, EGFR/ERBB2 kinase activity was significantly upregulated in LNCaP cells co-cultured with osteoblastic cells as determined by multiplex kinase activity profiling. This study suggests that EGFR activity is stimulated by the tumor-associated bone cells [25]. Activation of EGFR is also mediated by type 1 IGF and ECM which are produced by the tumor-associated microenvironments during PCa metastases to the bone marrow [19, 20, 24]. A recent intriguing study reports that EGFR can be activated by macrophage-specific factor: migration inhibitory factor (MIF) through complex formation [26]. Since MIF is mostly produced by the immune cells, this finding suggests that EGFR activity in cancer cells can be modulated by the infiltrating immune cells, and cancer cells may utilize EGFR to suppress immune cells in order to gain advantages to facilitate cancer metastases [26].

EGFR regulates multiple downstream pathways including the MAP kinases, PI3K/AKT, MET, and cell cycle pathways, leading to activation of cell proliferation and migration [17, 27]. EGFR-mediated activation of AKT is in part through dimerization of EGFR with HER3 [28, 29] and in part via interaction of EGFR with the intracellular adaptor Gab1 [30]. Abnormal activation of PI3K/AKT pathways is associated with the loss of tumor suppressor PTEN gene. PTEN mutations are observed in 70% metastatic PCa [29]. This suggests that EGFR and its downstream PI3K/AKT pathways play important role in development of CRPC and PCa metastases. Although the precise mechanisms underlying the interplay between EGFR and PTEN remain largely unknown, it is clear that constitutive activation of EGFR in cooperation with PTEN mutation is responsible for elevated activity of PI3K/AKT pathways, thus rendering PCa cells to gain survival and invasive advantages under castration-resistant conditions.

It is known that amplification, mutation, or enhanced phosphorylation in AR may also allow PCa cells to bypass androgen-dependent pathways [32, 33]. Increased AR expression has been observed in PCa metastatic tissues compared with primary cancer [34]. Increasing evidence suggests that EGFR signaling cross talks with AR axis and renders PCa cells resistant to castration treatment [35, 36]. AR is a transcriptional factor that regulates a panel of genes controlling growth of prostate cells; EGFR and its ligands may replace androgens to enhance phosphorylation in AR or act as AR coregulators to promote activation of downstream genes.

It has been shown that forced overexpression of HER2 kinase increases AR expression and promotes growth of androgen-independent PCa cells through AR pathway [37, 38].

Mutations are often found in the catalytic kinase domain in EGFR in lung cancer, resulting up to a 50-fold increase in kinase activity leading to oncogenic transformation of lung epithelial cells [39, 40]. However, the rate of somatic mutations or amplifications in EGFR appears to be low in PCa, suggesting that EGFR mutations in lung cancers represent a biologically distinctions in different types of cancers [17, 39, 41].

Tyrosine Kinase Inhibitors that Target EGFR for Treatment of Cancer

The crucial importance of EGFR in tumor cell survival and invasion made them the ideal targets for small molecule-based targeted therapy and immunotherapy. Monoclonal antibodies (mAbs) including cetuximab (a chimeric mouse-human IgG1 antibody) and panitumumab (a fully humanized IgG2 antibody) have been developed to mediate killing of the cancer cells which express high level of EGFR [18, 42]. Because proliferation and survival of tumor cells are dependent on EGFR and its downstream signaling, inhibition of EGFR blocks the growth and survival signaling of tumor cells, leading to tumor cell death. Cetuximab and panitumumab block ligand binding of the extracellular domain of EGFR, thus inhibit internalization of EGFR [18, 42]. Cetuximab and panitumumab are approved by FDA for treatment of colorectal cancer with wild-type KRAS, but they have little or no effect in metastatic or advanced colorectal cancer harboring KRAS mutations [43].

The use of these EGFR inhibitors for treatment of CRPC and metastatic PCa remains to be investigated in preclinical and clinical settings. Since the growth and survival of CRPC cells may be dependent on EGFR signaling, blocking EGFR by cetuximab and panitumumab will prevent activation of downstream cell proliferation and survival pathways, leading to cytotoxicity in cancer cells. As mentioned above, PCa is a heterogenic tumor with various types of cells. PCa cells may use their microenvironment and multiple signaling to escape drug-specific killing. Cetuximab is more effective as compared to panitumumab, as the IgG1 subclass is more effective than IgG2. Cetuximab induces G1 arrest and subsequent induction of apoptosis, followed by tumor shrinkage in mice and humans [18, 40, 42].

Although antibody-based therapies lack efficacy for treatment of CRPC, nevertheless PCa, like other highly vascularized tumors, produces excess of EGFR which triggers the intracellular signaling pathway. In addition, PCa bone metastases express elevated levels of EGFR [25]. Thus, small molecule-based cancer therapy may be used for treatment of CRPC. EGFR tyrosine kinase inhibitors (TKIs) including gefitinib, erlotinib, and afatinib are approved by FDA for treatment of non-small-cell lung cancer (NSCLC) [18]. Gefitinib, erlotinib, and gefitinib reversibly

inhibit the EGFR tyrosine kinase domain by competitively binding with ATP, thereby inhibiting proliferation of cancer cells, leading to apoptosis in NSCLC cells expressing elevated level of EGFR [40, 42]. It remains to be determined whether gefitinib, erlotinib, and gefitinib will be suitable drugs to target CRPC and metastatic PCa [44, 45].

Expression of FGFR Is Associated with Progression of Castration-Resistant and Metastatic Prostate Cancer

FGFs are ubiquitously expressed in the majority of human tissues and play important roles in a variety of normal homeostasis and pathological processes, including development, wound healing, and oncogenic process [46]. At least 23 different FGF genes encoding the FGF family of polypeptides have been identified [46, 47]. There are four distinct receptors designated, FGFR-1, FGFR-2, FGFR-3, and FGFR-4, which share similar structures. FGFR consists of an extracellular portion of three immunoglobulin-like domains and an intracellular tyrosine kinase domain [46, 47]. The activation of FGFR kinase activity is trigged through binding of FGFR and FGFs. FGFR further activates downstream signaling that controls several key cellular events including proliferation, survival, and migration. FGFR is detected at low level in the basal epithelial cells of the normal prostate [48–50]. In contrast, elevated levels of FGFR-1 and FGFR-2 are observed in poorly differentiated PCa from patients and in cancer tissues of TRAMP PCa mouse model [46, 48, 51, 52]. This suggests that FGF/FGFR signaling may play a role in PCa development and progression. The alterations in genes encoding FGFR were detected, suggesting that alterations in FGFR gene may result in constitutively activation of FGFR kinase activity. Amplifications in FGFR-1 and FGFR-2 have been shown to be frequent events in invasive PCa [46, 53]. Forced expression of constitutively active mutant of FGFR-1 in nonmalignant cells results in development of high-grade PIN lesions. Inhibition of epithelial FGFR-1 signaling using a dominant negative FGFR-1 led to reversal of the cancer phenotype [53–55]. The clinical data and experimental studies provide evidence suggesting that aberrantly activated FGFR axis is involved in PCa progression. Similar to EGFR, abnormal activation of FGF promotes malignant transformation of PCa in autocrine- or paracrine-dependent manner [51, 52]. It is also known that FGFR-1 is in part responsible for activating PI3K/AKT and MAP/kinase pathways to promote cell proliferation [54, 55]. Thus, PCa cells utilize FGFR signaling for their uncontrolled growth and survival. It is less clear whether PCa cells also utilize EGFR signaling from stromal/fibroblasts and immune cells in tumor-associated microenvironment. It will be of importance to gain deeper understanding of the underlying mechanisms by which EGFR signaling may be enhanced via interplay between tumor cells and their microenvironment during progression of CRPC.

FGF Receptors and FGF Receptor Signaling as Therapeutic Targets in Prostate Cancer

The involvement of FGF pathways in CRPC provides a rationale for the therapeutic blockade of this pathway in PCa cells. Two small molecule tyrosine kinase inhibitors including dovitinib and nintedanib have shown promising effect for treatment of advanced PCa [56–58]. Preliminary results from these trials suggest that blocked FGF pathway in cancer cells represents a promising new strategy for targeting CRPC [46, 56–58]. Dovitinib (TKI258) has potent activity to block FGFR activity, thus preventing FGFR-mediated cell growth. The clinical studies show that dovitinib is an active therapeutic agent in certain patient populations with CRPC or with bone metastases [46, 56, 58]. Treatment of PCa with dovitinib inhibits ability of PCa cells to interact with stromal and epithelial cells [46, 56, 58]. This suggests that PCa cells may utilize tumor-associated cells to secret and produce EGF and EGFR for their growth and survival. Thus, dovitinib induces PCa cell death by blocking the interactions between PCa cells and tumor microenvironment.

Expression of VEGFR in Prostate Cancer

VEGF is one of the most potent facilitators of angiogenesis in cancers. VEGF is expressed at low level in prostatic glandular epithelial cells [59–63]. There are seven members in VEGF family including VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and placental growth factor (PLGF) [60, 61, 63]. The major receptors that bind to VEGF ligands include VEGFR1, VEGFR2, and VEGFR3 [64]. It is widely known that VEGFs and VEGFRs play important roles in angiogenesis of various types of cancers including PCa [65–68].

Expression and clinical importance of VEGFR in PCa have been investigated. In contrast to their low expression in endothelial cells of blood vessels, elevated expression of VEGF and VEGFR is often found in malignant prostate tissue [69–71] and in bone metastatic lesions from PCa patients [72–74]. Given that VEGF and VEGFR play important role in vascularization and angiogenesis, their abnormal levels in metastatic PCa cells indicate their role in PCa metastases. It has also been shown that increased VEGF plasma levels correlate with bone metastases and poor outcome in PCa patients [72, 74–76]. Activation of VEGFR1 and VEGFR2 results in the increased activity of PI3K/AKT and Ras pathways [64]. Abnormal activation of PI3K/AKT and Ras pathways which are triggered by VEGFR not only promotes growth of PCa cells but also increases tumor-associated densities of vessels, allowing tumor cells to disseminate and invade to distant organs including bones [17, 77–80].

VEGFR2 is best known for its role in promoting cancer metastases. Activation of VEGFR2 by VEGF-A induces PLC activity and triggers phosphorylation of MAPK, leading to increased proliferation and migration [59]. VEGFR2 forms a complex

with MET and regulates MET signaling [17, 80]. Further, the interaction between PCa cells with tumor-specific ECM is mediated through VEGFR2 and integrin pathways [81]. Such interaction between PCa cells and ECM leads to the rapid degradation of ECMs, allowing dissemination of cancer cells to distant organs [82].

VEGF Inhibitors and Prostate Cancer Therapy

The angiogenesis inhibitor bevacizumab (Avastin) is a ligand-trapping monoclonal antibody, and sorafenib and sunitinib target VEGFR2 [79, 80]. Bevacizumab is currently in Phase II clinical trials in relapsed PCa and is approved by the US Food and Drug Administration (FDA) for treatment of metastatic colorectal, renal, and breast cancer and other solid tumors [79, 80]. Sorafenib (Nexavar®, Bayer HealthCare and Onyx Pharmaceuticals, Emeryville, CA) is a multi-tyrosine kinase inhibitor that targets multiple receptors, VEGFR1, VEGFR2, and VEGFR3, as well as PDGF-β[beta], and has been evaluated in preclinical models and is able to inhibit wild-type and mutant b-Raf and c-Raf kinase isoforms in vitro [80, 83–85]. Similarly, aflibercept is another antibody which neutralizes VEGF and is currently being used in Phase II clinical trials for patients with recurrent or metastatic urothelial cancer [80, 83–85]. Semaxanib targets VEGFR2 [84–86]. Ramucirumab is a human IgG1 monoclonal antibody which binds to the extracellular domain of VEGFR2 and blocks the VEGF-A to VEGFR2 interaction and subsequent downstream signaling [86]. Blocking activity of VEGFR by these inhibitors reduces the growth ability of cancer cells and induces cytotoxic effects by blocking proliferation pathways which are associated with MAPK signaling [87, 88]. Most importantly, these inhibitors exert their effect on angiogenesis and metastatic growth of PCa [84]. However, multiple clinical studies in PCa patients using semaxanib and sorafenib or other inhibitors to VEGFRs are somewhat disappointing. The pronounced severe toxic effects and lacking drug specificity are the major problems. VEGF cross talks with other growth factors, and VEGFR2 can be replaced by platelet-derived growth factor receptor (PDGFR); thus, cancer cells may bypass VEGFR2 pathways to facilitate angiogenesis and metastases, despite blocking VEGFR2-dependent signaling [88]. Thus, bevacizumab or the small molecule inhibitors to VEGFR may be used in combination to gain an improved therapeutic efficacy.

PDGF Signaling in PCa

PDGF family mainly consists of three proteins, PDGF-AA, PDGF-AB, and PDGF-BB, which are encoded by two genes, *PDGF-A* and *PDGF-B*. PDGF binds to two isoforms of a TKR, a-PDGFR and h-PDGFR, which can either homodimerize or heterodimerize [89–91]. PDGF is highly expressed in several cancers including PCa [89–91]. It has been shown that a-PDGFR and h-PDGFR are undetectable in normal or nonmalignant hyperplastic prostate. In contrast, both primary prostate tumors and skeletal metastases exhibit

high level of PDGFR expression [89–91]. Androgen-dependent LNCaP cell line does not express a-PDGFR or h-PDGFR, while bone metastasis-derived PC3 cells and PC3-ML cells, a subline of the PCa cells with high metastatic potential, have high level of a-PDGFR [92–94]. Moreover, PDGF is expressed in bone cells and endothelial cells in tumor-associated microenvironment [95–97]. PDGF and PDGFR exert their effect on several mesenchymal and epithelial cell types and bone cells as well [89, 90]. PDGF-BB promoted the proliferation of MSCs in vitro, and this effect could be reversed by PDGF-BB siRNA. IFNγ, TNF-α[alpha], and TGF-α[alpha] significantly increased PDGF-BB production in MSC cells [91, 92, 95, 97]. The regulation of PDGF and PDGFR is mediated through autocrine-dependent manner. a-PDGFR expression renders PC3-ML cells particularly susceptible to the stimulation of the survival PI3K/Akt and MAPK signaling pathways by PDGF [97–99]. Similar to other family members of RTKs, PDGFR also serves as signal transducer for activation of mitogen-mediated protein kinases and STATs transcriptional activity, leading to cell growth and migration [98–100].

PDGF Inhibitors for Treatment of PCa

Inhibition of PDGF may provide antiangiogenic potential and target metastatic lesions. Several small molecules that inhibit tyrosine kinase activity of PDGF have shown promising effects in treatment of PCa. The tyrosine kinase inhibitor imatinib (Gleevec) is used for treatment of CRPC [101, 102]. Although the anticancer efficacy of imatinib as monotherapy was observed, combined inhibition of FGFR/PDGFR/VEGFR is proposed to improve the outcome of patients with CRPC. Sunitinib (PDGFR/VEGFR inhibitor) and sorafenib (Raf kinase/VEGFR inhibitor) have been evaluated for treatment of CRPC in preclinical models [101–106]. TKI258 is an inhibitor that targets FGFR/PDGFR/VEGFR pathways. TK1258 has antitumor activity by inducing apoptosis of endothelial cells of blood vessels, leading to inhibition of bone and lymph node metastases in PC-3 tumor xenograft mouse model [96, 98, 107]. Combination treatment using imatinib mesylate (STI571) and paclitaxel reduces bone and lymph node metastases by inhibition of PDGFR [98, 104–108]. Combination with chemotherapeutic agents may further increase growth inhibitory potential. STI571 and leflunomide (SU101) inhibit growth of PCa prostate [103, 108]. Inhibition of platelet-derived growth factor receptors reduces interstitial hypertension and increases transcapillary transport in tumors [103, 108].

Insulin Growth Factor Receptors

IGF consists of two members: IGF-I and IGF-II. There are two receptors IGF-IR and IGF-IIR that interact with IGF [109]. IGF-IR is expressed in normal prostate and benign prostatic hyperplasia (BPH) at low level. In contrast, elevated serum IGF-I is observed in men with PCa [110, 111]. Serum levels of IGF-I and IGF-II predict risk of PCa [112, 113]. IGF-I stimulates proliferation of PCa cells, whereas

antisense-mediated inhibition of IGF-IR expression suppresses in vivo tumor growth and prevents PCa cell invasiveness [111]. Progression to androgen independence is associated with increased expression of both IGF-IR and IGF-I in human PCa cell xenografts [114]. Since IGF/IGFR family is involved in cellular metabolism, differentiation, proliferation, transformation, anti-apoptosis, angiogenesis, bone metastases, and androgen-independent progression, these families of proteins may serve as prognostic markers to predict risk for development of CRPC [115]. IGF-IR signaling influences cell-cell contact and interaction of PCa cells with their microenvironment [113]. IGF-IR and IGF-IIR are expressed in the bone cells. PCa cells specifically metastasized to bone but not to other organs after being implanted into mouse via rail vein injection, indicating that human bone provides microenvironment for the growth of PCa cells [114, 116]. Altered IGF-IIR results in increased secretion of cathepsins and facilitates metastasis by degradation of basement membranes [110, 113]. It is proposed that IGF-I/IGF-IR induce VEGF expression, thereby facilitating angiogenesis and leading to metastatic spread to the bones [117–119]. FGF inhibits insulin-like growth factor-II (IGF-II) gene expression and increases IGF-I receptor abundance in BC3H [119–122]. Elevated IGF-I/IGF-IR signaling is also linked to angiogenic VEGF signaling [119]. It is suggested that PCa cells can alter the normal balance between bone cells and growth factors including IGF and VEGF and other key factors that modulate bone production and blood vessel formation including bone morphogenetic protein (BMP), FGF, TGF-PDGF, prostate-specific antigen (PSA), and urokinase-type plasminogen activator (uPA) [119, 122]. Osteoblasts also secrete IGF in bone cells. IGF-I may act as a coupling factor in bone remodeling by activating both bone formation and bone resorption [119]. Overexpression of IGF and IGF-IIR promotes organ-confined metastatic PCa in mouse model [119, 121, 123].

It has been shown that androgens upregulate IGF-IR expression and sensitize PCa cells to the biological effects of IGF-I [124, 125]. This suggests that AR activation may stimulate PCa progression through the altered IGF-IR expression and IGF action. It has been shown that IGF-IR signaling may modulate subcellular translocation of AR and thus affect AR activity on its target genes in PCa cells. Inhibition of IGF-IR signaling can result in cytoplasmic AR retention and a significant change in androgen-regulated gene expression [126]. IGFR is capable of triggering the activation of the phosphatidylinositol 3-kinase/Akt pathway and phosphorylates the AR. The activation of PI3K/AKT survival signaling in cooperation with inhibition of AR and its coregulators may allow cancer cell survival [115, 128]. In addition, activation of the Ras/mitogen-activated protein kinase pathway by IGF-I may sensitize the androgen effect on AR transcriptional activity in LNCaP PCa cells [123].

IGFR Inhibitors in Treatment of CRPC

Several anticancer agents that selectively inhibit activity of IGFRs have been developed for treatment of PCa [109]. IGF-IR antibodies including CP-751,871, AVE1642/EM164, IMC-A12, SCH-717454, BIIB022, AMG 479, MK-0646/

h7C10, and KM1468 have been developed to direct against human IGF-I and IGF-II [119, 127, 128]. The IGFR antibodies inhibit growth of various types of cancer cells including breast cancer, rhabdomyosarcoma, and Ewing's sarcoma in vitro and in multiple tumor xenografts [119, 129]. These antibodies block the binding of IGF-IR to their ligands, leading to the inhibition of IGF-IR downstream signaling pathway and inducing apoptosis. KM1468 inhibits the bone tumors in mouse model [119, 129, 130]. Mab 391, a mouse-neutralizing antibody against IGF-IR, inhibits the growth of DU-145 cells. Inhibition of tumor growth following administration of IMC-A14 as a single agent was observed in androgen-dependent and androgen-independent PCa xenograft models [119, 129]. EM164 and IMC-A14 in combination inhibit cell growth in various types of human cancer cell lines [129] in vitro suggesting that blocking the IGF-IR is a promising strategy for targeting cancer. h7C10 and A12 in combination inhibit IGF-mediated mitogenesis in a variety of tumor xenograft prostate tumor models with no significant toxicity. A12 blocks binding of IGFR to its ligand and inhibits receptor internalization and degradation [109, 131]. A12 enhances the effect of docetaxel in a preclinical human xenograft model of CRPC [131]. A recombinant humanized anti-IGF-IR antibody (h7C10) enhances the antitumor activity of vinorelbine and anti-EGFR therapy against human cancer xenografts [131, 132]. Mab 391, a mouse-neutralizing antibody to IGF-IR, inhibits growth of HT-29 colorectal and DU-145 cells [131, 133]. EM164, a purely antagonistic anti-IGF-IR monoclonal antibody, inhibits proliferation and invasion of diverse human cancer cell lines in vitro. This antitumor effect is enhanced by combining EM164 with the cytotoxic agent gemcitabine [119, 129]. IMC-A14 is a fully human antibody that binds to IGF-IR. In addition to binding to the receptor, this antibody induces receptor internalization and downregulation, thereby reducing the number of available IGF-IRs on the cell surface in androgen-dependent and androgen-independent PCa xenografts (LuCaP35 and LucaP35V) [127, 129, 134, 135]. IMC-A14 also has antitumor activity as a single agent or in combination with other chemotherapeutic agents such as melphalan and bortezomib in a multiple myeloma (MM) mouse models [119, 129].

The small molecule compounds targeting IGF-IR components have also been developed and evaluated in various types of cancers including PCa. IGF inhibitors include BMS-536942, BMS-554417, NVP-AEW541, NVP-ADW742, AG1024, potent quinolinyl-derived imidazo (1,5-a) pyrazine (PQIP), picropodophyllin (PPP), nordihydroguaiaretic acid INSM-18/NDGA [129, 135, 136]. These inhibitors prevent IGF-IR internalization, therefore blocking downstream activation of multiple pathways. NVP-ADW742 and NVP-AEW541 are the IGF-IR tyrosine kinase inhibitors that have potent inhibitory effect against IGF-IR [134, 137, 138]. NVP-ADW742 induces antiproliferative and proapoptotic effects in tumor cells. It is also effective to reduce growth of primary tumor cells from patients with multidrug-resistant disease [134, 136, 137]. IGF-IR kinase inhibitor, NVP-ADW742, sensitizes small cell lung cancer cell lines to the effects of chemotherapy [135, 138]. INSM-18 has demonstrated selective inhibition of the IGF-IR in preclinical studies of breast, lung, pancreatic, and prostate tumors. INSM-18 has shown promising effect for treatment of PCa in patients with recurrent cancer [135, 138]. Another selective inhibitor of IGF-IR kinase activity is a cyclolignan derivative picropodo-

phyllin (PPP). It inhibits phosphorylation of phosphorylated Akt, leading to cell death and tumor regression in xenograft mice [119, 138].

Cross Talk Among Different RTKs in CRPC

AR signaling plays an important role in the development of CRPC. It is believed that AR expression may be modulated by RTKs and cytokines which bypass androgen-dependent pathways [109, 116]. Because AR activity is facilitated by RTKs and their complexes, it is conceivable that these proteins serve as cofactors to regulate downstream factors [19]. Upon AR activation, AR can bind to its cofactors to form protein-protein complexes and assembly at their target gene promoter regions and regulate the transcription of these targets [139]. There is a cross talk between EGFR, VEGFRs, and IGF-IR [140]. In addition, both IGF-IR and EGFR are simultaneously elevated in a wide range of cancers, including PCa. Recent studies have demonstrated that tumor cells may gain resistance to anti-EGFR therapies without altering EGFR expression but rather through upregulation and activation of other RTKs, including PDGF, FGF, and IGF-IR [119, 140, 141]. These observations suggest that a combinational regimen targeting simultaneously EGFR and other RTKs, such as IGF-IR, may exert higher anticancer activity than the monotherapy using inhibitor to individual growth factor.

Acknowledgments This work was supported by the grants from the Swedish Cancer Society, the Swedish National Research Council, the Swedish Children Foundation, Malmö Hospital Cancer Foundation, Malmö Hospital Foundation, and Gunnar Nilsson Cancer Foundation. Crafoord Foundation to JLP.

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Chapter 25
Microtubules in Prostate Cancer

Paraskevi (Evi) Giannakakou and Giuseppe Galletti

Contents

Microtubule Biology in Prostate Cancer

The cytoskeleton is a dynamic, intricate network of filaments and tubules that spans the entire cytoplasm connecting the nucleus to the plasma membrane. It consists of three polymeric fibers that are composed of distinct protein subunits: the microfilaments (made of actin), the microtubules (made of tubulin), and the intermediate filaments (composed of more than 60 different subunits). The cytoskeleton is responsible for establishing cell shape, providing structural support, and facilitating cell movement, mitosis, and trafficking pathways.

Microtubules are long, hollow, cylindrical protein polymers composed of α[alpha]/β[beta]-tubulin heterodimers that assemble in a head-to-tail fashion to form a protofilament; 12–13 protofilaments then associate longitudinally to form the hollow microtubule. Microtubules are polarized structures, with the β[beta]-tubulin monomers oriented toward the faster-growing "plus" end, while the α[alpha]-tubulin monomers are oriented toward the slower-growing "minus" end.

P.(Evi) Giannakakou, Ph.D. (✉) • G. Galletti, M.D., Ph.D.
Hematology-Medical Oncology, Department of Medicine, Weill Cornell Medical College, New York, NY, USA
e-mail: pag2015@med.cornell.edu

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*, Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_25

Microtubules undergo cycles of assembly and disassembly, a phenomenon called "dynamic instability"; this ability to rapidly switch from a "growth" state to "shrinkage" allows them to readily rearrange their network in response to cellular needs [1]. Thus, their biological functions and their polymerization dynamics are interdependent.

Due to their intrinsic high dynamicity and ability to undergo rapid remodeling, microtubules are directly involved in essential cellular functions; they form the mitotic spindle that aligns and separates chromosomes during mitosis, and they provide a dynamic platform for protein-protein interaction and signal transduction pathways during interphase [2]. As a result, drugs that target and disrupt the microtubule network affect both mitosis and signaling pathways and represent one of the most successful therapeutic strategies in cancer treatment.

Microtubules are very heterogeneous in length, dynamicity, and functions, all characteristics that are tightly regulated through several biological mechanisms. Microtubules' functional diversity is achieved largely by differential expression of several tubulin isotypes, by interactions with numerous regulatory proteins (motor proteins and non-motor microtubule-associated proteins-MAPs), and through post-translational modifications [3].

The complexity and dynamic nature of the microtubule cytoskeleton also affect cancer progression; particularly, they play a crucial role in prostate cancer where distinct alterations of the microtubule network have been identified.

In most eukaryote cells, α[alpha]- and β[beta]-tubulins consist of isotypes encoded by different genes and differing in amino acid sequence; there are 8 human α[alpha]-tubulin isotypes and 9 β[beta]-tubulin ones. The differential tissue distribution of each isotype and their high evolutionary conservation suggest that these differences have potentially functional significance [4]. Even though a significant amount of preclinical and clinical data indicates that microtubules composed of different tubulin isotypes differ in assembly characteristics, dynamics, and sensitivity to microtubule-targeting drugs [5, 6], the actual biological function of most of these tubulin variants remains unknown.

Immunohistochemical tubulin profiling evaluation of healthy and malignant human prostate tissues showed that the expression of β[beta]IV-tubulin isotype is expressed at higher levels than β[beta]II and β[beta]III in both benign hyperplastic epithelium and in moderate and poorly differentiated prostatic carcinomas [7]. Increased expression levels of β[beta]IV-tubulin in Gleason 3–4 tumors suggest its association with tumor dedifferentiation; however, the impact of the differential expression of tubulin isotypes in prostate cancer prognosis and progression has still to be clarified.

Microtubules can also acquire several posttranslational modifications (PTM) occurring on both α[alpha]- and β[beta]-tubulin subunits such as acetylation, detyrosination, polyglutamylation, and phosphorylation [8, 9]. The functions of these evolutionary-conserved modifications have not yet been fully elucidated. It has been proposed that PTMs could be involved in the recruitment of microtubule-binding proteins, thus mediating microtubule-based functions [10]. The outline of tubulin posttranslational regulation has been explored in prostate cancer cell line models

revealing a significant change of the PTM profile in both hormone-sensitive and hormone-resistant prostate cancer specimens, potentially related to cell differentiation and cancer progression [11].

The microtubule network has been implicated in facilitating the intracellular trafficking and the nuclear import of several cancer regulatory proteins, including p53, HIF-1α[alpha], and RB [12–15]. In prostate cancer, numerous observations indicate that microtubules are also required for ligand-dependent and ligand-independent AR nuclear translocation and its downstream transcriptional activity [16, 17]. The therapeutic impact of these findings is significant as they suggest that microtubule-targeting drugs (taxane) affect cancer progression not through mitotic arrest but by impairing AR signaling activity, by blocking its nuclear translocation and inhibiting the downstream transcriptional activation of androgen response element (ARE)-containing target genes. Interestingly, the interaction between AR and microtubules is partially altered for the AR splice variants; in particular, ARv567, which lacks the ligand-binding domain, still displays a microtubule-dependent nuclear accumulation, while ARv7 does not bind microtubules, as it lacks the hinge region [18].

Taxanes in the Treatment of Castration-Resistant Prostate Cancer

Chemotherapy has been used to treat prostate cancer for decades; however, only taxanes, a class of agents that target the microtubule cytoskeleton, proved to prolong survival of patients with metastatic castration-resistant prostate cancer (mCRPC). Two large phase III clinical trials (TAX327 and SWOG 99–16) investigated the role of docetaxel in the treatment of CRPC revealing that docetaxel prolonged overall survival when compared with the previous standard-of-care mitoxantrone, thus becoming the current gold standard treatment for chemo-naïve mCRPC patients [19, 20]. In addition to taxanes, the current algorithm for CRPC treatment includes FDA-approved second-generation hormonal therapies and immunotherapies and bone-targeting agents. Taxanes who are traditionally administered to PC patients after progression with androgen deprivation therapy are currently one of the most clinical relevant strategies among several available treatment options.

At a molecular level, taxanes stabilize the microtubule cytoskeleton after binding to β[beta]-tubulin, leading to mitotic arrest and consequently to apoptosis in rapidly dividing cells [3]. Furthermore, taxanes have been shown to affect microtubule dynamics during interphase, resulting in a significant compromise of intracellular trafficking [12, 13, 15]. It has been widely shown that, in prostate cancer cells, AR associates with and traffics on microtubules to translocate to the nucleus after ligand binding. Consequently, in prostate cancer, the interphase effect of taxane manifests mainly by AR cytoplasmic sequestration and by inhibition of the downstream transcriptional activation of AR target genes that are crucial for tumor progression [16] (Fig. 25.1a).

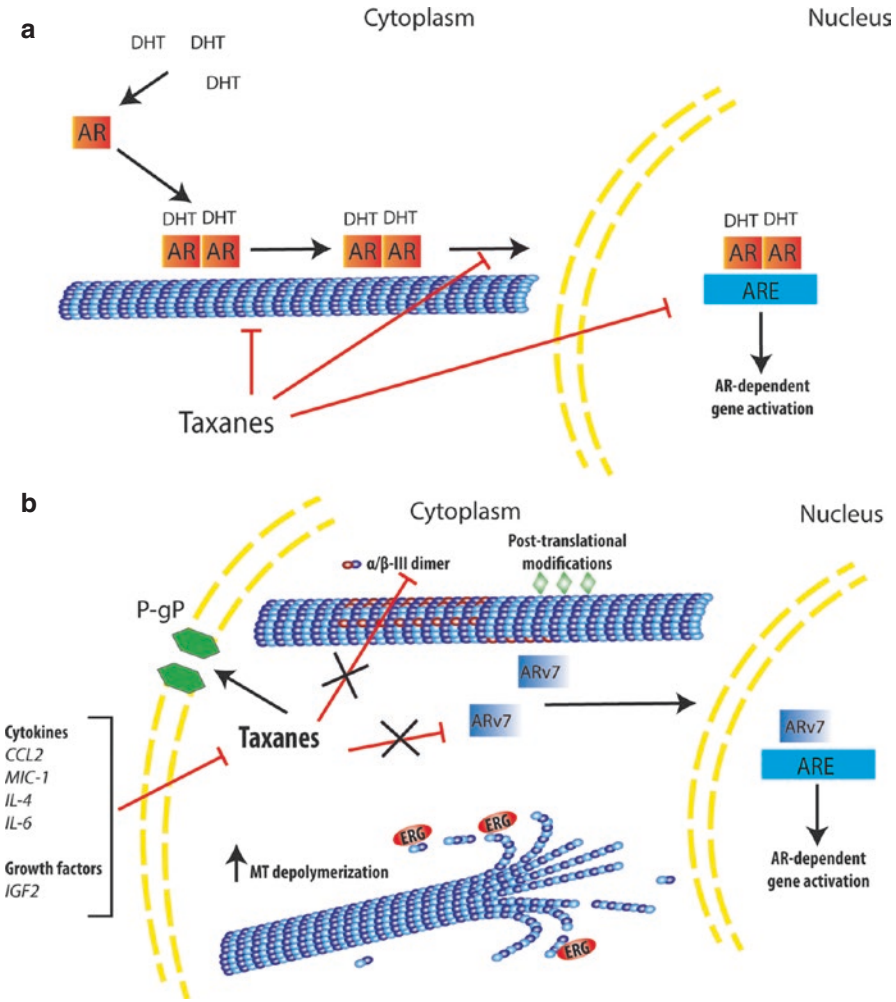

Fig. 25.1 (**a**) This schematic model describes the mechanism of action of taxane treatment in prostate cancer. Taxanes bind and stabilize microtubules, consequently inhibiting AR nuclear translocation and the activation of the androgen-dependent downstream signaling. (**b**) This model represents the main mechanisms of resistance to taxane antitumor activity in prostate cancer. Overexpression of P-glycoprotein (P-gP) results in reduced intracellular taxane accumulation. Increased levels of specific β[beta]-tubulin isotypes, such as $\beta[\text{beta}]_{\text{III}}$-tubulin, the overexpression of destabilizing microtubule-associated proteins (MAPs, such as ERG), and the enrichment in microtubule posttranslational modifications (PTMs) can alter microtubule dynamics, reducing taxane activity on their target. Aberrant activation of the androgen receptor signaling through AR slice variants (ARv7) or the activation of other intracellular pathways by cytokines and growth factors can eventually counteract taxane activity. *DHT* dihydrotestosterone, *AR* androgen receptor, *ARE* androgen-responsive element

Cabazitaxel is a third-generation taxane that elicited the scientific attention after it exerted in vitro activity in prostate cancer models, especially in docetaxel-resistant cell lines [21], and a tolerable toxicity profile in phase I studies [22]. Cabazitaxel was granted FDA approval for treatment of docetaxel-refractory mCRPC patients in 2011 after the results of the randomized, multicenter phase III TROPIC trial that demonstrated the superiority of cabazitaxel over mitoxantrone in improving overall survival, time to progression, and both biochemical and radiographic responses [23, 24].

Even though both docetaxel and cabazitaxel share the same target, the results of the TROPIC clinical trial demonstrated that patients who progressed to docetaxel still could respond and have a durable disease control when treated with cabazitaxel. In particular, these data emphasize the need to understand the molecular mechanisms underlying the differential response to both taxanes and to identify biomarkers to guide the choice between these two drugs.

Recent observations indicate that cabazitaxel has a more pronounced antiproliferative and pro-apoptotic effect than docetaxel in in vitro and in ex vivo models and that the two taxanes elicit distinct gene expression changes in prostate cancer cell lines, suggesting a not completely overlapping mechanism of action [25]. The difference in mechanism of action is further supported by data that showed that cabazitaxel exerts a superior antitumor activity compared to docetaxel in enzalutamide-resistant mouse models, independently from an effect on the AR pathway [26]. Interestingly, retrospective clinical analyses in abiraterone-pretreated CRPC subjects suggest that cabazitaxel retains its antitumor activity, in contrast with docetaxel, whose efficacy appeared lower than expected in this subset of patients [27, 28]. Taken together, these intriguing preclinical data with further results obtained by cabazitaxel as second-line treatment prompted the use of cabazitaxel in the first-line setting. The multicenter phase III clinical trial FIRSTANA compared head-to-head docetaxel to cabazitaxel as first-line cytotoxic treatment, to assess the potential superiority of one taxane over the other for the treatment of chemo-naive CRPC patients. No significant difference was observed between the two drugs in terms of overall survival and progression-free survival; nevertheless, the two drugs showed a different toxicity profile, which can help guide treatment decision [29, 30].

The introduction of second-generation AR-targeted drugs abiraterone and enzalutamide induced a paradigm shift in the treatment of mCRPC patients. The availability of novel hormonal therapies prior to chemotherapy prompts an urgent need to optimize the treatment sequence and drug selection, also taking into account the molecular profile of the disease in the individual patient; in this new exciting scenario, the identification and development of predictive biomarkers is of the utmost importance.

Within this context, the international multicenter correlative phase II clinical trial TAXYNERGY (NCT01718353) investigated the potential clinical differences between docetaxel and cabazitaxel, with a special focus on the evaluation of predictive biomarkers of response. This study prospectively evaluated molecular markers and investigate the mechanisms of taxane resistance in men with metastatic CRPC.

In this trial, subjects were randomized 2:1 to first-line docetaxel or cabazitaxel, and circulating tumor cells (CTCs) were used as a source of tumor tissue to monitor

longitudinally potential predictive biomarkers including AR nuclear localization, AR variants, and presence of intratumoral drug-target engagement [31]. Of the 63 patients enrolled in the study, 26 had CTC evaluable before the first cycle of treatment (C1D1) and after 1 week (C1D8); in these subjects, taxane-induced decrease in AR nuclear localization (C1D1 vs. C1D8) was associated with a higher rate of biochemical response (≥50% PSA decrease at C4, $p = 0.009$), suggesting that AR nuclear localization assessment can serve as early biomarker of clinical benefit in patients treated with taxanes [32].

Additional observations revealed that loss of RB appears to hypersensitize CRPC to cabazitaxel in prostate cancer in vitro models, suggesting RB status as potential biomarker for treatment outcome prediction [25]; the phase II clinical trial ABICABAZI (NCT02218606) will test this hypothesis, evaluating retrospectively the impact of RB expression in cabazitaxel sensitivity in CRPC.

Taxanes in Castration-Naïve Prostate Cancer

Using taxane chemotherapy earlier in the treatment sequence of hormone-naïve prostate cancer is currently under scrutiny in large clinical trials in light of the success of taxane such as docetaxel in later stages of treatment of PC patients.

Currently, three randomized phase III clinical trials aim to assess whether administering chemotherapy (docetaxel) to men with metastatic hormone-sensitive prostate cancer (mHSPC) in combination with androgen deprivation therapy could improve patient outcomes.

In the European GETUG-AFU-15 study, 385 men with mHSCP were randomized to receive either ADT alone or ADT in combination with docetaxel and, after a median follow-up of 82.9 months, a statistically significant difference was observed in favor of the combination in PFS (23.5 months vs. 15.4 months, HR: 075, $p = 0.015$) but not in OS, the primary endpoint of the study (60.9 months vs. 46.5 months, HR: 0.9, $p = 0.44$) [33, 34]. A final analysis reassessed the data in the cohorts of patient with high- and low-volume disease, but no significant difference in OS was found for either subgroup.

The US E3805 CHAARTED (ChemoHormonal therapy versus Androgen Ablation Randomized Trial for Extensive Disease) trial also investigated the effect of the addition of docetaxel to ADT in 790 men with mHSPC. OS was the primary endpoint, and after a median follow-up of 29 months, it was found to be significantly longer for the group of patients treated with ADT plus docetaxel (57.6 months vs. 44.0 months, HR: 0.61, $p = 0.0003$). The greatest difference was observed in patients with high-volume disease [35, 36].

The contrasting results from these two high-profile trials fuelled a still ongoing debate on the role of taxanes as front-line treatment in mHSPC.

The positive CHAARTED trial although is a larger study powered to detect differences between the two treatments, it requires a longer follow-up before drawing a final conclusion; however, it strongly points to docetaxel in combination with

ADT for the treatment, in particular, for those patients with a higher burden disease. Moreover, the synergistic benefit of taxanes and androgen deprivation therapy was largely anticipated by in vitro mechanistic data (will elaborate on later in the chapter) that indicate that docetaxel may act as a hormonal agent, interfering with AR nuclear translocation on microtubules.

The negative trial GETUG-AFU-15, although has a longer median follow-up, is a significantly smaller trial, probably underpowered to observe potential differences between treatment groups. Thus, further clinical trials are needed to clarify the role of taxanes treatment in mHSPC [37].

Recently, the results of the interim analysis of another large randomized phase III Systemic Therapy in Advancing or Metastatic Prostate cancer: Evaluation of Drug Efficiency (STAMPEDE) trial have been released; the preliminary data include the outcome of four of the nine different treatment arms of this study (standard of care, at least 3 years of ADT, standard of care plus docetaxel, standard of care plus zoledronic acid, and standard of care plus docetaxel and zoledronic acid). After a median follow-up of 42 months, the median OS was 10 months longer in the docetaxel arm compared with the standard of care (81 months vs. 71 months, HR: 0.78, $p = 0.0006$) with an improvement of 15 months in the subset of patients with metastatic disease (60 months vs. 45 months) [38]. These findings strongly suggest that men with newly diagnosed mHSPC should receive docetaxel as part of their initial therapy; however, a longer follow-up is needed before coming to final conclusions.

Additional data for the benefit of the addition of docetaxel to ADT were reported in locally advanced treatment-naïve prostate cancer patients participating in the GETUG-12 trial, in which docetaxel-based chemotherapy improved relapse-free survival compared to ADT alone in men with high-risk localized prostate cancer [39].

Overall, these data support a promising role of docetaxel as part of first-line treatment regimens in combination with AR-targeted therapies, especially in patients with high-volume disease. Nevertheless, even though these results are very encouraging, further investigations are required before medical practice is changed.

Biomarkers of Activity of Taxane Therapy

Taxanes are the only chemotherapy drugs that demonstrated clinically a significant benefit for the treatment of CRPC patients progressing antiandrogen therapy. Nevertheless, only 50% of patients durably respond to treatment and any survival benefit usually comes at the cost of significant toxicity. Cancer cells could potentially acquire numerous biological alterations during cancer progression, and genetic and biologic modifications might occur as a consequence of cell adaptation to drug exposure; all these changes can contribute to the onset of drug-resistant phenotypes, circumventing the antitumor activity of taxanes. Most likely most of these mechanisms don't act separately but as a complex multifactorial process that complicates the understanding of the actual biology of taxane resistance (Fig. 25.1b).

Nevertheless, a more comprehensive knowledge of the mechanisms that drive drug response and/or resistance is required to improve clinical decision-making and maximize patient outcomes.

Over the last two decades, accumulating reports show that overexpression of multidrug transporters in in vitro cancer cell lines represents a major mechanism of drug resistance to cancer therapy. Through the action of these molecular pumps, cancer cells manage to lower the intracellular concentrations of different classes of drugs, including the taxanes, thus, impairing their anticancer activity. Several of these transporters are overexpressed in prostate cancer [40, 41], and numerous lines of evidence indicate that the phenotype of multidrug resistance (MDR) could play a clinically relevant role in the onset of taxane resistance in prostate cancer. However, further retrospective and prospective analyses will be required to validate the clinical impact of MDR phenotype in taxane resistance. Interestingly, a specific single nucleotide polymorphism of the P-glycoprotein-encoding gene mdr1/ABCB1 has been retrospectively associated with a worse clinical outcome and a higher probability to develop neurologic and hematologic toxicities in docetaxel-treated CRPC patients [42].

Numerous studies have repeatedly associated the expression profile of β[beta]-tubulin isotypes with the response to microtubule-targeting drugs in several cancer types; in particular, β[beta]III-tubulin overexpression correlates with increased resistance to taxane treatment and a worse prognosis in solid tumors [43, 44]. Although different ratios of tubulin isotypes in microtubule polymerization can affect microtubule dynamics in vitro [3], their impact on microtubule biological behavior remains unclear. β[beta]III-tubulin expression is believed to play a crucial role in prostate cancer progression, as it is predominantly expressed in CRPC and can be further upregulated by androgen ablation treatment in prostate cancer cells [45]. Moreover, a retrospective analysis assessing β[beta]III-tubulin expression in 37 docetaxel-treated prostate cancer patients demonstrated that β[beta]III-tubulin positive subjects experienced a significantly shorter overall survival together with a lower PSA response rate and a shorter time to progression compared to negative patients [46].

These results are in line with similar observations in other solid tumors, supporting the role of β[beta]III-tubulin as a biomarker of resistance to taxane treatment in prostate cancer. However, clinical prospective studies are needed in order to verify these observations and establish the role of tubulin isotype expression profile in clinical decision-making.

Interestingly, a recent study reports a potential link between high β[beta]III-tubulin levels and ERG overexpression in prostate cancer [47]. Even though the biological meaning of this association has still to be clarified, this connection could open interesting clinical scenarios, as both biomarkers have been independently associated with taxane resistance, as we will discuss later in this chapter [48].

Active AR signaling is an essential driver of prostate cancer growth and progression, as AR translocates from the cytoplasm to the nucleus where it binds to and activates its target genes [49, 50]. Interestingly, in order to exert its function as transcriptional factor, AR has to associate with microtubules so that it can be

efficiently trafficked to the nucleus after ligand stimulation [16]; these observations are intriguing and illustrate that taxanes can exert their antitumor activity in prostate cancer by interfering with microtubules not only during mitosis but also in interphase by inhibiting AR nuclear accumulation and the subsequent activation of the AR axis.

This novel mechanism of taxane activity offers the molecular basis to interpret the synergistic effect exhibited by the combination of taxanes with antiandrogen therapies, as it has recently been suggested by phase III clinical trials (CHAARTED trial, STAMPEDE trial). The clinical impact on patient survival achieved by combining the standard androgen deprivation therapy with taxane chemotherapy in hormone-naïve prostate cancer patients could be further improved by exploring additional treatment options, for instance, including also more active second-generation antiandrogen drugs (abiraterone, enzalutamide) aiming to improve the clinical management of patients.

Upregulation of constitutively active AR splice variants represents one of the molecular mechanisms at the basis of disease progression during androgen deprivation therapy [51]; several splice variants have been described, but ARv567 and ARv7 are predominantly present in clinical specimens [52, 53]. In vitro studies and animal models have demonstrated that ARv567, but not ARv7, relies on dynamic microtubules to shuttle into the nucleus and exert its transcriptional function; in contrary, ARv7 seems to be microtubule independent, as it lacks the microtubule-binding domain, and its presence has been thus proposed as a potential marker for taxane resistance [18].

Recently, the clinical impact of ARv7 has been evaluated in a small cohort of 37 CRPC patients receiving taxane-based chemotherapy. In this single institutional study, mRNA derived from circulating tumor cells (CTCs) was examined for the presence of ARv7 transcript. Seventeen patients (46%) were considered as ARv7 positive, but no differences were observed between ARv7+ and ARv7- subjects in terms of biochemical response and PFS. Moreover, a significant correlation between ARv7 status and treatment type was observed, with higher biochemical response and longer PFS observed in taxane-treated compared to enzalutamide- or abiraterone-treated ARv7 patients [54]. These clinical findings seem to contradict the role of ARv7 as a biomarker of taxane resistance observed in vitro and may point to the potential function of ARv7 as a driver for clinical decision-making in CRPC patients; ARv7-negative patients are treated with second-generation ADT, while ARv7-positive subjects directed to a taxane-based chemotherapy. However, the results of this trial cannot be conclusive, as the very small number of subjects analyzed might have masked a not statistically significant but still detectable difference in response to taxane with ARv7+ men less likely to benefit from the treatment [55]. Large multi-institutional prospective clinical trials are currently ongoing to further evaluate the role of ARv7 in taxane-treated CRPC patients [TAXYNERGY trial NCT01718353; PCF Challenge trial NCT02269982].

Proteins that regulate microtubule dynamics and functions by interacting with polymerized microtubules or soluble dimers can also potentially interfere with the activity of microtubule-targeting drugs in cancer cells. Several examples have been

reported in prostate cancer, and, importantly, some of the proteins involved are now considered as biomarkers of response to taxane treatment.

ERG is a transcriptional factor that is found overexpressed in at least 50% of prostate cancer specimens as the result of gene fusions with the 5′ promoter of AR-driven genes (TMPRSS2, SLC45A3, and NDRG1) [56].

Mass spectrometric analysis indicated a potential interaction between ERG and tubulin suggesting an extranuclear function of the factor [57]; recent efforts investigated the relationship between ERG, microtubules, and taxane treatment in prostate cancer and demonstrated that ERG induces taxane resistance in vitro, in animal models, and in CRPC patients, by directly interacting with tubulin and shifting microtubule dynamics toward an increased catastrophe rate [48]. These changes render cancer cells more resistant to taxane treatment but, in turn, more sensitive to microtubule depolymerizing drugs.

These findings strongly suggest that ERG can be a potential predictive biomarker of taxane response in prostate cancer, pointing to an alternative therapy for the ERG-positive group of patients who could benefit from other FDA-approved drugs (vinca alkaloids, eribulin). Prospective validation of these results is currently in progress.

Kinesin motor proteins are proteins that move along microtubules and are involved in several cellular processes including transport of cellular cargoes and mitosis [58]. Several studies have associated members of the kinesin family with resistance to taxane-based treatment in solid tumors, as a consequence of their close interaction with microtubules [59].

Recently, the potential impact of kinesin Eg5 on taxane treatment outcome was explored in prostate cancer patients; Eg5 (KIF11/kinesin-5) plays a central role in mitotic spindle formation, regulating the separation of spindle poles [60]. In a retrospective analysis of 110 PC patients, nuclear IHC positivity for Eg5 was associated with a higher objective response to DTX treatment [61]. Although intriguing, these results need further clinical validation; moreover, a more comprehensive investigation of the relationship between taxane treatment and Eg5 is still missing, hampering the clinical value of this observation.

Overexpression of the mitotic centromere-associated kinesin (MCAK) has been associated with resistance to taxane therapy in ovarian cancer cells [62], and gene expression analyses found MCAK upregulated in multiple CRPC chemotherapy-resistant datasets [63]. However a direct mechanistic association between MCAK and docetaxel resistance in CRPC is still missing, and clinical validation of its role on the onset of chemoresistance is needed.

Cytokines have been directly involved in tumor growth and metastatic expansion in prostate cancer; it has been shown that CCL2 is implicated in PC cells proliferation in vivo by inducing microphage tumor infiltration and angiogenesis. In addition, it was demonstrated that AR suppression could cause an increase of CCL2 levels, mediating tumor progression [64]. Intratumor levels of CCL2 have been also associated with the development of drug resistance after taxane treatment in PC; in particular, taxane-mediated disruption of microtubule dynamics could induce an

increase of intratumor CCL2 levels as stress response, which, in turn, activates PI3K/AKT signaling promoting cell survival [65]. These in vitro observations still require clinical validation; nevertheless, they open interesting clinical scenarios suggesting the potential combination of taxane therapy with anti-CCL2 approaches to optimize the antitumor activity of taxanes.

Furthermore, in vitro and clinical data demonstrated a relationship between the inflammatory response and docetaxel resistance, manifesting with a significant increase of circulating pro-inflammatory cytokines involved in macrophage recruitment and activation (MIC-1, IL-4, and IL-6) in CRPC patients not responding to docetaxel [66]. However, the mechanisms that link these pro-inflammatory changes and resistance to treatment of cancer cells remain elusive. Overall, intratumor and circulating cytokines may play a significant role on the onset of taxane resistance and could serve as predictive biomarkers of response and as potential therapeutic targets.

Deregulation of growth factors and the intracellular pathways that they activate downstream represents one of the main mechanisms of cancer progression and metastasis, and it has been repeatedly associated with chemotherapy resistance in solid tumors [67, 68].

Recent studies shed light into the potential role of IGF2 on the onset of taxane resistance in prostate cancer; it has been well documented that GATA2 expression levels increase during progression from primary PC to disseminated CRPC, with the highest levels observed in taxane-treated patients. GATA2-induced upregulation of IGF2 would activate the PI3K/AKT and ERK1/2 pathways through IGF1 and INSR inducing cell survival and consequently chemotherapy resistance [69, 70]. The clinical implications of these findings support the combination of docetaxel treatment with anti-IGFR1 targeted therapies as a potential strategy to overcome the IGF2-mediated onset of taxane resistance.

Other studies indicate that also the Notch and hedgehog pathways are involved in docetaxel resistance; in in vitro models and CRPC patients, docetaxel treatment tends to enrich for a subpopulation of PC cells characterized by the lack of differentiation markers (CKs 18–19, AR, PSMA) and HLA-I antigens and by the overexpression of the Notch and hedgehog signaling [71]. Inhibition of these pathways abrogated docetaxel resistance.

Conclusions

For decades taxanes have represented the mainstay of treatment of men with mCRPC and are likely to maintain their crucial role in cancer therapeutics; the approval of hormonally acting drugs abiraterone and enzalutamide for the treatment of this subset of patients enriches the arsenal of available tools for clinicians. The best approach to meet the urgent clinical need to select the optimal treatment sequence can be realized by a biomarker-informed clinical decision.

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Part V
Precision Medicine Approach: Diagnosis, Treatment, Prognosis

Chapter 26
Prostate Cancer Tissue Diagnosis

Joshua I. Warrick and Scott A. Tomlins

Contents

Introduction

The majority of prostate cancers in the USA are diagnosed by prostate needle core biopsy. In contemporary practice, 12 biopsy needle cores are taken from the posterior and lateral prostate via a transrectal approach. More extensive, "saturation" biopsies may also be taken in some cases, frequently via a perineal approach. Likewise, multiparametric magnetic resonance imaging (MRI), particularly when "fused" with transrectal biopsy, is gaining more attraction particularly in the repeat

J.I. Warrick, M.D.
Department of Pathology, Penn State Milton S. Hershey Medical Center, Hershey, PA, USA

S.A. Tomlins, M.D., Ph.D. (✉)
Department of Pathology, University of Michigan Medical School, Ann Arbor, MI, USA

Department of Urology, University of Michigan Medical School, Ann Arbor, MI, USA

Michigan Center for Translational Pathology, Ann Arbor, MI, USA
e-mail: tomlinss@umich.edu

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*,
Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_26

biopsy setting. A smaller subset of cancers is diagnosed incidentally on transurethral resection of the prostate (TURP) performed for benign prostatic hyperplasia (BPH). Diagnosis of prostate cancer is based on histologic examination under light microscopy. The specific morphologic changes of prostate cancer are beyond the scope of this chapter; however, the majority of prostate cancers appear as crowded small glandular foci, with an appearance similar to the prostatic acinus, but with cytological (e.g., nucleoli) and architectural (e.g., an infiltrative appearance) features with lack of basal cells indicative of malignancy. Several benign entities also appear as crowded small acinar foci, including prostatic adenosis (atypical adenomatous hyperplasia), sclerosing adenosis, basal cell hyperplasia, and prostatic atrophy. Given this morphologic overlap, ancillary testing may be performed on suspicious foci, most commonly in the biopsy setting, to distinguish prostate cancer from its benign mimics.

Classic Immunohistochemical Markers: Basal Cell Markers and α[alpha]-Methylacyl-CoA Racemase/P504S (AMACR/P504S)

Benign prostatic glands and ducts are lined by a dual cell population: a basal cell layer and a luminal secretory cell layer. Gene expression in the basal cell layer differs from the luminal cell layer, as the basal cell layer expresses p63 and high molecular weight keratins (HMWKs), such as CK5/6, among other markers. These may be used as immunohistochemical markers to distinguish prostate cancer from its benign mimics, because the mimics will retain expression of basal cells, while prostate cancer will not [1–4]. However, some benign mimics, such as adenosis and partial atrophy, may have a discontinuous basal cell layer. Basal cell markers may therefore fail to identify this cell population on microscopic sections [5–10].

AMACR is overexpressed in 90% of prostate cancers and is unexpressed in the majority of benign prostatic tissues [11, 12]. This marker may thus be utilized as an immunohistochemical marker in the distinction of prostate cancer from its benign mimics. As with loss of basal cell marker expression, AMACR is not entirely specific for prostate cancer, as atrophy and adenosis lesions may be AMACR+ [5–10]. This is particularly true of a variant pattern of atrophy referred to as partial atrophy. These cases usually show less intense AMACR expression in comparison to prostate cancer. Importantly, the expression of AMACR limits its utility in distinguishing prostate cancer from benign lesions, though it is overexpressed in the vast majority of prostate cancers.

HMWCKs, p63, and AMACR may be performed as individual immunohistochemical stains or in a combined multiplex “cocktail” including all three antibodies (PIN4 triple stain, HMWCK/p63/AMACR). Prior studies have shown that the performance of these markers is not significantly improved in the multiplexed assay in comparison to individual antibodies [7, 13]. However, the “cocktail” of antibodies has proven to add significant convenience to interpretation and has thus become the preferred testing modality in many laboratories (Fig. 26.1).

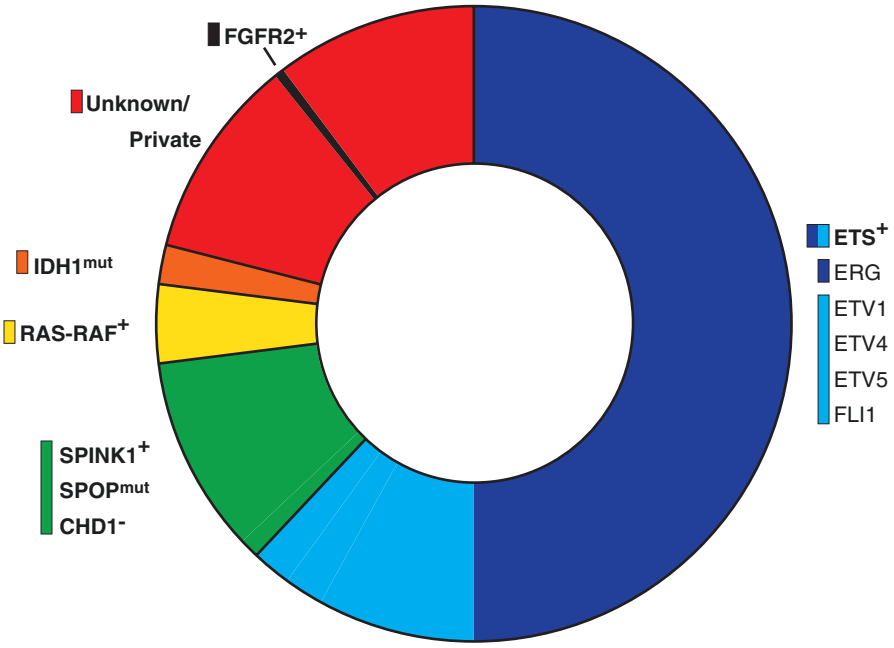

Fig. 26.1 Prostate cancer molecular subtypes. The approximate distribution of prostate cancer molecular subtypes in PSA-screened, predominantly Caucasian populations is shown. ETS gene fusions (ETS+, including those involving ERG, ETV1, ETV4, ETV5, and FLI1) are mutually exclusive with tumors harboring SPINK1 outlier expression (SPINK1+), disruption of CHD1 (CHD1-), and/or SPOP mutations (SPOPmut). Likewise, subsets of tumors lacking these alterations are characterized by activating gene fusions or mutations in RAS and RAF family members (RAS-RAF+) or IDH1 R132 mutations. Approximately 20% of prostate cancers have unclear or private drivers, such as fusions involving FGFR2 (FGFR2+)

ETS Rearrangements in Cancer Diagnosis

Approximately 50% of screening-detected prostate cancers harbor genetic rearrangements involving the ETS genes, a family of transcription factors inactive in benign prostate tissue (Fig. 26.2) [14–19]. Greater than 90% of ETS+ prostate cancers result from fusion of the ETS gene ERG on chromosome 21 to the upstream gene TMPRSS2, a constitutively expressed protein in the prostate. Approximately 50% of TMPRSS2:ERG (T2:ERG) fusions result from a deletion between TMPRSS2 and ERG on chr 21, which creates a fusion gene under control of the TMPRSS2 gene promoter. Rearrangements may also occur via more complex insertions. T2:ERG rearrangements result in marked overexpression of full length, or near full length, ERG protein product. ERG rearrangements may be detected by fluorescence in situ hybridization (FISH), most commonly using break-apart probes targeting the 5′ and 3′ ends of the ERG gene [17, 18, 20]. ERG protein is readily demonstrated by immunohistochemistry, which appears as a strong, diffuse pattern

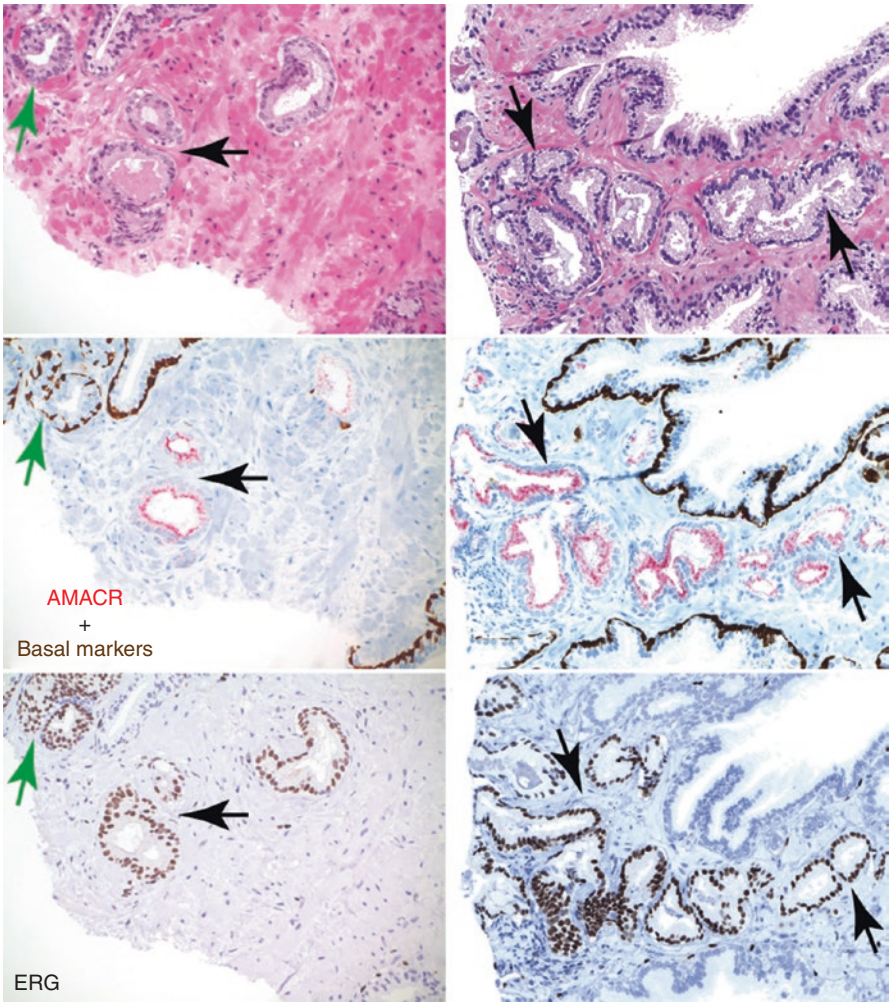

Fig. 26.2 Diagnostic utility of immunohistochemistry (IHC) for AMACR, basal cell markers and ERG. The use of ancillary immunohistochemistry (IHC) can aid in the interpretation of small atypical foci, particularly on diagnostic biopsy. Hematoxylin and eosin staining (*top panels*), AMACR + basal cell markers in a cocktail (*middle panels*, AMACR in *red* chromogen, basal markers in *brown*) and ERG immunohistochemistry (*bottom panel*) for suspicious foci in two diagnostic biopsy cores from two cases. In both cases, areas of cancer, with positive AMACR expression, negative basal cell marker expression, and positive ERG expression, are indicated by *black arrows*. Note ERG expression in adjacent high-grade prostatic intraepithelial neoplasia (HPGIN, retained basal cell markers) in the case on the *left*. Images courtesy of Drs. Makul Divatia and Mahul Amin (Cedars-Sinai Medical Center)

of nuclear staining. ERG expression by immunohistochemistry has been shown to be >95% sensitive and specific for ERG rearrangement as detected by FISH [14–16, 21–23]. Less commonly involved ETS genes include ETV1 and ETV4, and less common 5' partners include SLC45A3 and NDRG1 (Fig. 26.2).

Immunohistochemistry for the ERG protein product is emerging as a useful marker in prostate cancer diagnosis in selected scenarios. Benign prostatic tissue does not overexpress ERG, making ERG expression highly specific for prostate cancer in the context of a crowded acinar focus. This high specificity is superior to that of basal cell markers and AMACR. However, ERG immunohistochemistry is limited by sensitivity, being only expressed in 50% of cases. Despite this, there is an evidence of ERG overexpression offers a utility beyond basal cell markers and AMACR in correctly classifying difficult small acinar foci. For example, Shah et al. and Lee et al. separately showed immunohistochemistry for ERG aids in establishing the diagnosis of prostatic carcinoma in atypical glandular foci in which the diagnosis would not have been established confidently otherwise [24–26]. ERG immunohistochemistry in combination with AMACR and basal cell markers is shown in Fig. 26.1.

ETS rearrangements have not been identified in non-prostatic carcinoma (outside of Ewing sarcoma and leukemias where they had been identified previously) [27–30]. ERG is usually constitutively expressed in ERG+ prostate cancers via the TMPRSS2 promoter, which is under androgen receptor (AR) control. Expression of many genes expressed in both benign prostatic tissue and prostate cancer is androgen dependent. Prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA), and NKX3.1 are examples, which are also highly specific for prostate cancer as immunohistochemical markers in the context of carcinoma. Thus, if a prostate cancer is negative for expression of PSA, PSMA, and NKX3.1, androgen signaling is likely weak or inactive [31]. As ERG expression is under control of the AR-dependent TMPRSS2 promoter, ERG protein expression will likely also be negative [31, 32], even if the cancer in question harbors a T2:ERG rearrangement. The addition of ERG to an IHC panel including PSA, PSMA, and NKX3.1 is thus of questionable utility. Importantly, angiosarcoma, epithelioid sarcoma, and Ewing family tumors may also be ERG+ [33–35], in part dependent on the specific anti-ERG antibody used. These other ERG+ entities must be considered in a malignant neoplasm that expresses ERG.

As described above, poorly differentiated prostatic carcinomas harboring an ERG rearrangement may lack ERG expression, owing to diminished or absent androgen signaling. This is particularly true of prostatic neuroendocrine small cell carcinoma, which has a similar frequency of ERG genomic rearrangements at the genomic level as conventional acinar carcinoma [31, 36–38]. Importantly, shared presence or absence of ERG genomic rearrangements in concurrent conventional acinar carcinoma and small cell carcinoma supports a transdifferentiation model. Importantly, although small cell carcinoma (or poorly differentiated carcinomas) lacking AR signaling usually lack ERG expression, ERG rearrangements are still detectable by FISH. Hence, FISH for ERG rearrangement may distinguish prostatic

adenocarcinoma from other cancers, such as poorly differentiated urothelial carcinoma or small cell carcinoma of the bladder. This is not a trivial distinction, as poorly differentiated prostatic adenocarcinoma may be responsive to aggressive androgen deprivation even in cases with decreased androgen signaling, while other carcinomas will not.

Determining Tumor Clonality

ETS rearrangements are early events in prostate cancer development and are thus expressed in the entirety of ETS+ prostate cancer foci when clearly demarcated. Expression of ERG can therefore be used as a marker of tumor clonality. For example, in a prostate needle core biopsy showing two small foci of prostate cancer on opposite sides of the core, ERG expression by one but not the other is strong evidence the patient has multifocal disease. In scenarios where both foci are positive or negative, their clonal relationship cannot be determined. The utility of this is unclear at present, though it may have future applications in estimating prostate cancer burden based on needle biopsy parameters.

It has become apparent that ETS rearrangements are mutually exclusive of other common molecular findings. For example, ~10% of prostate cancers harbor mutations in the SPOP gene [39]. These mutations are essentially mutually exclusive with ETS rearrangements. SPINK1 overexpression is seen in ~10% of prostate cancers and is similarly mutually exclusive of ETS rearrangement, with overlap of SPOP mutated and SPINK1+ prostate cancers [40–43]. Assessment for these alterations, or other molecular subtype defining alterations (Fig. 26.2), may thus add utility to ERG immunohistochemistry for determining tumor clonality. An immunohistochemical cocktail of ERG and SPINK1 has been developed for this purpose and has shown utility in documenting multiclonality of prostate cancer foci, classifying the vast majority of cancer foci as ERG+/SPINK1+, ERG+/SPINK1-, ERG-/SPINK1+, or ERG-/SPINK1- [40].

High-Grade Prostatic Intraepithelial Neoplasia

High-grade prostatic intraepithelial neoplasia (HGPIN) is a common non-obligate precursor to prostatic adenocarcinoma. Morphologically, it has the architecture of benign prostatic tissue but has enlarged nuclei with prominent nucleoli. It is frequently identified on prostate needle core biopsy material. Patients with elevated serum PSA undergoing prostate needle core biopsy who have multiple positive cores for HGPIN (at least >2 of 12 cores) have a 40–60% chance of prostate cancer on rebiopsy, compared to a ~20% chance of cancer with elevated PSA and no HGPIN [44–46]. HGPIN usually expresses AMACR and often has discontinuous basal cell marker expression.

While 90% of HGPIN foci are AMACR+ [47], only ~10–15% are ERG+ [15, 48–50], with ERG+ varying significantly depending on criteria used to select HGPIN lesions (i.e., close or distant to carcinoma). HGPIN foci expressing ERG have been shown to be associated with prostate cancer more frequently than ERG- HGPIN foci [15, 48, 50]. For example, Furastro et al. reported that 96.5% of ERG+ HGPIN foci were adjacent to ERG+ prostate cancer [15]. A similar association with ERG- HGPIN was not seen in this study. A recent biopsy study by Park et al. reported similar findings in a cohort of 1,590 patients from a phase III clinical trial with isolated HGPIN on needle core biopsy treated with either toremifene (a selective estrogen receptor modulator) or placebo, followed with repeat biopsy for 3 years. Study entry biopsies with isolated HGPIN were evaluable for ERG in 461 cases. In the 11% of cases with ERG+ HGPIN, 53% had prostate cancer on follow-up, in contrast to 35% of patients with ERG- HGPIN ($p < 0.05$) [48]. Lee et al. reported similar findings in a series of prostate needle core biopsies [24]. Though further study is necessary for confirmation of these findings, it appears ERG may be useful in risk stratifying cases of HGPIN, although subsequent risk of cancer is likely confined to the immediate area of the ERG+ HGPIN and does not inform on unsampled tumor size or aggressiveness. HGPIN, that is, AMACR+, has similarly been shown to be associated with prostate cancer more frequently than HGPIN, that is, AMACR-, although not in the context of a clinical trial cohort [47]. Additionally, the high rate of AMACR expression in HGPIN limits the potential utility of AMACR in this context.

Intraductal carcinoma of the prostate is associated with aggressive, high-grade prostate cancer [51–55]. A diagnosis of intraductal carcinoma on needle core is therefore considered by most to be an indication for definitive treatment, such as prostatectomy or radiation therapy. Intraductal carcinoma is defined as high-grade carcinoma within the prostatic ductal acinar structure. Thus, cases morphologically appear as high-grade carcinoma with surrounding basal cells, which express p63 and HMWCK. Cases show high-grade features, including markedly enlarged nuclei and nonfocal comedonecrosis. The majority of cases are seen in the context of associated invasive carcinoma, even on the limited material afforded on needle core biopsy. However, rare cases exist in which a single focus is identified that has features of intraductal carcinoma but cannot be confidently distinguished from HGPIN. PTEN may be of value in such cases, as deletion of this gene has been shown to be specific for intraductal carcinoma in this differential [56]. For example, Morias et al. recently performed FISH for PTEN on a series of prostate needle core biopsies demonstrating HGPIN, isolated intraductal carcinoma of the prostate, or a borderline lesion in which the differential diagnosis included HGPIN and intraductal carcinoma [57]. Intraductal carcinoma had PTEN loss in 76% of cases, while PTEN loss was seen in none of the HGPIN foci. The borderline foci had PTEN loss in 52% of cases. Rebiopsy showed prostate cancer in 64% of borderline cases with PTEN loss, compared to 50% of cases without PTEN loss, though statistical significance of this observation was not reported.

Prostate Cancer Early Detection

Screening for prostate cancer with serum PSA is controversial, with most professional societies recommending against routine screening (reviewed in [58]). Serum PSA has issues of sensitivity and specificity for detection of prostate cancer and is often elevated in benign prostatic hyperplasia or benign inflammatory conditions. However, prostate cancer remains the second most common cause of cancer-associated mortality in American men, and screening with serum PSA likely saves lives, albeit at the cost of high rates of overdiagnosis of clinically insignificant cancers and subsequent overtreatment [59–61]. An accurate screening test thus remains a desirable goal.

Various modifications to serum PSA measurement or related kallikrein enzymes have been proposed to distinguish elevations due to prostate cancer vs. benign prostatic hyperplasia (Fig. 26.3). These includes free PSA, PSA velocity (the rate of rise of the PSA over time), PSA density (serum PSA divided by prostate size measured at ultrasound), age-adjusted PSA, pro-PSA, intact PSA, and KLK4. These tests, and total serum PSA, are immunoassays in the majority of cases. Assays for pro-PSA have gained recent interest. PSA is first synthesized as a propeptide, with pro-leader peptide sequences of various amino acid lengths. The propeptide with two amino acids leading, named [-2]pro-PSA, has been shown to be more specific for prostate cancer than total serum PSA. This test is currently Food and Drug Administration (FDA) approved for risk stratification in the prebiopsy setting. Regarding performance, [-2]pro-PSA has been shown to have better accuracy than serum PSA for the diagnosis of prostate cancer. For example, Sokoll et al. show improved accuracy of this assay over serum PSA in men with serum PSA between 4 ng/mL and 10 ng/mL for the prediction of prostate cancer at biopsy (area under the curve (AUC), 0.70 vs. 0.58, respectively), though [-2]pro-PSA did not outperform free PSA [62].

The Prostate Health Index (PHI) is a model incorporating total PSA, free PSA, and [-2]pro-PSA, which has been shown to have superior specificity for prostate cancer than serum PSA. For example, in a study of men with total serum PSA ranging from 2.0 to 10, Ito et al. showed fixed at a sensitivity of 95%; PHI demonstrates 28% specificity for detection of prostate cancer on needle core biopsy, compared to 3% specificity of serum PSA at this sensitivity [63]. However, it is not clear how PHI would have performed with the sensitivity equivalent to that of serum PSA at 4.0 ng/mL, the most commonly used cutoff. Furthermore, [-2]pro-PSA has been shown to be expressed at low levels in the majority of benign prostate epithelium, while prostate cancer shows high level expression [64]. It thus appears the marker is itself not specific for prostate cancer.

Likewise, the 4Kscore represents another kallikrein-based panel that uses a logistic regression model that incorporates clinical information, total PSA, free PSA, intact PSA, and KLK2 to report individualized high-grade prostate cancer risk estimates. This assay, or the four kallikreins assessed in the assay, has been extensively validated in European cohorts and was recently validated in a large, prospective American cohort [65–72]. For example, when compared directly to a modified

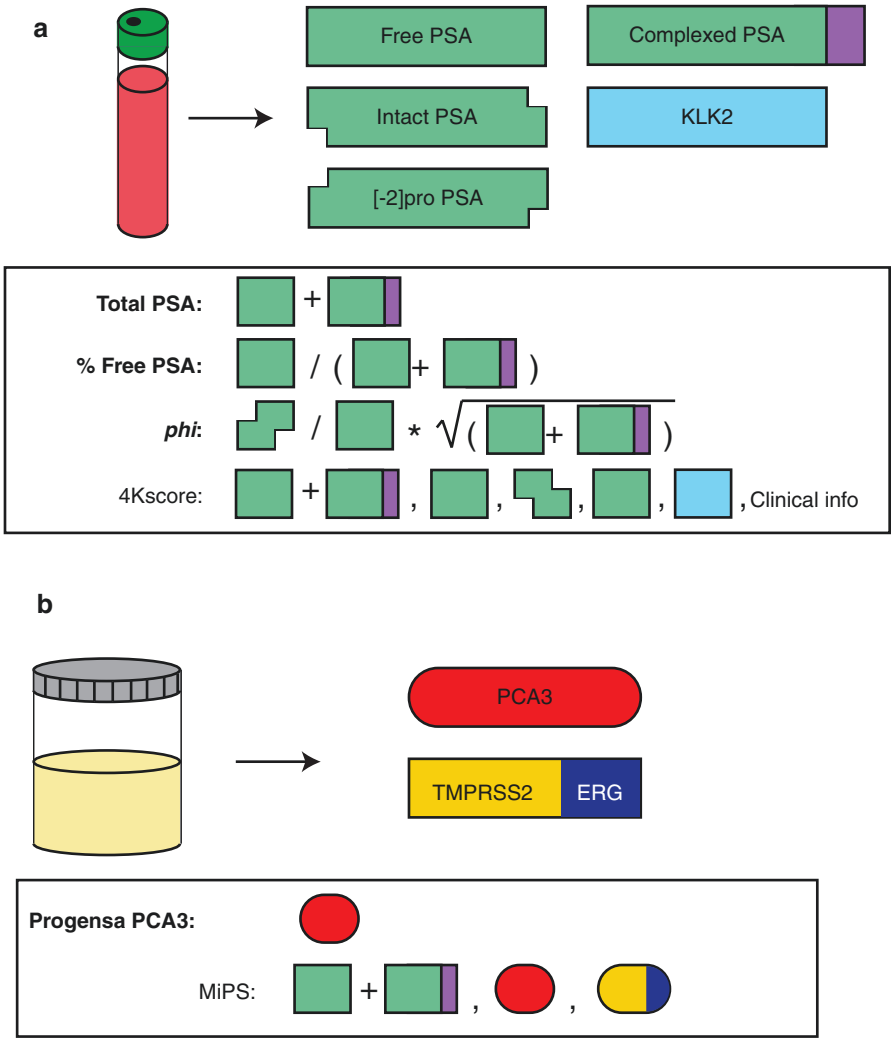

Fig. 26.3 Clinically available protein- and transcript-based biomarkers for prostate cancer early detection. Both serum- (**a**) and urine-based (**b**) prostate cancer early detection biomarkers are clinically available. (**a**) Multiple forms of prostate-specific antigen (PSA, KLK3) protein are quantifiable in serum, including free PSA, intact PSA (a free PSA form), [-2]pro PSA (a different free PSA form), and complexed PSA. KLK2 (hK2) is a kallikrein related to PSA that is also quantifiable in serum. Multiple combinations are used clinically, including total PSA, phi, and 4Kscore, using combinations of the indicated biomarkers. (**b**) Urine-based prostate cancer biomarkers include transcripts of PCA3 (a long noncoding RNA) and the TMPRSS2:ERG gene fusion. The Progensa PCA3 assay quantifies urine PCA3 transcripts normalized to urine PSA transcripts using transcription-mediated amplification yielding a PCA3 score, while the Mi-Prostate Score (MiPS) test incorporates total serum PSA, urine PCA3 score, and urine TMPRSS2:ERG score (generated by an assay analogous to the Progensa PCA3 assay). In (**a**) and (**b**), FDA-approved biomarkers are *bolded*

prostate cancer prevention trial risk calculator (PCPTRC) 2.0 (without family history), decision curve analysis demonstrated that 4Kscore had higher net clinical benefit at all threshold probabilities (the risk of high-grade cancer on biopsy at which a patient would be biopsied) [69]. Likewise, in the prospective, randomized prostate testing for cancer and treatment study (ProtecT), assessment of the four kallikreins from the 4Kscore panel increased the AUC for detecting high-grade cancer on biopsy from 0.738 for PSA alone to 0.820 [67, 68, 72].

Urine biomarkers are also available for determination of prostate cancer risk (Fig. 26.3). The two most advanced and well studied are prostate cancer gene3 (PCA3) and T2:ERG. PCA3 is a noncoding RNA that is dramatically overexpressed in prostate cancer compared to benign prostatic tissue [73–75]. PCA3 is detectable in the urine of many patients with prostate cancer [76–91]. The test is currently clinically available as the Progensa PCA3 assay, which calculates a risk score based on the ratio of urine PCA3 mRNA to urine PSA mRNA, measured from urine specimens taken after "attentive" digital rectal examination, to generate a PCA3 score. Various PCA3 scores have been proposed as an optimal cutoff for detection of prostate cancer, with most studies utilizing 35, although any cutoff is a compromise between sensitivity and specificity. Sensitivity and specificity of urine PCA3 have been 47–78% and 50–80%, respectively, using a cutoff of 35 [76–81, 83, 84, 86–88, 90]. Urine PCA3 has consistently been shown to offer greater diagnostic accuracy than serum PSA, particularly in specificity. Addition of PCA3 to the European randomized study of screening for prostate cancer (ERSPC) risk calculator (includes family history, patient age, specific symptoms, and serum PSA) has also been shown to improve the diagnostic accuracy of this model [86, 92]. The Progensa assay is currently FDA approved to determine if men with a negative prostate biopsy should undergo repeat biopsy. Using a Progensa score of 25 as a cutoff, to optimize sensitivity, a negative result has been shown to have a negative predictive value of 90% for cancer on rebiopsy [85]. Men with a negative test can thus avoid being rebiopsied. Most recently, PCA3 was validated in a prospective, blinded National Cancer Institute (NCI) sponsored Early Detection Research Network (EDRN) cohort, where it was shown to significantly improve the positive predictive value (PPV) for an initial biopsy (at a score >60) and the negative predictive value (NPV) for a repeat biopsy (at a score <20) [93].

Transcripts of the T2:ERG fusion are detectable in post-DRE urine specimens of patients with ERG+ prostate cancer [86, 94–96], and urine levels of T2:ERG correlate with ERG+ prostate cancer burden [50]. Similar to Progensa, a clinically available assay for this marker has been developed, which calculates a T2:ERG score based on the ratio of T2:ERG mRNA to urine PSA mRNA [50, 96–100]. At the tissue level, T2:ERG is essentially never expressed in benign tissue; hence detection of elevated urine T2:ERG should be specific for prostate cancer, and sensitivity should be lower than PCA3. For example, in the largest prospective study to date, Leyten et al. showed 24% of men with prostate cancer at biopsy had elevated prebiopsy urine T2:ERG (cutoff of 10 using a whole transcriptome amplification and qRT-PCR-based assay from urine sediment), while 68% and 81% had elevated urine PCA3 (cutoff 35 and 25, respectively) [86]. Conversely, only 6% of men without cancer at biopsy had elevated urine T2:ERG, compared to 48% and 40% for

PCA3 (cutoffs 25 and 35, respectively). The diminished sensitivity of urine T2:ERG likely relates to only half of prostate cancer foci being ERG+. However, it should be recognized that prostate cancer is a multifocal/multiclonal process in the majority of cases, and most men with prostate cancer have at least one ERG+ cancer focus [50].

Combined urine PCA3 and T2:ERG have been shown to have greater diagnostic accuracy than either marker alone. In their large prospective study, Leyten et al. evaluated urine PCA3 score and T2:ERG status in a multicenter cohort of men with T2:ERG assessed using a binary, whole transcriptome and qRT-PCR assay from sedimented prebiopsy urine specimens [86]. The study showed addition of both markers increased the diagnostic accuracy for prostate cancer over the ERSPC alone. Specifically, AUC for prediction of prostate cancer was 0.799 for ERSPC alone, which increased to 0.833 with the addition of PCA3 and to 0.842 with the addition of both PCA3 and T2:ERG. Though the addition of T2:ERG added a relatively small amount of predictive ability for prostate cancer diagnosis, it also added important prognostic information regarding disease aggressiveness, while PCA3 did not. Likewise, Tomlins et al. recently reported the validation of the Mi-Prostate Score (MiPS) assay, which utilizes validated logistic regression models incorporating serum PSA, urine PCA3 score, and urine T2:ERG score (using an assay analogous to the Progensa PCA3 assay) to report individualized risk estimates of cancer (and high-grade cancer) on biopsy. Compared to the PCPTRC alone, MiPS models using PCPTRC in place of serum PSA showed significantly increased AUC and net clinical benefit (by decision curve analysis) for predicting cancer or high-grade cancer on biopsy [100].

Importantly, head-to-head comparisons of the various serum- and urine-based prostate cancer early detection biomarkers/assays are limited in contemporary cohorts. For example, Vedder et al. recently found that PCA3 and the 4 kallikreins in the 4Kscore assay showed similar performance when added to the ERSPC risk calculator in the Dutch ERSPC cohort, with the 4-kallikrein panel improving the predictive value of the ERSPC risk calculator in participants with PSA $\geq$3.0 ng/ml, while PCA3 improved the predictive value in prescreened men, regardless of total PSA [67]. Lastly, Gronberg et al. demonstrated the potential of combining multiple classes of early detection biomarkers in the STHLM3 study, which tested the utility of a combination of plasma protein biomarkers (PSA, free PSA, intact PSA, KLK2, MSMB, and MIC1) with 232 genetic polymorphisms and clinical variables [101]. They found that all classes of biomarkers were associated with increased risk of high-grade disease on biopsy. Importantly, the recent clinical implementation of PHI, 4Kscore, and MiPS should enable additional studies into the optimum combination of early detection biomarkers, which may include both serum- and urine-based biomarkers.

Conclusions

Molecular studies have proven valuable in diagnosing prostate cancer and related lesions with greater confidence and precision, largely through the identification of biomarkers that can be assessed in combination. Importantly, immunohistochemistry

with standard markers (AMACR and basal cell markers) in supplementation with ERG enables a confident diagnosis in the vast majority of biopsies. Hence, major advances in diagnostic biomarkers will likely need to occur noninvasively (e.g., imaging, blood, or urine) as described herein. Importantly, while serum PSA has proven disappointing as a diagnostic screen for prostate cancer, combining serum PSA with more specific isoforms/related kallikreins or urine-based biomarkers may enable more accurate noninvasive early detection of prostate cancer.

Acknowledgments SAT is supported in part by the Evans Foundation/Prostate Cancer Foundation, the A. Alfred Taubman Medical Research Institute, and the National Institutes of Health (R01 CA183857).

Disclosures

The University of Michigan has been issued a patent on the detection of ETS gene fusions in prostate cancer, on which SAT is listed as a co-inventor. The University of Michigan licensed the diagnostic field of use to Hologic/Gen-Probe, Inc., which sublicensed some rights to Ventana Medical Systems, Inc. The University of Michigan has filed a patent on SPINK1 in prostate cancer, on which SAT is listed as a co-inventor. The University of Michigan licensed the diagnostic field of use to Hologic/Gen-Probe, Inc., which sublicensed some rights to Ventana Medical Systems, Inc. SAT has received honoraria from and served as a consultant to Ventana Medical Systems.

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Chapter 27
Molecular Imaging of Prostate Cancer: Radiopharmaceuticals for Positron Emission Tomography (PET) and Single-Photon Emission Computed Tomography (SPECT)

Shankar Vallabhajosula, Berna D. Polack, and John W. Babich

Contents

Introduction

Prostate cancer is the most common noncutaneous malignancy and second leading cause of cancer death among men in the USA [1]. The disease is characterized by evolution from a clinically localized hormone-naıve state to an eventually metastatic castration-resistant prostate cancer (CRPC) [2]. Based on initial screening with prostate-specific antigen (PSA), approximately 80% of men diagnosed in the USA present with localized disease [2–4]. National Comprehensive Cancer Network (NCCN) panel recommends that digital rectal exam (DRE) as a complementary test with serum PSA in asymptomatic men and DRE should be performed in men who have abnormal serum PSA [5, 6]. Systematic prostate biopsy under the guidance of transrectal ultrasonography (TRUS) is the recommended technique for prostate biopsy [7, 8]. NCCN panel recommends an extended pattern of at least 12-core biopsies with sextant (6 cores) and lateral zone (6 cores) directed palpable nodule or suspicious image. Pathological evaluation of prostate cancer is done according to Gleason grading system. Cancers with Gleason score (GS) 6 or less are often well

S. Vallabhajosula, Ph.D. • B.D. Polack, M.D. • J.W. Babich, Ph.D. (✉)
Division of Radiopharmaceutical Sciences and Molecular Imaging
Innovations Institute, Department of Radiology,
Weill Cornell Medical College of Cornell University, New York, NY, USA
e-mail: job2060@med.cornell.edu

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*,
Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_27

differentiated or low grade, with GS 7 are moderately differentiated or intermediate grade, and with GS 8 to 10 are poorly differentiated or high grade [9]. Recent prognostic group was recommended by International Association of Urological Association (ISUP) as group 1 (GS 6, 3 + 3), group 2 (GS 7, 3 + 4), group 3 (GS 7, 4 + 3), group 4 (GS 8, 3 + 5, 4 + 4, and 5 + 3), and group 5 (GS 9 and 10) [10]. According to TNM stage, PSA level, life expectancy, and Gleason score, patients can be classified in different risk groups such as very low, low, intermediate, high, and very high. At the initial evaluation, if the patients have life expectancy more than 5 years, further imaging tests such as bone scan (planar and SPECT using 99mTc-MDP), computed tomography (CT), and/or magnetic resonance imaging (MRI) are recommended for staging of the disease.

Prostate cancer can be treated with a very wide range of options according to the patient's risk group and life expectancy. These treatment options can be active surveillance or observation as well as definitive or palliative treatment methods such as radical prostatectomy (RP), RP + pelvic node dissection, primary external beam radiation therapy (EBRT), primary/salvage brachytherapy, palliative radiotherapy, radionuclide therapy using Radium-223 in symptomatic bone metastases, androgen deprivation therapy (ADT), immunotherapy, or chemotherapy. Determining whether the prostate cancer has spread to the lymph nodes or other parts of the body, however, is critical for making accurate decisions on whether and how to treat prostate cancer. For men with localized prostate cancer, treatment with EBRT or radical prostatectomy (RP) is largely successful at controlling disease [11]; however, approximately a third of men develop biochemical failure (BF) typically defined by a rise in serum level of PSA of >0.2 ng/ml after RP or nadir +2 ng/ml after EBRT [12–14]. Accurate detection of recurrent disease is important because it allows for appropriate treatment selection and early delivery of the directed therapy, which favor patient outcome.

Role of Imaging in Prostate Cancer

Currently, imaging plays an important role in many aspects of prostate cancer, but its role will need to evolve to accurately answer key clinical questions at various phases of the disease in a cost-effective manner. These clinical decision-making landmarks include (a) accurate primary diagnosis, (b) characterization and staging of cancer at the time of initial presentation, (c) determination of local recurrence or distant disease at the time of biochemical recurrence of prostate cancer to select the most appropriate therapy, (d) accurate assessment of therapy response to various treatment regimens under the new practice paradigm, and (e) prediction of patient outcome (e.g., time-to-event endpoints such as time to hormone refractoriness in castration-sensitive disease, time to progression, and overall survival).

The imaging techniques in prostate cancer can be classified into two different methods: structural imaging and molecular imaging. The structural imaging provides details about the anatomy and anatomical relations such as size, local invasions, tumor borders, and anatomical distortions. In contrast, molecular imaging

shows molecular content, biochemistry, physiological dynamics, and the biology of the tumor tissue, noninvasively. In order to make medicine "personalized," the clinicians need to know both structural and molecular information about the tumor.

Structural Imaging of Prostate Cancer

Transrectal Ultrasonography (TRUS)

It is the most commonly used imaging method and essential tool for image-guided biopsy of prostate cancer. In TRUS, cancer usually appears as hypoechoic relative to normal peripheral zone of the prostate. The size, location, and grade of the tumor may affect sensitivity of the test [7, 15]. TRUS has limited value in detecting and staging of prostate cancer because of low sensitivity and specificity which is modestly increased using TRUS with the color duplex Doppler and color power Doppler ultrasound [16, 17]. In order to evaluate local recurrence, TRUS has a sensitivity 76% and specificity 67%. TRUS also plays an important role as a guide for brachytherapy and cryotherapy of the prostate cancer [18, 19].

CT and MRI

In NCCN guidelines, pelvic CT and MRI are recommended imaging tools at the initial clinical assessment of the patients who have life expectancy >5 years or are symptomatic, and T3 and T4 tumors or T1–T2 tumors and nomogram indicated probability of lymph node involvement >10. While CT has almost no role for detecting primary cancer in the prostate, MRI may have diagnostic value in detecting primary tumor of the prostate. The criterion of positive nodal disease with CT and MRI is based on the size of the nodal metastasis. The sensitivity and specificity in detecting nodal metastases with CT and MRI vary: 42% and 82% with CT and 39% and 82% for MRI, respectively. Reliance on either CT or MRI, however, may cause misevaluation of the patient's lymph node status and misdirect the therapeutic strategies [20].

Optimal MRI for the detection and local staging of prostate cancer requires the use of an endorectal coil in conjunction with a pelvic phased-array coil on a mid- to high-field-strength magnet. Thin (3-mm) sections and a small (14-cm) field of view are required to obtain submillimeter-resolution T2-weighted images necessary for local staging. The detection of prostate cancer also depends on the type of imaging sequence used. On T1-weighted images, the prostate demonstrates homogeneous medium signal intensity, and tumors are impossible to discern. On T2-weighted images, cancer most commonly demonstrates decreased signal intensity relative to the high-signal-intensity normal peripheral zone [21, 22]. Detection of primary prostate cancer with MRI is the most effective for the tumors that are located in the peripheral zone. MRI combined with MR spectroscopic imaging can also be useful for transition zone tumors [23]. MRI is also useful for evaluating extracapsular

extension (ECE) and seminal vesicle invasion (SVI). A recent meta-analysis reported that MRI has 49% median sensitivity and 82% specificity for detection of ECE and 45% median sensitivity and 96% specificity for detection of SVI [24]. In a consensus report from the United Kingdom, "multiparametric MRI" (combining T2-weighted, diffusion-weighted and dynamically enhanced sequences) is recommended for clinical use of MRI before biopsy. After biopsy, a staging MRI should be performed at least 10 weeks after biopsy and if possible after 20 weeks [25].

After therapy, MRI, especially the functional imaging sequences showed very promising results in detecting local recurrence [26]. T2-weighted MRI and 3D 1H-MR spectroscopy sequences showed a sensitivity of 57% and 53%, respectively, in patients with radical prostatectomy (group A) and 71% and 78%, respectively, in subjects with external beam therapy (group B). Dynamic contrast-enhanced MRI (DCE-MRI) alone, however, showed a sensitivity of 100% and 96%, respectively, for groups A and B. Diffusion-weighted imaging (DWI) alone had a higher sensitivity for group B than for group A (96% vs. 71%). The combination of T2-weighted imaging plus DWI plus DCE-MRI provided a sensitivity as high as 100% in group B [26].

Overall, the structural/anatomic imaging modalities, although they are used commonly in clinical practice, have a low to moderate sensitivity and specificity in detecting primary tumor, staging, and follow-up patients with prostate cancer.

Molecular Imaging of Prostate Cancer

The complex and heterogeneous biology of prostate cancer poses major challenges and opportunities for the development of molecular imaging probes based on radioisotopes or radionuclides. Molecular imaging based on single-photon emission computed tomography (SPECT) and positron emission tomography (PET) radiopharmaceuticals is a type of medical imaging that provides detailed pictures of what is happening inside the body noninvasively at the molecular and cellular level and offers unique insights into the human body that enable physicians to personalize patient care. SPECT/CT, PET/CT, and PET/MRI are the combined fusion imaging that can be obtained in a single imaging session. The molecular imaging radiopharmaceuticals target specifically biologically relevant molecules such as enzymes involved in the metabolism of glucose and fatty acids, receptors such as androgen receptors (AR), and antigens such as prostate-specific membrane antigen (PSMA). The current FDA-approved molecular imaging radiopharmaceuticals include 99mTc-labeled bone imaging agents and 111In-labeled anti-PSMA antibody (ProstaScint™) for SPECT and [18F]fluorodeoxyglucose, [18F]sodium fluoride (NaF), and [11C]choline (CH) for PET. Emerging agents under clinical development include radiolabeled analogs of lipid, amino acid, and nucleoside metabolism, as well as other small molecules more specifically targeting prostate cancer biomarkers including AR and PSMA [27–29]. Several important radiopharmaceuticals and the mechanisms of tumor uptake and localization are summarized in Table 27.1 and Fig. 27.1.

Table 27.1 Radiopharmaceuticals for molecular imaging of prostate cancer

	Biochemical target/mechanism	Radiopharmaceutical	Imaging technique
1	Bone matrix	99mTc-MDP[a] 99mTc-HDP[a]	Planar/SPECT
		[18F]Sodium fluoride[a]	PET
2	Glucose metabolism	[18F]Fluorodeoxyglucose (FDG)[a]	PET
3	Lipid metabolism	[11C]Choline (CH)[a] [18F]Fluorocholine (FCH) [11C]Acetate [18F]Fluoroacetate	PET
4	Amino acid transport	[11C]Methionine	PET
		[18F]FACBC	PET
5	Androgen receptor	[18F]FDHT	PET
6	Gastrin-releasing peptide receptor (GRPR)	68Ga-BAY86–7548 64Cu-CB-TE2A-AR06	PET
7	Prostate-specific membrane antigen (PSMA)		
7a	PSMA-antibody binding	111In-capromab pendetide (ProstaScint™)[a] 111In-DOTA-J591 mAb 177Lu-DOTA-J591 mAb	Planar/SPECT
		89Zr-DFO-J591 mAb 89Zr-DF-IAB2M (J591 minibody)	PET
7b	Small-molecule PSMA inhibitors	99mTc-MIP-1404 123I-MIP-1095	Planar/SPECT
		68Ga-PSMA 68Ga-MIP-1588 18F-DCFBC 18F-DCFPyL	PET

[a]FDA approved for routine clinical use

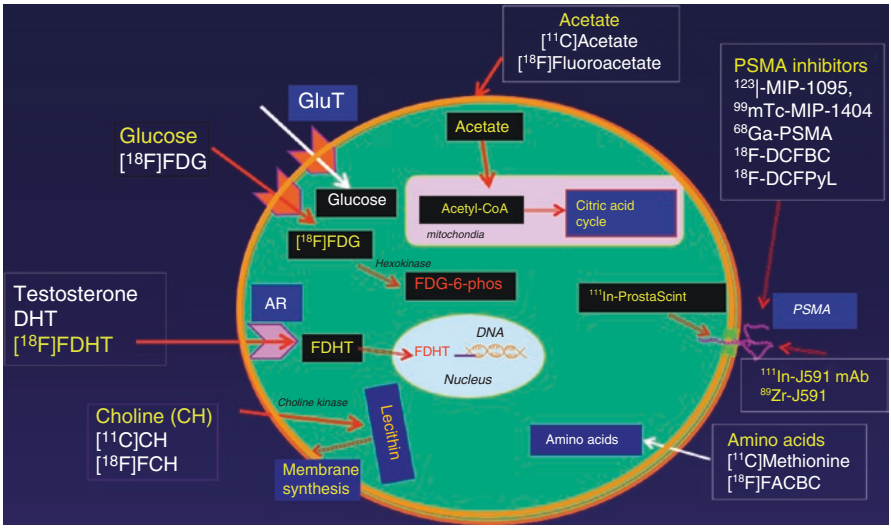

Fig. 27.1 Biochemical targets of radiopharmaceuticals for molecular imaging of prostate cancer

In general, the radioisotope-based molecular imaging technology has the following unique advantages:

- Provides information that is unattainable with other imaging technologies or that would require more invasive procedures such as biopsy or surgery
- Identifies disease in its earliest stages and determines the exact location of a tumor, often before symptoms occur or abnormalities can be detected with other diagnostic tests
- Determines the extent or severity of the disease, including whether it has spread elsewhere in the body
- Assesses disease progression and identifies recurrence of disease
- Selects the most effective therapy based on the unique biologic characteristics of the patient and the molecular properties of a tumor or other diseases
- Accurately assesses the effectiveness of a treatment regimen and determines a patient's response to specific drugs

Bone Matrix

Prostate cancer most frequently metastasizes to the bone with a predominantly osteoblastic (sclerotic) pathogenesis. Bone scan is the oldest and well-known imaging modality to investigate bone metastases in prostate cancer. 99mTc-labeled bisphosphonates such as methylene diphosphonate (MDP) and hydroxyl diphosphonate (HDP) have been used to evaluate bone metastases since the 1970s. Bone scan agent's uptake mechanism in metastatic sites depends on blood flow and osteoblastic activity [30]. The binding of radiotracer to the bone is due to physicochemical adsorption (chemisorption) to the hydroxyapatite structure of bone tissue. Bone scan is used for initial staging of intermediate to high-risk disease and for restaging after PSA relapse. It has high sensitivity and the ability to survey the entire skeleton with a simple planar scan. However, it has limited specificity and is not sensitive enough to detect micrometastases. SPECT and SPECT/CT have been shown to improve the sensitivity and reduce the number of equivocal reports for detection of bone metastases in prostate cancer [31, 32]. Besides metastatic lesions, infectious lesions and traumatic and degenerative changes also show increased uptake of bone agents. As healing process of metastatic sites may cause an increased osteoblastic activity of the bone which is called "flare phenomenon," evaluating of therapy response with bone scan is not reliable [33, 34]. A quantitative parameter known as the bone scan index (BSI) has been shown to be prognostic for survival and was proposed for stratifying patients entering tumor protocols to measure the extent of tumor involvement of the bone [35] and for the assessment of tumor response [36, 37].

18F-labeled sodium fluoride (18F-NaF) is a marker of bone perfusion and turnover in which 18F fluoride (F−) ions exchange with hydroxyl groups in the hydroxyapatite crystal of the bone to form fluorapatite with higher uptake in the new bone because of higher availability of binding sites [30, 31]. NaF-PET/CT is a highly sensitive and specific modality for the detection of bone metastases in

patients with high-risk prostate cancer. It is a more sensitive and specific imaging technique than planar and SPECT bone scan and NaF-PET alone [31]. Similar to 99mTc-MDP bone scan, flare phenomenon has also been observed with NaF-PET imaging studies [38].

Dynamic bone scanning with 99mTc-MDP or 18F-NaF provides functional information sensitive for subtle changes in bone turnover and perfusion, which assists the clinical management of numerous osseous pathologies [30].

Glucose Metabolism

Accelerated glucose metabolism is one of the phenotypic or functional changes observed in cancer tissue and was first recognized by Warburg [39] more than 80 years ago. The phosphorylation of glucose, an initial and important step in cellular metabolism, is catalyzed by the enzyme hexokinase (HK), which converts glucose to glucose-6-phosphate, and helps to maintain the downhill gradient that results in the transport of glucose into cells through the facilitative glucose transporters. Malignancy-induced glucose hypermetabolism is due to the overexpression of cellular membrane glucose transporters (mainly GLUT-1) and enhanced hexokinase enzymatic activity in tumors [40, 41]. 2-Deoxy-D-glucose (DG) was first developed in 1960 as a chemotherapeutic agent to inhibit glucose use by cancer cells. After conversion to 2-DG-6-phosphate by hexokinase, further metabolism of DG is inhibited, and DG is trapped in the cell. [18F]Fluoro-2-deoxyglucose (FDG), similar to glucose, enters the cells, is converted to FDG-6-phosphate, and gets trapped in the cell. Elevated glucose metabolism in malignant tissue in comparison to normal tissue is the underlying mechanism for the accumulation of FDG in malignant tissue. In vitro studies have shown that GLUT1 expression is higher in the poorly differentiated prostate cancer cell lines than in the well-differentiated hormone-sensitive cell lines, suggesting that the level of GLUT1 expression increases with progression of malignancy grade [42]. GLUT1 expression in prostate tumor is also correlated directly to GS and androgen level [43, 44]. Therefore, FDG uptake is lower in well-differentiated, low-GS, and androgen-sensitive prostate cancer than poorly differentiated, high-GS, and androgen-resistant tumors.

Evaluation of the prostate gland on FDG-PET/CT studies is challenging because of overlap of FDG uptake in normal, benign, and malignant tissues; the multifocal distribution of cancer deposits mixed with noncancerous cells; and the proximity of the gland to the urinary bladder [27]. FDG may also show increased uptake in BPH or prostatitis. FDG-PET/CT is not recommended in detecting primary focus of the cancer and staging of patients with clinically organ-confined prostate cancer because it has low sensitivity and specificity [45]. It also has relatively low uptake in the setting of biochemical recurrence or castrate-dependent disease. However, there is evidence that FDG-PET may be useful for restaging after PSA relapse and for assessment of treatment response (Fig. 27.2) in CRPC [46–50]. In particular, FDG-PET is most useful for evaluating lymph node and bone metastases in patients with PSA > 2.4 ng/mL and PSA velocity > 1.3 ng/mL/year [48]. In summary, FDG-PET/CT has a very limited diagnostic value in well-differentiated,

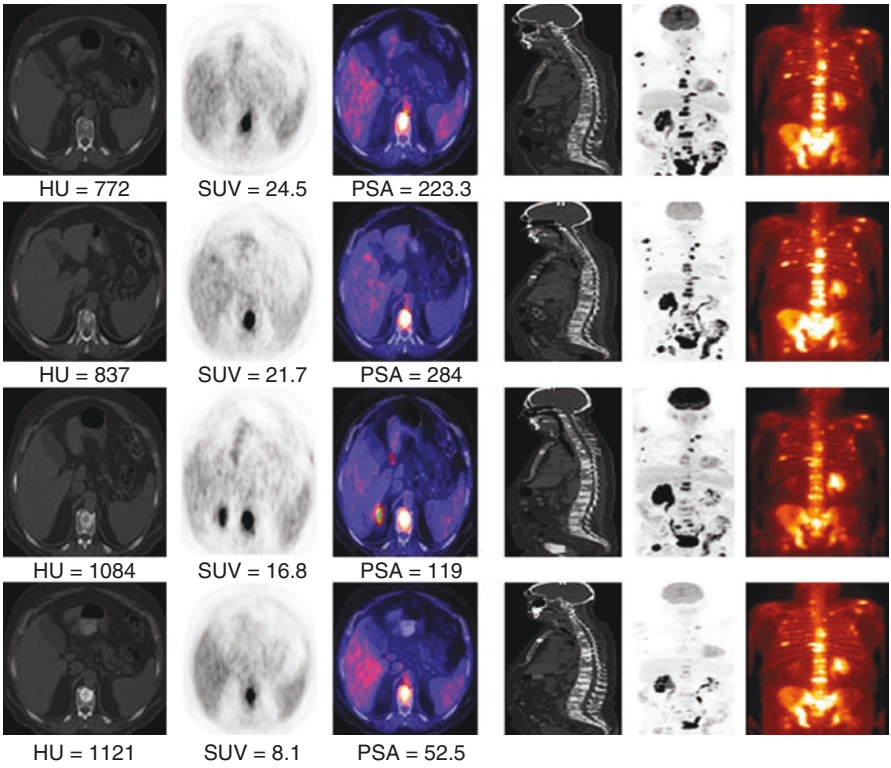

Fig. 27.2 Serial FDG-PET/CT and bone scans of a 63-year-old man with castration-resistant metastatic prostate cancer (original primary cancer Gleason score of 9). *Rows* from *top* to *bottom* are scans at baseline (before chemotherapy) and at 4, 8, and 12 months after initiation of chemotherapy. *Columns* from *left* to *right* are axial CT scans (bone window level), 18F-FDG-PET scan, fused PET/CT scans, midsagittal CT scan (bone window level), PET maximum-intensity projection images, and Tc-MDP bone scans. Concordant decline in overall metabolic activity of metastatic lesions and PSA level is seen with treatment. Sclerosis of osseous lesions increases as corresponding metabolic activity declines with treatment (Reprinted with permission from Jadvar H. Prostate Cancer: PET with 18F-FDG, 18F- or 11C-Acetate, and 18F- or 11C-Choline. J Nucl Med 2011; 52:81–89)

androgen-sensitive, and low-GS prostate cancer. However, F-18 FDG-PET/CT may be useful in detection of aggressive tumors and metastases and obtaining prognostic information in patients with prostate cancer.

Lipid Metabolism

Malignancy-induced increased cellular membrane synthesis is the basis for imaging prostate cancer with radiolabeled choline and acetate [51, 52]. Choline enters the cell via choline transporters and is used for biosynthesis of phosphatidylcholine in the tumor cell membrane through malignancy-induced overexpression of choline kinase. Acetate is transported across the cellular membrane through

monocarboxylate transporter and participates in the de novo synthesis of fatty acids from acetyl-CoA and malonyl-CoA through the action of fatty acid synthase, which is upregulated in prostate cancer. [11C]Choline (CH) has rapid cancer cell uptake, rapid blood clearance, relatively minimal excretion in the urine, and high diffuse liver uptake. [18F]Fluorocholine (FCH) shows more urinary excretion and intense bladder activity compared to CH, but it has the advantage of longer half-life allowing commercial distribution.

The diagnostic potential role of both CH and FCH in detecting and staging or restaging of prostate cancer has been reviewed extensively [53–56]. These two radiotracers are not ideal for initial staging due to false positives in prostatitis and BPH and false negatives in small (<5 mm) or necrotic tumors [57]. However, they have shown promise for restaging after PSA relapse, with high sensitivity for local recurrence, nodal metastases, and bone metastases. Also CH uptake correlates with PSA velocity and doubling time in the setting of PSA relapse [58]. In addition, CH-PET has the potential application for personalized image-guided salvage radiation or lymph node dissection [59, 60]. Recently FDA approved the clinical indication of CH-PET after PSA relapse at Mayo Clinic. The current recommendation is for considering CH-PET/CT as the first-line diagnostic procedure in patients with biochemical relapse showing PSA levels greater than 1 ng/mL, PSA velocity higher than 1 ng/mL/year, or PSA doubling time <6 months [55, 61]. Overall, there is limited but promising evidence for the use of choline PET/CT to stage patients with untreated, high-risk prostate cancer (Fig. 27.3). Relatively robust evidence suggests that choline PET/CT can be helpful for the evaluation of locoregional and distant metastatic disease in men with biochemical

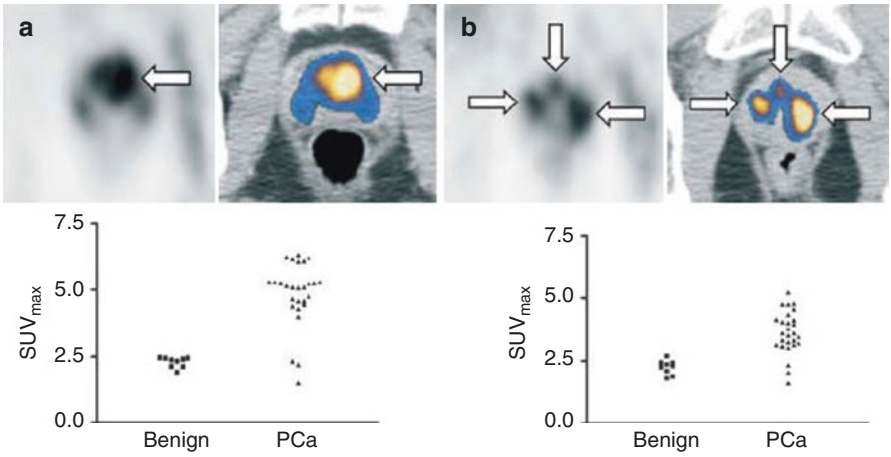

Fig. 27.3 [11C]Choline (CH)-PET/CT showing focal (**a**) and multifocal (**b**) distribution of prostate cancer within the prostate gland (*arrows*). Scatter plots of segmental CH SUVmax show higher values in most segments compared with segments with benign histopathologic lesions (Reprinted with permission from Reske SN, Blumstein NM, Neumaier B, Gottfried HW, Finsterbusch F, Kocot D, Möller P, Glatting G, Perner S. Imaging Prostate Cancer with 11C-Choline PET/CT. J Nucl Med 2006; 47:1249–1254)

relapse of prostate cancer with a detection rate that is positively associated with increasing PSA level, increasing PSA velocity, and decreasing PSA doubling time [27–29].

[11C]Acetate has been used for many years as a tracer for measuring oxidative metabolism in the myocardium. Both [11C]acetate and [18F]fluoroacetate have been evaluated for imaging prostate cancer [62–64]. Similar to acetate, fluoroacetate has been found to be a substrate for acetyl coenzyme A synthase but with a lower specificity. In addition, fluoroacetate is a toxic compound due to its conversion to fluorocitric acid in vivo [64]. [11C]Acetate tumor uptake is rapid but reversible. The average SUV of tumors was significantly higher than normal prostate tissue, but it was not significantly different that of BPH [65]. Acetate-PET may be useful for pelvic nodal staging and treatment planning in men with intermediate-risk (GS 7 or PSA 10–20 ng/mL) to high-risk (GS 8–10 or PSA >20 ng/mL) prostate cancer [66]. It may also be useful in the localization of tumor recurrence in men with biochemical failure, with a detection rate that tends to be positively associated with increasing serum PSA level [67]. Acetate-PET may be best used for restaging, and studies have shown enhanced sensitivity compared to 18F-FDG-PET [68, 69]. Direct comparison of 11C-acetate with choline PET showed no clear clinical differences between these two agents [70, 71].

Amino Acid Transport

The amino acids leucine, methionine, and glutamine are effectively taken up by many tumors due to increased amino acid transport and metabolism. [11C]-Methionine has recently shown potential for initial evaluation of low- and high-grade primary prostate tumors and for guidance of prostate biopsies in patients with elevated PSA and multiple negative biopsies [72, 73].

Anti-1-amino-3–18F-fluorocyclobutane-1-carboxylic acid (anti-18F-FACBC) is a synthetic non-metabolized L-leucine analog that accumulates in prostate cancer via overexpression of the ASC (alanine, serine, and cysteine) transport system and other amino acid transport systems [74]. FACBC-PET has shown early clinical success in imaging primary and recurrent disease in the prostate, pelvic lymph nodes, and bone, with relatively high tumor uptake with little urinary excretion and improved sensitivity compared to ProstaScint™ imaging (Fig. 27.4) [75–78]. A recent study suggested that FACBC-PET may be advantageous over CH-PET for localization of disease in biochemical failure [79].

Androgen Receptor

Prostate cancer growth and progression are stimulated by androgens, acting through the nuclear AR, which is a ligand-dependent (either testosterone or 5α[alpha]-dihydrotestosterone) transcription activator involved in cellular proliferation and differentiation and is present in all histologic types of prostate tumors,

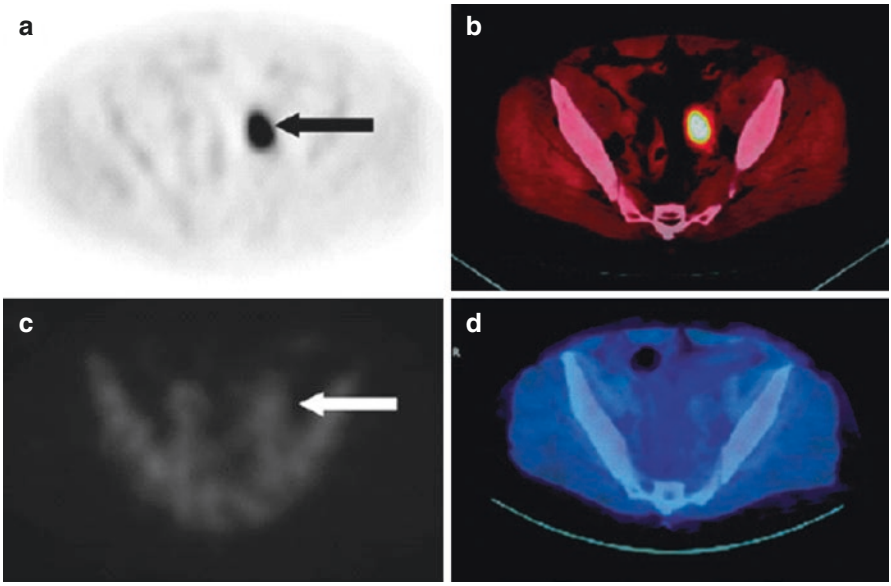

Fig. 27.4 Comparison of [18F]FACBC-PET with 111In-capromab pendetide (ProstaScint) SPECT in biochemical recurrence of prostate cancer. In a 67-year-old man, PET (**a**) and PET/CT (**b**) images show intense localization of FACBC in metastatic left external iliac nodal basin (*arrow*). ProstaScint SPECT (**c**) and fused SPECT/CT (**d**) demonstrate only low grade (Reprinted with permission from Schuster DM, Votaw JR, Nieh PT, Yu W, Nye JA, Master V, Bowman FD, Issa MM, Goodman MM. Initial experience with the radiotracer anti-1-amino-3–18F-fluorocyclobutane-1-carboxylic acid with PET/CT in prostate carcinoma. J Nucl Med 2007; 48(1):56–63)

in recurrent carcinoma, and in tumor metastases [80]. The AR is the key driver of prostate differentiation and prostate cancer progression, and androgen ablation is the cornerstone of advanced prostate cancer treatment. [18F]Fluoro-16β[beta]--*α*[alpha]-dihydrotestosterone ([18F]FDHT), a ligand that targets the ligand-binding domain of AR, was originally developed to assess AR occupancy [81–83]. FDHT-PET has shown a 78% tumor localization rate in patients with metastatic disease. Direct comparison with FDG-PET has suggested that there may be diverse metabolic phenotypes of castration-resistant cancers (androgen receptor predominant, glycolysis predominant, or androgen receptor/glycolysis concordant) and that 18F-FDHT is probably suited as a pharmacodynamic response marker, rather than a treatment response marker [27, 84]. Recent studies of [18F]FDHT-PET in CRPC patients treated with MDV3100 (AR-mediated drug) found that tumors in nearly all patients showed a decrease in [18F]FDHT binding, indicating that MDV3100 can occupy the AR ligand-binding domain and preclude radiotracer binding. However, these [18F]FDHT-PET "responses" did not correlate with declines in serum PSA or tumor response [81, 85]. Therefore, [18F]FDHT-PET may have utility in optimizing the dose of antiandrogen required for complete blockade of androgen binding to AR, but it cannot assess AR pathway downstream activity.

Gastrin-Releasing Peptide Receptor (GRPR)

Overexpression of the GRPR in prostate cancer, but not in the hyperplastic prostate, provides a promising target for staging and monitoring of prostate cancer [86, 87]. Overexpression of GRPR was found in 63–100% of primary prostate tumors and more than 50% of prostate cancer with lymph and bone metastases [88]. The GRPR density was reported to be 26-fold higher in prostate carcinoma than BPH [88]. Bombesin (BBN) is a 14-amino acid analog of the human gastrin-releasing peptide that binds to the GRPR. A variety of radiolabeled BBN analogs such as 68Ga-AMBA have been developed for targeting GRPR-positive tumors and were evaluated in preclinical and clinical studies [89]. Several recent reports have shown that GRPR antagonists show properties superior to GRPR agonists, affording higher tumor uptake and lower accumulation in physiologic GRPR-positive nontarget tissues [90–93]. GRPR agonists elicited side effects; however, GRPR antagonists are expected to have no adverse effects. Clinical evaluation of the 68Ga-labeled GRPR antagonist BAY86–7548 (68Ga-DOTA-4-amino-1-carboxymethylpiperidine-D-Phe-Gln-Trp-Ala-Val-Gly-His-Sta-Leu-NH_2) has shown a specificity, sensitivity, and accuracy of 88%, 81%, and 83%, respectively, for the detection of primary prostate carcinoma [92]. In another preclinical and clinical study, another GRPR antagonist, 64Cu-CB-TE2A-AR06, demonstrated the most favorable characteristics with respect to tumor uptake and tumor-to-organ ratios [93]. These encouraging preliminary clinical results suggest a potentially important role for the radiolabeled GRPR antagonists for the molecular imaging of prostate cancer.

Prostate-Specific Membrane Antigen (PSMA) Imaging

PSMA, also known as glutamate carboxypeptidase II (GCPII), *N*-acetyl-α[alpha]-linked acidic dipeptidase I (NAALADase), or folate hydrolase, is a type II transmembrane protein, which is anchored in the cell membrane of prostate epithelial cells [94]. The PSMA protein has a unique three-part structure: a 19-amino acid internal portion, a 24-amino acid transmembrane portion, and a 707-amino acid external portion [95]. PSMA is considered to be the most well-established target antigen in prostate cancer, since it is highly and specifically expressed at all tumor stages on the surface of prostate tumor cells [96, 97]. PSMA switches from a cytosolically located protein in the normal prostate to a membrane-bound protein in prostatic carcinoma. The majority of PSMA expression appears to be restricted to the prostate, with some expression seen in the brain, kidney, salivary glands, and small intestine [94, 96–98]. The level of PSMA expression is increased with increased tumor dedifferentiation and in metastatic and hormone-refractory cancers [96, 97, 99–101]. In addition to expression by prostate cells, it can be expressed also by non-prostate tissues such as the small intestine, proximal renal tubules, and salivary glands [99] albeit at levels 100- to 1000-fold less than in prostate tissue. PSMA expression was also found on the vascular endothelium of solid tumors and sarcomas, but not of normal tissues [98].

Anti-PSMA mAbs

The mAb 7E11-C5.3 was the first anti-PSMA mAb originally developed with a type of prostate cancer cell line known as LNCaP cells [96, 102]. This antibody was later conjugated with DTPA, radiolabeled with 111In, and commercialized as an imaging agent, known as 111In capromab pendetide (ProstaScint™) [103]. Since it recognizes and binds to an intracellular or cytoplasmic epitope of PSMA, only the fixed cells and necrotic cells, but not the intact viable cells, bind to the 7E11 mAb [101]. FDA-approved ProstaScint™ in 1996, as a staging agent indicated for the detection of recurrent prostate cancer in post-prostatectomy patients with a rising prostate-specific antigen (PSA) and negative or equivocal standard metastatic evaluation, in whom there is high clinical suspicion of occult metastatic disease, and for newly diagnosed patients with biopsy-proven prostate cancer thought to be at high risk for lymph node metastasis. In patients with prostate cancer who are at high risk for metastatic disease, the sensitivity was 77% and specificity was 86% [104]. Subsequent publications have revealed wide variance in the efficacy: sensitivity of 67% for disease detection in prostate bed but a sensitivity of only 10% for extraprostatic disease detection [77]. This agent repeatedly failed in the clinical setting, likely due to poor pharmacokinetics and failure to reach its target epitope on the intracellular portion of PSMA [105–107].

The monoclonal antibody J591, developed by Bander et al., targets the extracellular portion of PSMA and, therefore, binds to the viable tumor cells [108, 109]. Initially, the bifunctional chelating agent, DOTA, was conjugated to humanized J591 mAb and was labeled with 111In for imaging studies and 90Y and 177Lu for radioimmunotherapy. Planar and SPECT imaging studies with 111In and 177Lu DOTA-huJ591 have shown accurate detection of bone and soft tissue metastases of prostate cancer, as well as uptake in the tumor neovasculature of many solid tumors [110–114]. Recently, J591 mAb has been radiolabeled with Zirconium-89 (half-life 78.41 h) for PET imaging studies, and it showed excellent tumor localization with pathological correlation of disease [115–117]. 89Zr-DFO-J591 also detected tumor even when lesions were negative by standard imaging or FDG-PET imaging, allowing 89Zr-DFO-J91 to aide in directing biopsies. Other anti-PSMA antibody-based agents in preclinical development include 64Cu-J591, which has been used to demonstrate PSMA upregulation after androgen blockade, and 64Cu-3/A12, a mAb to the extracellular portion of PSMA [118, 119].

While imaging with radiolabeled whole IgG mAb approach is highly promising, the optimal time for patient imaging after injection, in terms of achieving adequate tumor-to-background ratios, was 7 ± 1 days. Although radiolabeled antibodies offer potential for tumor targeting, their effectiveness as diagnostic radiopharmaceuticals is limited by a long plasma half-life, poor tumor penetrability, and the nonspecific localization exhibited with immunoglobulins. The limitation of radiolabeled antibodies is therefore significant delay between injection and imaging and multiple visits to the imaging center by the subject. In addition, the radiation dosimetry with 89Zr radionuclide ($T½$ = 78.41 h) is not favorable for repeat studies to study the changes in PSMA expression before and after therapy.

Small-Molecule PSMA Inhibitors

Developing small molecules that interact specifically with PSMA and carry appropriate radionuclides may provide a promising and novel molecular imaging option for prostate cancer patients. Smaller molecular weight compounds with higher permeability into solid tumors will likely have a definitive advantage in obtaining higher percent uptake per gram of tumor tissue and a high percentage of specific binding. Smaller molecules will likely also display improved blood clearance and tissue distribution in normal tissues compared to intact immunoglobulins making lesion detection more conspicuous.

PSMA is highly homologous to *N*-acetylated R-linked acidic dipeptidase (NAALADase), a neuropeptidase that produces the neurotransmitter glutamate and *N*-acetylaspartate (NAA) [120, 121].

The "active substrate recognition site" of PSMA is composed of two structural motifs, one recognizing the glutarate moiety of glutamic acid inherent in NAAG while the other, a more promiscuous site lined with basic amino acids, allows binding to the free carboxylate of aspartate and can accommodate more bulky structural moieties. Analysis of the crystal structure of PSMA has aided in the understanding of the critical interactions of potent inhibitors within the active site of the enzyme and has led to the design and synthesis of several classes of NAALADase inhibitors with high specific binding to PSMA [122, 123]. Synthetic-aperture radar (SAR) reveals that a urea containing at least one glutamate residue plus a second residue bearing a carboxyl group in addition to another group (SR or C02H) represents the minimum requirement to achieve effective GCPII inhibition. Several groups have reported on the development of small-molecule inhibitors of PSMA based on the structural motifs of various NAALADASE inhibitors comprising two amino acids joined through their NH_2 groups by a urea linkage (glutamate-urea heterodimers) and labeled with 123I, 99mTc, 68Ga, 124I, 18F, 124I, and 64Cu [124–143].

Among these agents, 99mTc-labeled MIP-1404 (developed by Molecular Insight Pharmaceuticals, Inc., a subsidiary of Progenics Pharmaceuticals, Inc.) has completed phase I and II clinical studies [130, 131]. In patients with metastatic prostate cancer, 99mTc agents (MIP-1404 and MIP-1405) localized to lesions in the bone and soft tissue that correlated with radiologic evidence of metastatic disease identified by the bone scan [129]. In a 71-year-old patient who had prior prostatectomy and with a rising PSA (1.37—8.9 ng/ml over a period of 4 months), PSMA imaging with 99mTc-MIP-1404 (in March) detected more metastatic lesions earlier compared to the two bone scans performed either before (in January) or after (in June) the PSMA scan (Fig. 27.5). This observation suggests that PSMA-targeted molecular imaging may have the potential to identify disease progression earlier than the standard bone scan. In addition, in several patients, significant uptake was also observed in lymph nodes smaller than 10 mm, considered normal by size threshold criteria used in cross-sectional imaging such as CT and MRI. Based on these results, a preliminary phase I study and a multicenter phase II study were conducted in high-risk prostate cancer patients scheduled for prostatectomy and extended pelvic node lymph node dissection. In all subjects with GS >7, 99mTc-MIP-1404 SPECT clearly identified the prostate carcinoma foci in the prostate gland, confirmed by histopathology (Fig. 27.6) and PSMA staining [131].

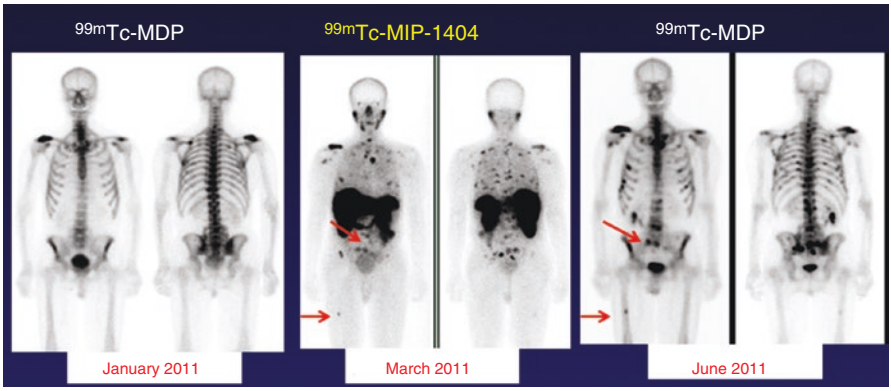

Fig. 27.5 Comparison of 99mTc-MIP-1404 PSMA scan with bone scans in patient with metastatic prostate cancer. Anterior and posterior images with 99mTc-MIP-1404 (in March) detected more metastatic lesions than the two bone scans obtained either before (in January) or after (in June) PSMA scan. Metastatic sites (*arrows*) shown in PSMA scan were only detected by the bone scan performed 3 months after PSMA scan (Reprinted with permission from Vallabhajosula S, Nikolopoulou A, Babich JW, Osborne JR, Tagawa ST, Lipai I, Solnes L, Maresca KP, Armor T, Joyal JL, Crummet R, Stubbs JB, Goldsmith SJ. 99mTc-labeled small-molecule inhibitors of prostate-specific membrane antigen: pharmacokinetics and biodistribution studies in healthy subjects and patients with metastatic prostate cancer. J Nucl Med 2014; 11:1791–1798)

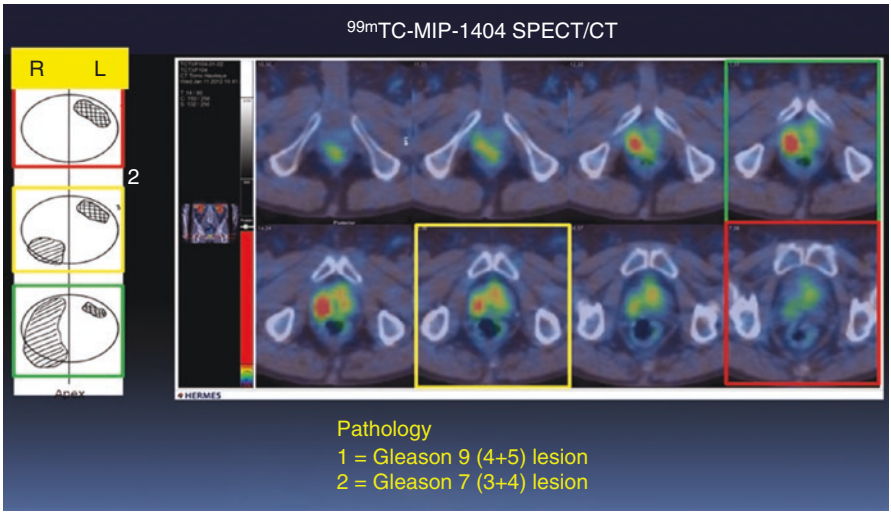

Fig. 27.6 Comparison of 99mTc-MIP-1404 PSMA scan with histopathology in a patient scheduled for prostatectomy. PSMA scan clearly identified the lesions in the prostate gland with GS >7. The uptake of the radiotracer was also correlated with PSMA expression in the primary cancer foci in the prostate gland (Images provided by Molecular Insight Pharmaceuticals, Inc.)

Molecular Insight Pharmaceuticals, Inc. has also reported on the development of 123I-labeled small-molecule PSMA inhibitors (MIP-1072 and MIP-1095) based on the glutamate-urea-lysine moiety modified with iodine containing aromatic substituents at the Ɛ[epsilon]-amine of lysine. The results of a phase I clinical study demonstrated that targeting PSMA with both these radiotracers facilitates the detection of radiologically proven prostate cancer in the bone, lymph nodes, and the prostate gland [128]. Direct comparison with ProstaScint imaging clearly documented that 123I-MIP-1072 identified several metastatic lesions in the pelvic lymph nodes not detected by anti-PSMA antibody, ProstaScint imaging (Fig. 27.7). Based on these early clinical results, MIP-1095 was labeled with 131I (a radionuclide that emits a beta particle) for targeted therapy of metastatic prostate cancer [132].

Eder et al. in Germany first reported the synthesis of another urea-based inhibitor, Glu-NH-CO-NH-Lys-(Ahx)-[68Ga(HBED-CC)] (known as 68Ga-PSMA) [133, 134]. The first clinical results using 68Ga-PSMA for PET imaging studies suggest a high potential for the detection of small recurrent prostate cancer lesions in patients presenting with low PSA values. As expected from the biological characteristics including the PSMA expression pattern, and the potential to internalize upon binding prostate cancer cells, the 68Ga-PSMA shows high accumulation in small metastases and is cleared rapidly from background tissue. In direct comparison to 18F-labeled choline, PSMA-targeted imaging is able to detect lesions much earlier in patients with low PSA values and shows a reduced background activity in healthy tissue (Fig. 27.8). In the last 4 years, 68Ga-PSMA-PET imaging was performed in thousands of patients in Europe, and the potential diagnostic utility was well documented [124, 134–136]. A recent retrospective analysis of Ga-68 PSMA imaging studies of 319 patients resulted in lesion-based sensitivity, specificity, and NPV and PPV values of 76.6, 100, 91.4, and 100% and a patient-based sensitivity of 88% [137].

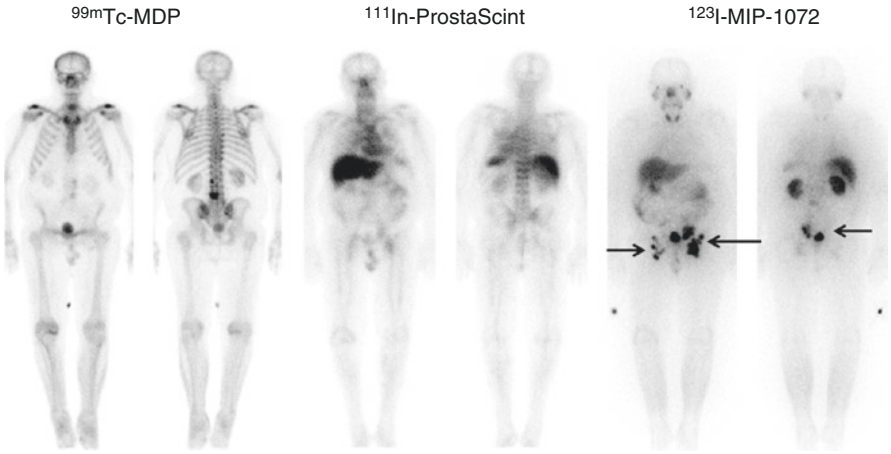

Fig. 27.7 123I-MIP-1072 PSMA imaging in a patient with prostate cancer: comparison with bone scan and ProstaScint imaging (Images provided by Molecular Insight Pharmaceuticals, Inc.). 123I-MIP-1072 images (at 3 h) show intense uptake in the lymph node metastatic lesions not seen on ProstaScint images obtained 6 days after the injection of the radiotracer

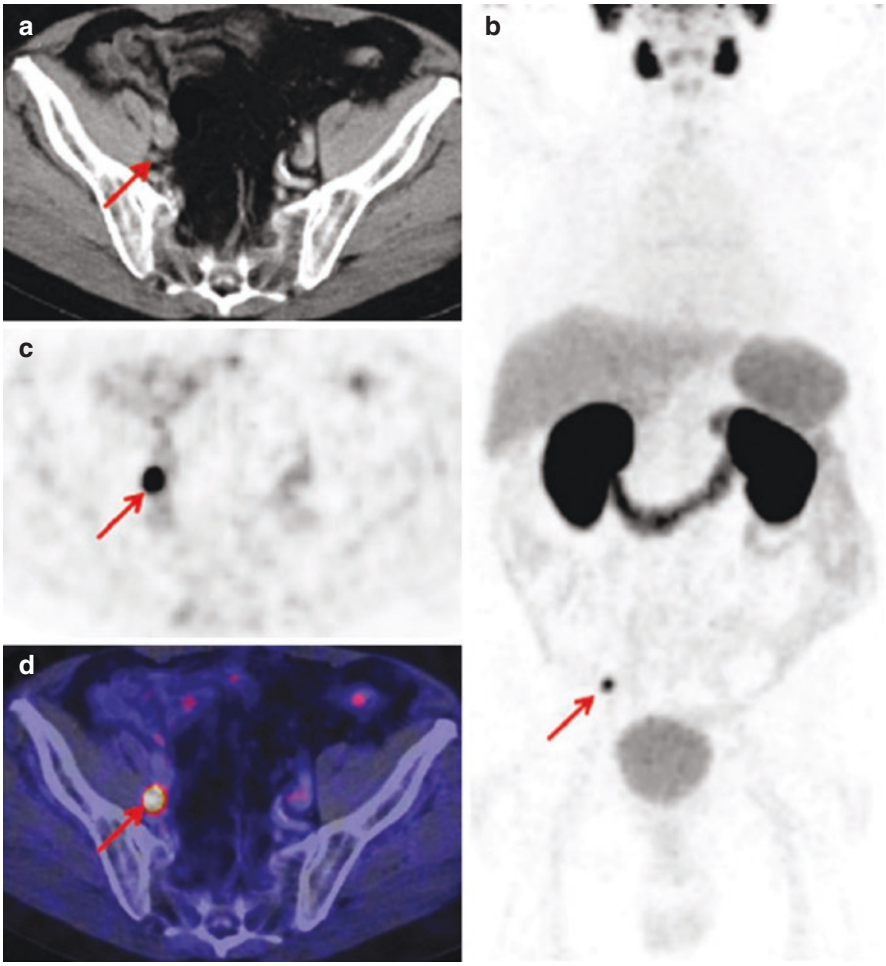

Fig. 27.8 68Ga-PSMA-PET/CT in patients with biochemical recurrence after radical prostatectomy (RP): in a 75-year-old patient after RP (2000; GS, 5; pT3b, pN1), radiation therapy (September 2011), and with a rising PSA value of 1.09 ng/mL (October 2013), CT images (**a**) reveal no suggestive finding, with a 5-mm lymph node behind the right external iliac vein. Corresponding PSMA-PET (**c**) and fused PET/CT images (**d**) show intense uptake, with high lesion-to-background ratio in this small lymph node indicating lymph node metastasis. Whole-body maximum-intensity projection (**b**) displays this lymph node and demonstrates no other suggestive lesions. Selective lymph node picking was performed in December 2013, confirming single lymph node metastasis. Subsequently, PSA value dropped below detection limit (0.07 ng/mL) without antihormonal treatment (last measurement April 2014) (Reprinted with permission from Eiber M, Maurer T, Souvatzoglou M, Beer AJ, Ruffani A, Haller B, Graner FP, Kübler H, Haberhorn U, Eisenhut M, Wester HJ, Gschwend JE, Schwaiger M. Evaluation of hybrid 68Ga-PSMA ligand PET/CT in 248 patients with biochemical recurrence after radical prostatectomy. J Nucl Med 2015; 56:668–674)

Since 18F radionuclide may provide higher image resolution (compared to 68Ga) with the current clinical PET scanners, the investigators at Johns Hopkins Medical Institutions reported the development of a low-molecular-weight, urea-based inhibitor of PSMA, 18F-*N*-[*N*-[(*S*)-1,3-dicarboxypropyl]carbamoyl]-4-fluorobenzyl-Lcysteine (18F-DCFBC) [138]. In the first clinical studies in patients with progressive metastatic prostate cancer, bone and soft tissue metastases were successfully visualized by PET, including probable early bone lesions that were not seen on CT or 99mTc-MDP bone scan [139]. Subsequently, a second-generation 18F-labeled PSMA targeting agent, 2-(3-[1-Carboxy-5-[(6-[18F]fluoro-pyridine-3-carbonyl)-amino]-pentyl]-ureido)-pentanedioic acid (18F-DCFPyL), demonstrated high tumor uptake (39.4 ± 5.4% injected dose) within the PSMA-expressing tumor, at 2 h postinjection [140].

Recently the first in man evaluation of 18F-DCFpyl clearly showed (Fig. 27.9) that the high accumulation in putative foci of prostate cancer was rapid and very high with some lesions demonstrating standardized uptake values (SUV) max >100 and comparable to the uptake reported in some metastatic prostate cancer sites evaluated with 68Ga-PSMA-targeted ligands [141]. Imaging at 1–2 h postinjection would be likely to evaluate the full extent of disease in most patients. In addition, other urea-based agents labeled with 64Cu have also been reported [142–144].

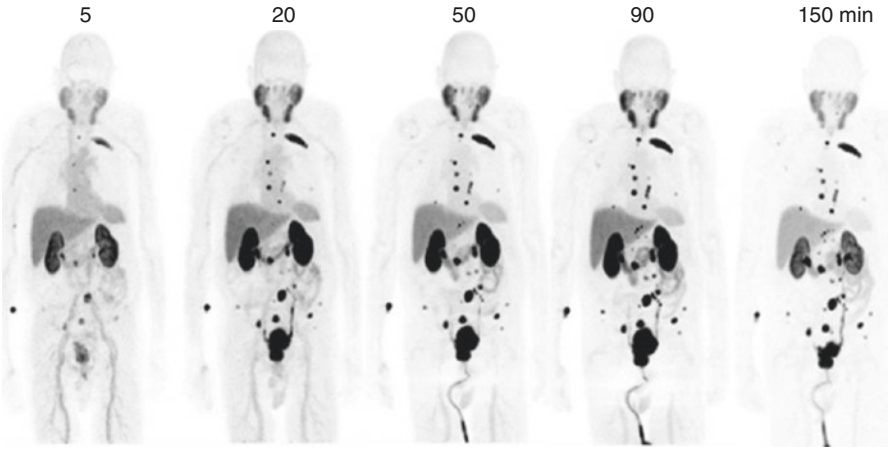

Fig. 27.9 [18F]DCFPyL for PSMA-targeted PET imaging in prostate cancer: maximum-intensity projection (MIP) PET image sequence in a patient with metastatic prostate cancer. The scan shows intense uptake of the tracer in focal metastatic lesions. In addition, this patient demonstrated physiologic tracer uptake in the salivary glands, lacrimal glands, kidneys, liver, spleen, small intestine, and urinary excretion. There was uptake also in a histologically confirmed metastatic lesion involving the rectal wall (Reprinted with permission from Szabo Z, Mena E, Rowe SP, Plyku D, Nidal R, Eisenberger MA, Antonarakis ES, Fan H, Dannals RF, Chen Y, Mease RC, Vranesic M, Bhatnagar A, Sgouros G, Cho SY, Pomper MG. Initial Evaluation of [18F]DCFPyL for Prostate-Specific Membrane Antigen (PSMA)-Targeted PET Imaging of Prostate Cancer. Mol Imaging Biol 2015; 17(4):565–74)

Summary

Molecular imaging based on SPECT and PET radiopharmaceuticals is a type of medical imaging that provides detailed pictures of what is happening inside the body noninvasively at the molecular and cellular level and offers unique insights into the human body that enable physicians to personalize patient care. The current FDA-approved molecular imaging radiopharmaceuticals include 99mTc-labeled bone imaging agents and 111In-labeled anti-PSMA antibody (ProstaScint™) for SPECT and [18F]fluorodeoxyglucose, [18F]sodium fluoride (NaF), and [11C]choline (CH) for PET. Emerging agents under clinical development include radiolabeled analogs of lipid, amino acid, as well as other small molecules more specifically targeting prostate cancer biomarkers including AR and PSMA. In the last 5 years, several small-molecule PSMA inhibitors labeled with 123I or 99mTc (for SPECT) and 18F or 68Ga (for PET) have been evaluated in phase I and II clinical studies and show significant diagnostic potential for molecular imaging studies of prostate cancer. As the management of prostate cancer becomes more personalized and new treatments become available, there is increasing clinical demand for molecular imaging.

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Chapter 28
Prostate Cancer Molecular Prognosis

Joshua I. Warrick and Scott A. Tomlins

Contents

Introduction

Prostate cancer is a genetically and clinically diverse disease. Tumors range from indolent tumors that go entirely unnoticed to aggressive cancers causing diffuse metastasis and patient death. The behavior of a given prostate cancer strongly

J.I. Warrick, M.D.
Department of Pathology, Penn State Milton S. Hershey Medical Center, Hershey, PA, USA

S.A. Tomlins, M.D., Ph.D. (✉)
Department of Pathology, University of Michigan Medical School, Ann Arbor, MI, USA

Department of Urology, University of Michigan Medical School, Ann Arbor, MI, USA

Michigan Center for Translational Pathology, Ann Arbor, MI, USA
e-mail: tomlinss@umich.edu

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*,
Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_28

correlates with several clinical and pathologic parameters, including Gleason score, pathologic tumor stage, and serum prostate-specific antigen (PSA) level. These predictive features have been assembled into predictive nomograms and scoring schemata that have proven invaluable in prognostication (reviewed in [1]). The contemporary treatment of prostate cancer is thus driven by these clinical and pathologic features. Gleason score is a major pathologic variable. In brief, specific morphologic patterns are designated as Gleason pattern 1 to Gleason pattern 5. The most common and worst components are added to give a Gleason score [2, 3]. Pathologic tumor stage is based on confinement within prostate and extension of cancer beyond the prostate, with increased stage assigned with increased extension beyond the prostate. Specific clinical parameters and other prognostic pathologic factors at biopsy or prostatectomy include the presence of a palpable mass on digital rectal examination, PSA levels, number of cores with cancer on biopsy, extent of cancer on biopsy, and tumor volume/maximum dimension.

In keeping with the diversity of clinical behavior, treatment practices for prostate cancer change drastically based upon clinical and pathologic features. On the indolent end, patients with small foci of low-grade cancer identified at biopsy, without other features of clinically aggressive disease, are candidates for active monitoring (by serial PSA, biopsy, or imaging) without undergoing definitive treatment unless evidence of aggressive disease emerges. Such protocols are termed "active surveillance" or "watchful waiting," depending the protocol, and have shown success in preventing overtreatment of men with indolent disease. Men with intermediate or aggressive disease are treated with definitive therapy, typically with either radical prostatectomy or primary radiotherapy. Men with aggressive disease at diagnosis may be treated with a combination of surgery, radiotherapy, and/or androgen deprivation therapy. Once definitive treatment has been performed, patients are monitored for evidence of recurrence, including serial PSA measurements. Men with evidence of true recurrent disease (i.e., distant metastasis or prostate bed recurrence) are treated in most cases with androgen deprivation therapy, wherein circulating androgen levels are pharmacologically decreased and cancer growth is thus suppressed. In cases of elevated PSA without evidence of true recurrence (termed biochemical recurrence), treatment is tailored based on the above-mentioned clinical and pathologic variables. For example, a rapidly rising PSA immediately after surgery is considered more pressing than a slowly elevating level. Patients with high-grade or high pathologic stage disease at prostatectomy are treated more aggressively for biochemical recurrence compared to those with low-grade and low-stage disease, given the increased pretest probability of clinically recurrent disease.

Molecular Markers of Aggressive Disease

MYC/PTEN/TP53

Several single gene events have been associated with aggressive behavior in prostate cancer, including MYC gain/amplification, TP53 mutation, and PTEN deletion. MYC is the prototypical member of a group of transcription factors implicated in

cancers of many organ systems. Prostate cancers harboring MYC gain/amplification are more often high Gleason grade [4, 5]. MYC gain/amplification has also been found to be a marker of earlier progression, earlier cancer-related death following prostatectomy, metastatic disease, and early recurrence following radiation therapy [5–7]. Of note, MYC gain/amplification is usually part of a broader gain of chromosome 8q24 (or all of 8q), with true high-level MYC amplification usually only seen in advanced castration-resistant prostate cancer (CRPC), which is unresponsive to traditional antiandrogen therapy. TP53 mutation is associated with high-grade, aggressive prostate cancer. It is also the most commonly mutated gene in CRPC [8]. PTEN is the most commonly deleted gene in prostate cancer, and it is similarly associated with aggressive disease [9–13]. Prostate cancers with PTEN loss have higher Gleason grade and stage, are at increased risk for upgrading at prostatectomy, and have lower progression-free survival and overall survival [9, 10, 12, 14–16]. They have been shown to have more frequent biochemical recurrence following prostatectomy than tumors with normal PTEN copy number [17, 18]. PTEN loss is also enriched in advanced, CRPC [8, 10].

The well-studied clinical and pathologic features of aggressive prostate cancer, namely, serum PSA, tumor stage, and Gleason grade, are highly correlated with MYC amplification, TP53 mutation, and PTEN loss. Thus, although these molecular events are by themselves predictive of aggressive disease, their value in clinical decision-making has not been established. For example, in a recent study with good follow-up data, PTEN deletion status was statistically significant association with increased risk of biochemical recurrence in a multivariate model incorporating Gleason score, pathologic tumor stage, and serum PSA [10]. However, the hazard ratio (HR) for PTEN loss in this model was low (HR 1.2) compared to Gleason score (maximum HR 6.1 and 5.9), raising doubts about its value in the model. Likewise, the performance of an optimized clinicopathological model (incorporating grade, stage, etc.) was not compared to the same model with PTEN status. Lastly, PTEN loss is frequently heterogeneous [19, 20], complicating assessment on biopsy specimens with multiple positive cores (Fig. 28.1).

High-grade, high-stage prostate cancers are diagnosed as such based on standard histologic evaluation and are known to be aggressive irrespective of molecular status. Thus, in order to be adopted into clinical practice, newly developed prognostic biomarkers must add new information to that already known from optimized models of these standard clinical and pathologic parameters. The main challenge with prognostic biomarkers in prostate cancer is thus raised. That is, while many markers of both aggressive and indolent diseases have been identified, and many are statistically, independently significant on multivariate analysis, a marker must prove valuable in clinical decision-making to be incorporated into practice. This is best done by likelihood ratios, area under the curve (AUC), comparison of receiver operating characteristic (ROC) curves, and/or decision curve analysis, in which a standard clinicopathological model is compared to the performance of the model plus the new biomarker [21–23]. For example, Zu et al. found that adding PTEN and IGF-IR protein expression to a base clinicopathological model increased the AUC from 0.837 to 0.864 [15]. Whether this improvement represents a clinically meaningful and cost-effective prognostic test for an individual patient remains to be

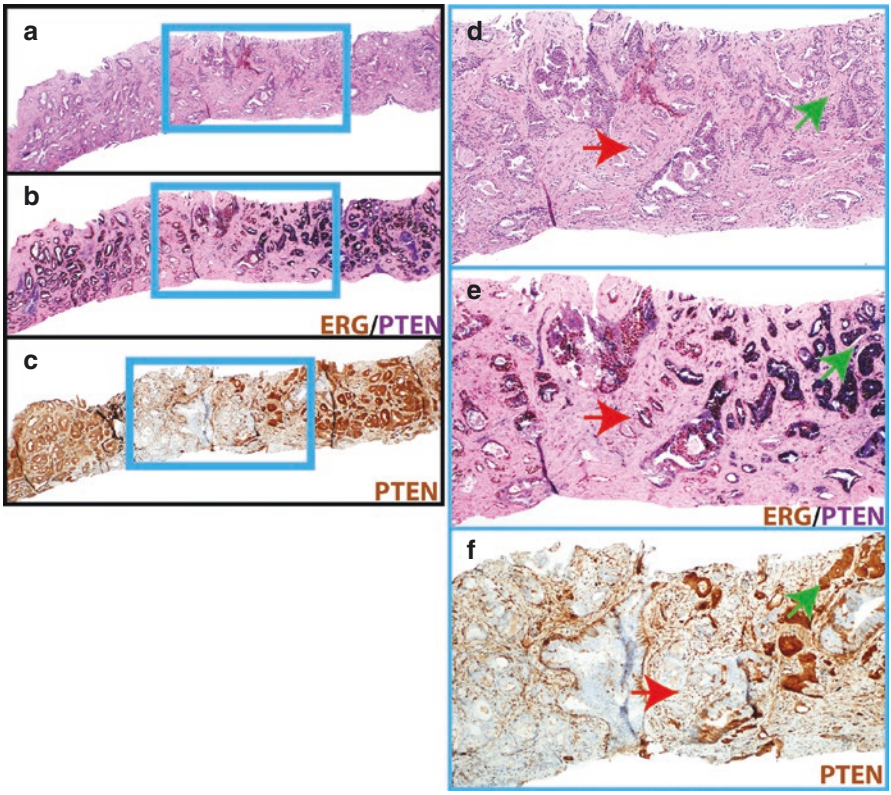

Fig. 28.1 Heterogeneity of PTEN expression/deletion is a potential confounder of its use as a prognostic biomarker. (**a**) A diagnostic prostate biopsy demonstrated diffuse involvement by prostatic adenocarcinoma as assessed by hematoxylin and eosin staining. (**b**) Dual ERG (brown chromagen) and PTEN (purple chromagen) immunohistochemistry (IHC) demonstrated diffuse ERG expression but focal loss of PTEN staining. (**c**) PTEN IHC alone confirmed focal loss of PTEN expression. Areas in blue boxes are shown in (**d**)–(**f**). Areas of ERG+/PTEN retained expression are indicated by green arrows. Areas of ERG+/PTEN loss are indicated by red arrows. Focal PTEN loss was confirmed by fluorescent in situ hybridization (FISH). 4× and 10× original magnification in (**a**)–(**c**) and (**d**)–(**f**), respectively

determined. Nevertheless, among single gene markers, PTEN appears to represent the most promising single protein-based marker for prostate cancer prognosis.

ETS Rearrangements

Fusion genes resulting from rearrangements involving member of the ETS family of transcription factors are the most common molecular alteration in prostate cancer, present in ~50% of cases detected with PSA screening [24–29]. ERG is the most commonly involved ETS member, accounting for >90% of cases. Most ERG rearrangements involve fusion to TMPRSS2, an androgen-regulated gene located ~3MB from ERG on

chromosome 21. Fusion results from deletion of the intervening genetic material or insertion of the intervening region to a different chromosome (each occurring at approximately equal frequency). Importantly, these mechanisms give rise to different fluorescent in situ hybridization (FISH) results when using split probes flanking ERG (5′ signal deletion in fusion through deletion, 5′/3′ break apart in fusion through insertion). Regardless of mechanism, TMPRSS2:ERG fusion result is a massive overexpression of complete, or near complete, ERG protein in prostate cancers harboring these fusions. In keeping with this, immunohistochemistry (IHC) for ERG is sensitive and specific for ERG rearrangement in prostate cancer [24–26, 30–32]. Other involved ETS genes include ETV1, ETV4, and ETV5, and less common fusion partners include SLC45A3 and NDRG1.

Results from large PSA screened, predominantly Caucasian series demonstrated that neither ERG expression by IHC nor rearrangement by FISH is prognostic for post-prostatectomy outcome [33, 34]. The majority of studies have shown no association between ERG status and clinical and pathologic variables, including Gleason score, tumor stage, and clinical outcome. Studies reporting association with Gleason score are contradictory, with an approximately equal number of studies showing association with low and high Gleason score. While most studies show no association with pathologic tumor stage, several have shown ERG+ cancers are more frequently associated with extraprostatic extension (although not with seminal vesicle involvement), while no study has shown an association with low stage. Importantly, however, prognostic utility of ERG status, like many markers, has not been comprehensively evaluated in non-prostatectomy cohorts. Of interest, Berg et al. recently showed that ERG positivity by IHC in diagnostic biopsy specimens was a strong predictor of pathologic disease progression in an active surveillance cohort [35]. Although this finding will need to be replicated in additional cohorts, it highlights the importance of cohort and outcome measure in assessing a “prognostic” biomarker.

SPINK1

SPINK1 is a trypsin inhibitor first described in the pancreas and later shown to be overexpressed in ~10% of prostate cancers. SPINK1 expression is essentially mutually exclusive of ETS gene rearrangements, suggesting SPINK1+ tumors evolve by a pathway different from ETS+ cancers [36–41]. Early studies suggested SPINK1+ tumors are more aggressive than SPINK1- cancers, showing increased biochemical recurrence after prostatectomy (HR = 2.65) [38]; however larger series do not support SPINK1 as a strongly prognostic factor after prostatectomy [42].

lncRNAs

Recent high-throughput studies have dramatically increased our understanding of the role of noncoding transcripts to disease biology. In particular, a wealth of novel long noncoding RNAs (lncRNAs) has been identified, including those that are

disease specific. For example, a large RNAseq pan-cancer study identified numerous prostate cancer and aggressive prostate cancer-specific lncRNAs. One lncRNA, SCHLAP-1, was shown to promote aggressive prostate cancer phenotypes in vitro [43]. In a multicenter study using data from the Decipher assay (see below), it was independently associated with metastatic progression [44]. RNA in situ hybridization (ISH) assessment of SCHLAP-1 by two independent groups likewise demonstrated associations with biochemical recurrence and pathologic tumor stage [45, 46].

Multigene Expression Assay to Predict Cancer Behavior

As single gene markers in general have not been found to be significantly prognostic to impact clinical practice, intense efforts have been made to develop multigene prognostic assays. Multigene markers may have more utility. Prostate cancer, like other cancers, is a highly diverse disease process, with many pathways activated and inactivated by multiple different mechanisms. Multigene markers are more able to account for this diversity. For example, in breast cancer, the Oncotype Dx assay evaluates gene signatures of cellular proliferation and ER signaling, among others, and has demonstrated clinical value in deciding if chemotherapy is appropriate in a subset of patients (reviewed in [47]). Several similar multigene panels have been developed for prostate cancer, although such panels to date have largely been evaluated as prognostic biomarkers, rather than predictive biomarkers (see Table 28.1).

Table 28.1 Commercial multigene and multi-protein tissue-based prognostic tests for prostate cancer

Test	Decipher	Oncotype DX	ProMark	Prolaris
Company	GenomeDx	Genomic Health	Metamark	Myriad Genetics
Predictor	Genomic classifier score	Genomic Prostate Score	N/A	CCP score
Test prediction	Post-RP metastasis	Freedom from adverse pathology at RP	Favorable vs. nonfavorable pathology at RP	Post-RP biochemical recurrence
Specimen type	FFPE RP	FFPE biopsy	FFPE biopsy	FFPE biopsy
Assay	Affymetrix Human Exon 1.0 ST Microarray	RT-PCR with preamplification	Quantitative multiplex proteomic imaging	RT-PCR with preamplification
Targets	22 target genes	12 target genes, 5 housekeeping genes	8 proteins	31 target genes, 15 housekeeping genes
Target types	Multiple pathways	Multiple pathways	Multiple proteins	Cell cycle/proliferation
Range	0–1	0–100	0–1	~−2 to 3

GenomeDx has developed the Decipher assay, an expression profiling-based assay performed on the Affymetrix Human Exon 1.0 ST array that uses a random forest classifier to generate a 22-gene genomic classifier (GC) score from formalin-fixed paraffin-embedded (FFPE) radical prostatectomy tissue samples [34, 48–56]. The GC score ranges from 0 to 1, representing the predicted risk of prostate cancer metastasis, and includes genes involved in cellular proliferation, cell cycle, immune response, other pathways, and single gene markers. Several case-control studies have been performed using this assay, which have consistently shown that GC offers superior accuracy for the prediction of distant metastasis on univariate analysis compared to Gleason score, pathology staging parameters, serum PSA, and various clinical models [48–50]. Several multivariate analyses, which include clinical and pathologic parameters, have shown GC alone is statistically significant for prediction of distant metastasis. For example, in a study with the endpoint of clinical metastasis within 5 years of radical prostatectomy (i.e., early metastasis), ROC analysis revealed an area under the curve (AUC) of 0.75 for GC, compared to 0.69 for a model incorporating several standard clinical and pathologic parameters, and 0.65 for Gleason score alone [48]. Addition of GC to the clinical model did not improve upon the AUC for GC alone. A multivariate model including GC, Gleason score, pathologic staging parameters, and serum PSA demonstrated only GC retained statistical significance (odds ratio (OR) 1.36 per 10% increase in GC, $p < 0.001$), though Gleason score approached statistical significance (OR 1.91, $p = 0.11$). GC has also demonstrated superior diagnostic performance for prediction of metastasis on decision curve analysis compared to several clinical models, such as the Cancer of the Prostate Risk Assessment Score (CAPRA-S) and Stephenson 5-year [48, 57, 58]. Additionally, GC retained predictive value within Gleason score groups in this study. For example, in patients with Gleason score 7 prostate cancers, dichotomized GC into >0.5 and ≤0.5 was predictive of both early metastasis (50% vs. 17%) and prostate cancer-specific mortality (14% vs. 5.7%). The GC has also showed utility in predicting outcome following adjuvant radiation therapy [54, 56] as well as the ability to perform ETS-based subtyping [34], supporting utility beyond routine post-prostatectomy prognosis.

Likewise, Myriad Diagnostics has developed a qPCR assay, Prolaris prostate cancer testing, that measures expression of 31 cell cycle genes and 15 housekeeper genes to give a cell cycle progression (CCP) score [59–67]. Like Decipher, it is performed from FFPE tissue, with the CCP score ranging from ~−1.3 to 4.7. CCP has been shown to retain statistical significance on a multivariate model for prediction of biochemical recurrence following radical prostatectomy [61]. The model included serum PSA, Gleason score, pathologic tumor stage, and surgical margins, all of which retained statistical significance. HR for CCP was 1.74 per increase in CCP score of 1 in this model, compared to 2.24 for serum PSA and 2.69 for Gleason score >7. CCP has also been shown to add to CAPRA-S for the prediction of prostate cancer recurrence [59]. Like Decipher, Prolaris has also shown utility in predicting outcome after primary radiation therapy [66].

Active Surveillance

Given a large fraction of prostate cancer identified on needle biopsy will never become clinically manifest, several active surveillance (AS) protocols have been developed to distinguish men with indolent vs. aggressive disease [68–76]. For example, the Epstein et al.'s criteria state a man is a candidate for AS if his PSA density is <0.15, and his prostate cancer sampled on needle biopsy is Gleason score $\leq 3 + 3 = 6$, involves <3 cores (in at least 12 needle core biopsies), and involves ≤50% of any core [70]. These protocols have decreased the number of men undergoing definitive treatment and have thus likely reduced the morbidity associated with prostate cancer overtreatment. However, their utility is limited in that they tend to be conservative, allowing in a small fraction of men with new prostate cancer diagnoses. They also likely miss a small fraction of aggressive tumors [77, 78]. Molecular markers are able to identify men who qualify for AS but have unsampled high-grade disease and would therefore be highly desirable. Equally desirable would be markers that could expand the pool of AS candidates. Several molecular tests have been developed for this purpose.

Multigene Expression Assays in AS

Multigene qPCR panels have been studied in AS. The Polaris assay has been reported to be predictive of death from prostate cancer in a cohort of conservatively managed patients diagnosed by needle biopsy in Great Britain [60]. In brief, 349 patients were evaluated. All had long-term follow-up (median 11.8 years), and clinical and pathologic data were available, including Gleason score, serum PSA, age at diagnosis, clinical stage, and hormonal treatment. CCP score was predictive of death from prostate cancer on univariate analysis, as were Gleason score, PSA, % of cores positive, clinical stage, and hormone use. Only CCP, Gleason score, and serum PSA remained statistically significant on multivariate analysis. Median CCP assigned was 1.03 (IQR 0.41, 1.74). An increase in CCP of 1 equated to increased HR of 2.02 on univariate and increased HR of 1.65 on multivariate analysis. The study concluded CCP may be valuable in managing men with prostate cancer diagnosed on needle biopsy.

The Genomic Health has developed the Oncotype Prostate assay, a qPCR assay that evaluates expression of 12 genes associated with stromal response, androgen signaling, cellular organization, and proliferation, along with five housekeeping genes (17 genes in total) [79]. The assigned score is referred to as Genomic Prostate Score (GPS) and reports out individualized estimates of freedom from adverse pathology at prostatectomy (defined as primary pattern 4, any pattern 5, or >pT2). A recent study has investigated this assay in predicting upgrading and upstaging of prostate cancer in men qualifying for AS [80]. The study was large, utilizing two training groups, used to create the predictive model. A validation cohort comprised a prostate biopsy cohort of 514 patients who fulfilled AS criteria at the time of needle biopsy but who opted for immediate prostatectomy. The validation cohort was reflective of a typical AS patient, as patients had low PSA (86% <10 ng/mL)

and biopsy Gleason score ≤3 + 4 = 7 (76% with Gleason score = 6), though central review of biopsies upgraded many cases, assigning Gleason score = 6 in 48% and Gleason score ≥8 in 2%. The study endpoint was an evidence of aggressive disease in the prostatectomy specimen, such as Gleason score ≥4 + 3 = 7 or high pathologic stage. The study reports several multivariate models, which include standard clinical and pathologic variables available at the time of prostate biopsy. The most complex model included GPS, age, serum PSA, clinical stage, and biopsy Gleason score, all of which demonstrated statistical significance for prediction of aggressive disease. In this multivariate model, each increase in GPS of 20 was associated with an increased OR of 1.9 for finding high-grade or high-stage cancer at prostatectomy. The majority of patients (~60%) had GPS 20–40.

Addition of GPS to CAPRA-S increased the AUC for discovery of aggressive disease from 0.63 to 0.67 [80]. Decision curve analysis showed improved net benefit in a model utilizing GPS and CAPRA-S, as opposed to CAPRA-S alone. Likewise, in an independent American cohort, biopsy GPS was associated with high-grade and stage tumors at prostatectomy after multivariate analysis, as well as increased risk of biochemical recurrence after univariate analysis [81].

Senescence Markers in AS

A novel approach to risk stratification using markers of cellular senescence has recently been reported [82]. In this study, a 377-gene panel of genes associated with cellular processes of aging and senescence was derived from meta-analyses of senescence-related genes. From this, a 19-gene "indolence" gene expression signature was defined from available prostate cancer data sets, trained to distinguish aggressive from nonaggressive cancers, with an emphasis on low-grade cancers. The 19-gene signature was then reduced to a 3-gene set: FGFR1, PMP22, and CDKN1A. IHC for these three markers was performed on a retrospective cohort of prostate needle biopsies from patients on AS. The cohort comprised 14 patients who had failed AS and 29 who had not failed AS for at least 10 years. Criteria for AS were strict, requiring Gleason score ≤6, <3 cores positive, cancer involving <50% any core, and serum PSA<10 ng/mL. The cancers from patients who did not fail AS reportedly all had strong expression of all three markers, while those that failed AS had significantly reduced expression. Though this latter part of the study is small, it is unique among studies of prognostic markers. It may find utility on further evaluation.

Multiplexed Protein-Based Markers in AS

Recently, the Metamark Genetics reported validation of ProMark, an eight-biomarker multiplexed immunofluorescence (IF)-based assay from FFPE prostate biopsy tissues that reports a biomarker risk score (range 0–1) for stratifying "favorable" (Gleason score 6 and <pT3a) vs. "nonfavorable" pathology (Gleason score > 6 or ≥pT3a or N1,

M1 disease) at prostatectomy [83]. This panel, which uses quantitative multiplex proteomic imaging (QMPI), integrates morphological object recognition and molecular biomarker measurements from tumor cells on individual slides. In a blinded study of 276 cases using trained models, the eight-biomarker multiplexed IF assay improved the AUC for predicting favorable disease at prostatectomy from 0.69 by the National Comprehensive Cancer Network (NCCN) guideline classification to 0.75. Net reclassification index and decision curve analysis demonstrated benefit from the combined eight-biomarker IF assay and NCCN classification vs. NCCN classification alone [83].

Urine Biomarkers and Prognosis

A major weakness of all tissue-based assays in AS is the problem of tumor clonality. Cancerous prostates contain multiple, clonally distinct carcinomas in the majority of cases. Sampling of one tumor is powerless in informing on the others within the prostate, which may be more aggressive. Markers that globally sample the prostate gland may thus serve as a better tool in quantifying risk in potential AS candidates.

Urine PCA3 and TMPRSS2-ERG are the most advanced, and best studied, urine markers in prostate cancer. PCA3 is a noncoding RNA that is prominently overexpressed in prostate cancer [84–86]. It is detectable in the urine post-digital rectal examination. Studies have shown urine PCA3 is a superior diagnostic test to serum PSA for the detection of prostate cancer [87–102]. The assay is clinically available as the Progensa assay, which calculates a ratio of urine PCA3 mRNA to urine PSA mRNA. Studies have reported contradictory findings regarding the association between elevated urine PCA3 and markers of aggressive prostate cancer, such as high Gleason grade and high tumor stage, although high levels have consistently been shown to correlate with increased tumor burden [87–102]. Recently, Leyten et al. showed urine PCA3 prior to prostate biopsy was predictive of clinically significant prostate cancer (per Epstein et al.'s criteria) at biopsy on univariate analysis [97]. However, PCA3 was not (statistically) significantly associated with high Gleason score or clinical tumor stage, indicating PCA3 predicted clinically significant cancer by detecting higher tumor burden.

The transcript of the TMPRSS2-ERG gene fusion is detectable in the urine of men harboring prostate cancer with this rearrangement [97, 103–105]. A clinically available laboratory test for this marker is available, which provides a TMPRSS2-ERG mRNA to PSA mRNA ratio, similar to the PCA3 assay [106]. Elevated urine TMPRSS2-ERG is highly specific for prostate cancer, although slightly less sensitive than urine PCA3 in most studies [97, 103–106]. Urine TMPRSS2-ERG has been shown to correlate with prostate cancer burden [106] and specifically ERG+ prostate cancer burden [107]. In a recent prospective study, urine TMPRSS2-ERG taken from men in the pre-biopsy setting was predictive of prostate cancer, clinically significant prostate cancer, Gleason score ≥ 7, and high clinical tumor stage (cT3-cT4) on univariate analysis [97]. The ability of urine TMPRSS2-ERG to

predict all of these remained on multivariant analysis incorporating the parameters in European Randomized Study of Screening for Prostate Cancer (ERSPC) risk calculator [97, 108]. Urine TMPRSS2-ERG was the strongest predictor of high Gleason score in the multivariate model (OR 7.16). Importantly, this study treated urine TMPRSS2-ERG as a binary variable using a cutoff of 10. While this is a reasonable approach, using levels as a continuous variable would likely yield more information, given the correlation between urine TMPRSS2-ERG and cancer burden. For example, a man with a TMPRSS2-ERG of 250 almost surely has high tumor burden, while this is less certain for a man with a level of 12. This quantitative relationship between urine TMPRSS2-ERG scores using a quantitative transcription-mediated amplification assay was recently validated in a study assessing over 1200 patients using a combined PCA3- and TMPRSS-ERG-based urine test [106]. Given the diagnostic performance of this test, it is highly possible it will find use in multiple settings, including potential risk stratification for men on AS, where it has been shown to be associated with cancer burden at the time of AS entry [109].

Circulating Tumor Cells in Advanced Prostate Cancer

Great interest has been generated in the potential utility of identifying and classifying cancer cells harvested from the peripheral blood, termed circulating tumor cells (CTCs). Since liquid biopsy is getting popularity, CTC evaluation will be popular in clinical practice. CTCs have been detected in the blood of patients with metastatic prostate cancer using multiple approaches and have shown prognostic ability [110, 111]. Importantly, the CellSearch system is Food and Drug Administration (FDA) approved in metastatic prostate cancer (among other cancer types) for prediction of patient survival. Molecular driver events including TMPRSS2-ERG rearrangement, AR amplification, and PTEN loss have been identified in CTCs [110, 112–115]. There is a great need for a surrogate prognostic biomarker that can be used in advanced prostate cancer clinical trials, given the long follow-up needed to demonstrate overall survival benefits. A recent study demonstrated that CTCs in combination with serum lactic acid dehydrogenase (LDH) levels fulfilled the Prentice criteria for individual level surrogacy for overall survival in phase III trial of abiraterone plus prednisone vs. prednisone (COU-AA-301) [116]. If confirmed in additional studies, CTCs may be increasingly used as prognostic markers, particularly in clinical trials.

Limitations

For a prognostic test to be clinically useful, it must not only be analytically and clinically validated—meaning the test is robust and reproducible and accurately predicts a clinical outcome of interest—but it must also change medical decision-making.

An initial estimation of the ability to change practice can be performed by comparing the performance of an optimized clinicopathological model containing all routine clinicopathologic variables at the time the test would be ordered vs. the same model with the test. Likewise, decision curve analysis can be used to quantify how much clinical benefit a test would provide while incorporating patient-specific preferences representing the harms of false-negative and false-positive tests. Importantly, all of the above multigene expression and protein tests have been shown to improve the performance of multivariate models. Additionally, both the Prolaris and Decipher tests have been shown to change practice when physicians are presented with clinicopathological parameters and then results of those tests [51, 52, 64, 65].

Other important considerations are the clinical benefit in the patient population receiving the test and the potential confounder of multifocality for biopsy-based tests (Fig. 28.2). For example, the absolute risk of patients with very low-risk NCCN guideline prostate cancer on biopsy having adverse pathology at prostatectomy (particularly using the definition used in the Oncotype DX prostate) test means that

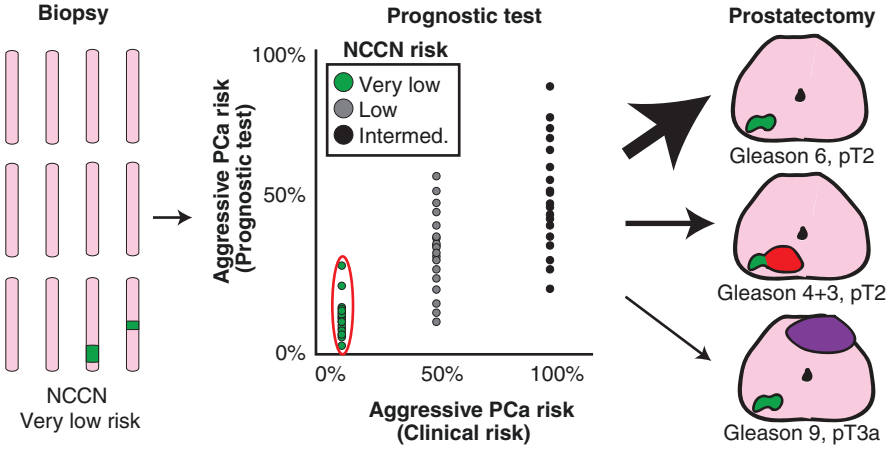

Fig. 28.2 Multifocality and clinical cohort assessed can confound prognostic prostate cancer tests. There is enormous interest in developing clinical tests to identify candidates for active surveillance in men diagnosed with prostate cancer (PCa). Multiple multigene expression and protein-based tests are clinically available. Both the clinical group assessed and the multifocality of prostate cancer can complicate the clinical utility of these tests. For example, as a group, men with NCCN very low-risk PCa on biopsy have a very low risk of having "aggressive" prostate cancer (variably defined across tests) at prostatectomy. Likewise, prognostic tests performed on men with NCCN very low-risk PCa nearly always report very low risk of "aggressive prostate" cancer. However, at prostatectomy, while the vast majority of these men will have insignificant disease (e.g., Gleason 6, pT2), a small number will have more aggressive disease (e.g., Gleason 4 + 3 = 7, pT2), while ~1–2% will have very aggressive disease (e.g., Gleason 9, pT3a) that was most likely not sampled on the diagnostic biopsy. Prospective studies are needed to ensure that prognostic tests performed on diagnostic biopsies of men with unsampled very aggressive disease accurately predict this risk. Likewise, prospective studies will be needed to demonstrate that results from prognostic tests in this very low-risk group actually meaningfully impact clinical decision-making given the cost of current prognostic tests

the vast majority of those patients will be predicted to have low-risk disease by the test, questioning the cost/benefit of testing this population. Yet, a small minority of these patients will have dramatically under- or unsampled disease at prostatectomy or on subsequent biopsy (e.g., biopsy Gleason 6 to prostatectomy Gleason 9, >pT2). Such cases nearly always represent true multifocality, as has been shown in a recent case report for a patient who developed aggressive disease while on AS [117]. Hence, if tested with an assay that is robust to true multifocality, these patients should presumably have a very high molecular risk on their initial biopsy specimen. Prospective studies and monitoring of outcomes from registries, which are lacking for all multigene prognostic tests, will thus be critical to evaluate the real-world performance of these tests in their actual area of intended and routine clinical use.

Conclusion

Our understanding of the molecular drivers of prostate cancer is expanding rapidly. Single gene evaluation or results from comprehensive sequencing/copy number assessment, multigene panels performed on limited FFPE biopsy tissue, molecular assays taken from urine specimens, and CTCs have each been shown to contribute to understanding prostate cancer prognosis at various disease states. The challenge now stands to apply these assays in clinically meaningful ways while focusing on improved outcomes for patients with cost-effective tests.

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Chapter 29
Molecular Targeted Therapies of Prostate Cancer

Panagiotis J. Vlachostergios, Marcia Paddock, and Ana M. Molina

Contents

Introduction

Prostate cancer (PCa) is a second leading cause of cancer mortality after lung cancer in men worldwide [1]. In the modern era, approximately half of the patients who die from PCa have metastatic disease at diagnosis, whereas the other half is represented by patients progressing from localized disease to metastases and eventually death [2]. An improved understanding of the underlying biology of PCa and molecular alterations in early and advanced stages has led to large advances in treatment

P.J. Vlachostergios, M.D., Ph.D. • M. Paddock, M.D., Ph.D. (✉) • A.M. Molina, M.D.
Division of Hematology and Medical Oncology, Department of Medicine, Meyer Cancer Center, New York Presbyterian Hospital, Weill Cornell Medical College, New York, NY, USA
e-mail: amm9052@med.cornell.edu

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*, Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_29

options, setting the stage for newer targeted therapeutics and combination strategies. The androgen receptor (AR) pathway provides a unique target as it remains active even during transition to the castration-resistant state, which represents the lethal form of PCa [3]. In addition, there are several other pathways, for example, PI3K/AKT, which may become overactivated in PCa, especially after the cancer cells adapt or evolve to survive in the setting of androgen deprivation [3]. These pathways are potential targets of newer therapeutics, and a plethora of several agents are currently being tested. There is also an increasing interest in identification of surrogate markers of response in an effort to better predict responders and target more clearly defined patient subpopulations.

Targeting Androgen Receptor Signaling

The AR is a ligand-dependent transcription factor important in cell survival and proliferation [4]. The link between testosterone and the prostate has been known since 1941, when Huggins and Hodges first treated men with metastatic PCa with surgical castration to effect rapid relief from pain and disability due to bony metastases [5]. Since the introduction of this first targeted therapy, significant advances in PCa molecular biology have broadened the potential for additional targeted approaches; however, the mainstay of treatment remains androgen deprivation.

Once dihydrotestosterone (DHT), the main AR ligand, is bound to the latter, the ligand-receptor complex translocates to the nucleus where it dimerizes and binds additional cofactors which together form a complex that binds to androgen response elements (AREs) and promotes transcription of an ever-growing list of genes, encoding for the prostate-specific PSA and TMPRSS2, cell cycle checkpoints cyclin D1 and CDK7, growth signals such as IGF-1 and EGFR, DNA repair factors (i.e. BRCA1 and RAD9), and regulators of cell survival such as phosphatase and tensin homolog (PTEN) and AKT1 (one of the three closely related serine/threonine-protein kinases) [3, 4].

Several strategies for inducing androgen deprivation have been developed and are associated almost uniformly with an initial response.

Targeting the Hypothalamic-Pituitary-Gonadal Axis

The first strategy for medical castration was administration of high doses of estrogens, which suppress gonadotropin-releasing hormone (GnRH) from the hypothalamus through negative feedback resulting in decreased LH and FSH from the pituitary and subsequently reduced testosterone levels. It is still occasionally used today; however, it has significant side effects including heart disease and stroke risk, as well as feminization including gynecomastia [6].

Understanding the function of the hypothalamic-pituitary-gonadal axis offered new opportunities for AR targeting with the use of leuprolide, a luteinizing hormone-releasing hormone (LHRH) agonist. Leuprolide and other LHRH agonists effectively prevent production of testosterone in the testes and adrenals without the need for orchiectomy and were the first pharmacologic-targeted agents approved by Food and Drug Administration (FDA) for the treatment of advanced PCa [7]. Leuprolide is an analog of the GnRH decapeptide and thus acts as an agonist at the receptor. Leuprolide disrupts the normal pulsatile release of GnRH and results in suppression of LH and FSH from the pituitary without the negative side effects of estrogens. Other GnRH agonists were also developed at the time, including goserelin, which was FDA approved in 1989 and continues to be used as well. Both of these agents exist as depot injections given every 1–4 months or implants which last even longer. However, due to their inherent mechanism of action, GnRH agonists initially increase the levels of testosterone over the first 1–2 weeks, before down-regulation of the GnRH receptor results in decreased levels of LH and thus testosterone [8]. This initial testosterone surge seen with GnRH agonists can cause a disease flare which may result in complications including renal obstruction, bone pain, and even precipitate spinal cord compression and neurologic deficits. As such, GnRH agonists need to be combined with an AR antagonist, such as bicalutamide, to avoid stimulation of tumor growth during the initial testosterone surge.

Development of the LHRH antagonist degarelix, which has a faster onset of action and is devoid of testosterone surge, has offered an additional option for medical castration [9]. In phase II and III studies, degarelix suppressed testosterone levels to <0.5 ng/mL in 96% of patients by day 3 with no detectable testosterone surge, which led to its approval by the FDA in 2008. Prospective randomized comparisons of degarelix with LHRH agonists provided evidence of their comparable side effect profile and therapeutic effects with respect to testosterone castration levels, PSA level variation, and PSA progression-free survival [10, 11].

Resistance to AR Pathway Targeting

In the absence of functional AR, there is a rapid shrinkage of tumors and improvement in symptoms in most patients. However, eventually resistance emerges. Several different mechanisms of tumor escape have been well characterized, including:

(a) AR overexpression due to AR gene gain or amplification
(b) AR splice variants (AR-V) resulting in constitutive AR activity in the absence of ligands
(c) Mutations in the AR ligand binding domain (LBD) allowing AR antagonists to take on an agonist conformation
(d) AR stabilization and increased AR-DNA binding through HER2 and HER3
(e) Increased AR transcriptional activity via phosphorylation, ubiquitylation, and methylation,

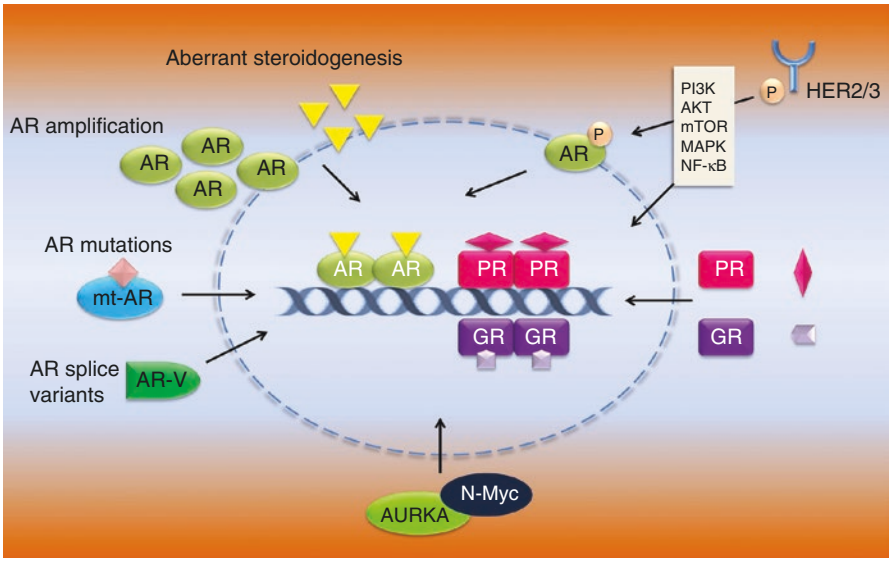

Fig. 29.1 Common resistance mechanisms to AR-targeted therapies at the cellular level

(f) Upregulation of steroidogenesis enzymes (intratumoral, adrenal)
(g) Bypassing of AR by glucocorticoid and progesterone receptors
(h) Activation of other signaling pathways, such as NF-κB and PI3K/AKT
(i) AR loss, inherent in *de novo* small cell prostate carcinoma (SCPC) or acquired after Myc/Aurora kinase A (AURKA)-driven transdifferentiation of adenocarcinoma to neuroendocrine PCa (NEPC) [3, 12, 13].

An overview of the most common mechanisms of resistance to AR signaling blockade is illustrated in Fig. 29.1. Currently approved therapies for CRPC based on phase III randomized trial data [14–21] are summarized in Table 29.1.

AR Antagonists

The first-generation AR antagonists were developed with the hope of blocking the AR directly through competitive inhibition. This class of drugs binds the ligand binding domain (LBD) of the AR. Bicalutamide and its predecessors nilutamide and flutamide are able to bind to the LBD of the AR without activating it. However, they do not achieve sufficient AR blockade to improve survival when used therapeutically as single agents [22, 23]. Bicalutamide has approximately a 50-fold lower affinity than DHT, and its effects can be overcome by increased expression of the AR or through mutations in the LBD which cause the antagonist to act as an agonist instead. This discovery justifies withdrawal of these AR antagonists as an additional

Table 29.1 Approved Therapies For CRPC

Study	Regimen	Mechanism of action	Comparator	Survival benefit	Hazard Ratio	Reference
TAX 327	Docetaxel/prednisone	Microtubule inhibitor	Mitoxantrone/pred	2.4	0.76	Tannock NEJM 2004 [14]
IMPACT	Sipuleucel-T	Immunotherapy	Placebo	4.1	0.78	Kantoff NEJM 2010 [15]
COU-AA-301 COU-AA-302	Abiraterone/prednisone	CYP 17 inhibitor	Placebo/pred	post-doc: 4.6 pre-doc: 4.4	0.65 0.75	De Bono NEJM 2011 [16] Ryan NEJM 2013 [17]
AFFIRM PREVAIL	Enzalutamide	AR antagonist	Placebo	post-doc: 4.8 pre-doc: 4.0	0.63 0.71	Scher NEJM 2012 [18] Beer NEJM 2014 [19]
TROPIC	Cabazitaxel/prednisone	Microtubule inhibitor	Mitoxantrone/pred	2.4	0.70	De Bono Lancet 2010 [20]
ALSYMPCA	Radium-223	Alpha emitter	Placebo	3.6	0.69	Parker NEJM 2013 [21]

therapeutic strategy upon progression, which may induce a significant but often short-lived antitumor effect [24, 25].

Enzalutamide is a second-generation AR antagonist that has five-fold higher affinity for the AR than bicalutamide. Its development is an example of rational drug design. The drug can bind the AR without activating it, thus reducing translocation to the nucleus and preventing DNA binding and coactivator recruitment of the ligand-receptor complex [26]. In contrast to bicalutamide, enzalutamide exhibits no agonistic properties and does not recruit coactivator proteins. In a human prostate cancer cell line that overexpresses AR, termed Vertebral-Cancer of the Prostate (VCaP), enzalutamide suppressed growth and induced apoptosis, whereas bicalutamide did not. Similarly, *in vivo* tumor suppression was evidenced in castration-resistant AR-overexpressing LNCaP/AR xenograft models. Mice treated with enzalutamide also had prolonged time to tumor progression relative to bicalutamide [26].

Enzalutamide was evaluated both in the chemotherapy-naive and post-chemotherapy setting of CRPC. The PREVAIL phase III study randomized 1717 patients to receive either enzalutamide or placebo with progression-free survival (PFS) and overall survival (OS) as coprimary endpoints [19]. Enzalutamide significantly decreased the risk of radiographic progression and death and delayed the initiation of chemotherapy in men with metastatic PCa.

Enzalutamide was also tested in a phase III trial (AFFIRM) involving patients with CRPC who had received prior treatment with docetaxel. The primary endpoint of the study was OS, with secondary endpoints of time to progression, radiographic PFS, and post-treatment PSA and circulating tumor cells (CTC) alterations. The study was positive, demonstrating that the patients who had received enzalutamide had a risk of death 37% lower than those who received placebo (HR 0.63, 18.4 months vs. 13.6 months median survival for each arm), conferring a 4.8-month OS advantage [18]. The FDA has approved enzalutamide for patients with metastatic CRPC who have received prior docetaxel.

ARN-509 or apalutamide is a newer, orally available selective antagonist of the AR. ARN-509 was discovered alongside enzalutamide but showed improved bioavailability and potency in phase I/II clinical trials. This allows the drug to be effective at lower circulating levels which may result in a larger therapeutic index and fewer side effects compared to enzalutamide. ARN-509 is currently undergoing advanced phase testing in patients with CRPC (non-metastatic and metastatic) and biochemically relapsed hormone-sensitive PCa. The first phase II results from 51 high-risk, non-metastatic patients after a median follow-up of 28 months support its activity, with a median time to PSA progression of 24 months. Eighty-nine percent of patients had ≥50% PSA decline at 12 weeks, while the most common treatment-related adverse event was fatigue (any grade 61%) [27]. Study ARN-509–003 is an ongoing phase III comparison of the drug with placebo in a similar patient population (NCT01946204).

Androgen Synthesis Inhibitors

Since androgen deprivation with use of LHRH agonists or antagonists inhibits gonadal androgen synthesis but does not affect extra-gonadal androgens, there was an unmet need at the castration state to inhibit low concentrations of testosterone and dihydrotestosterone of adrenal and intratumor origin which remain sufficient to stimulate AR [28–32]. Abiraterone was developed as a selective inhibitor of androgen biosynthesis that potently blocks CYP17, which is critical to testosterone synthesis by the adrenals, testes, and within the prostate tumor [33, 34]. Abiraterone acetate is the prodrug of the active drug abiraterone. Once absorbed after oral administration, abiraterone acetate is rapidly converted to the active form, abiraterone. In phase I/II trials, treatment with abiraterone resulted in significant antitumor activity in both taxane-naïve and taxane-treated patients [35, 36]. The most common adverse events were associated with increased mineralocorticoid levels, including hypokalemia, fluid retention, and hypertension; these were largely abrogated by co-administering low-dose prednisone.

Two phase III trials were conducted with abiraterone. In COUGAR 301, patients with progressive CRPC following treatment with docetaxel were treated with abiraterone and prednisone or prednisone alone. A total of 1195 men were randomized, with a primary endpoint of OS. Men who received abiraterone experienced a significant reduction in the risk of death, with a hazard ratio of 0.65, associated with a median OS of 14.8 months as opposed to 10.9 months [16]. As a result, abiraterone was approved by the FDA for patients with CRPC who have progressed despite prior treatment with docetaxel. In COUGAR 302, 1088 patients with CRPC who were chemotherapy- naïve were randomized to abiraterone and prednisone vs. prednisone alone. Patients who received placebo and prednisone had a median OS of 27.2 months. Patients who received abiraterone and prednisone had a 25% risk reduction for death (median OS not reached, HR 0.75) [17]. The FDA has thus expanded the indication of abiraterone to include patients with CRPC who are chemotherapy- naïve.

Orteronel is another inhibitor of CYP17A1 and androgen biosynthesis, targeting the 17,21 lyase activity without inhibiting 17 hydroxylase activity, and as such is able to effectively prevent testosterone synthesis without mediating the severe blockade on mineralocorticoid synthesis seen in abiraterone. Orteronel was assessed in chemotherapy-naïve or docetaxel-pretreated patients with mCRPC in randomized phase III trials; however, it did not meet its prespecified OS endpoint [37, 38].

Galeterone is an oral agent that disrupts AR signaling via AR degradation, CYP17 lyase inhibition, and AR antagonism. After encouraging preclinical and early clinical data, ARMOR3-SV was a phase III trial designed to test whether galeterone could improve radiographic PFS in mCRPC patients with AR-V7+ CTCs, as compared to enzalutamide. However, the study was closed early as it was unlikely to meet its primary endpoint [39].

Combined AR Targeting

Although ADT remains the standard first-line therapy for hormone-sensitive PCa (HSPC), there has been discussion over the years concerning the role of combining ADT with antiandrogens. A large meta-analysis supported a modest increase in expected 5-year survival (10 trials; HR = 0.871; 95% CI, 0.805–0.942) with first-generation AR antagonists against the increased risk of adverse effects [40]. In addition, only a minority of panelists at the Advanced Prostate Cancer Consensus Conference (APCCC) recommend their use [41].

Interest in combined androgen blockade (CAB) was reinvigorated after recent results of the STRIVE trial, which revealed a 76% reduction of the risk of progression or death with enzalutamide addition to ADT as compared with bicalutamide and ADT [42]. Abiraterone data from the STAMPEDE and LATITUDE trials are also supportive of this concept of CAB in HSPC. In STAMPEDE, adding abiraterone at the start of ADT in patients with high-risk locally advanced or metastatic PCa resulted in a clinically and statistically significant improvement in OS and failure-free survival (3-year OS improved from 76% to 83%) [43]. In LATITUDE, abiraterone addition to ADT in newly diagnosed, metastatic HSPC patients yielded significantly improved OS (not reached vs. 34.7 months) and radiographic PFS (33 months vs. 14.8 months) [44]. These are all significant advances to the already improved standard of care with addition of docetaxel to ADT in first-line treatment of HSPC patients, successfully tested in the STAMPEDE, CHAARTED, and GETUG phase III trials [45–47].

In contrast, in mCRPC patients combined use of enzalutamide and abiraterone post- PSA progression on enzalutamide in the PLATO phase IV trial did not result in a statistically significant improvement in composite PFS [48]. The results of an ongoing Alliance phase III study of enzalutamide with or without abiraterone and prednisone in patients with mCRPC are pending (NCT01949337).

Targeting Prostate-Specific Antigens

There are several cellular surface proteins that are preferentially or almost exclusively expressed in PCa. The ones with the highest clinical relevance based on prior associations with grade and stage of the disease, thus representing the most appealing targets, are prostate-specific membrane antigen (PSMA), prostate stem cell antigen (PSCA), and six-transmembrane epithelial antigen of the prostate-1 (STEAP-1) [49]. Agents that have been developed to target PSMA are considered an option to overcome treatment resistance in mCRPC [50]. For example, PSMA ADC is a fully human IgG1 antibody conjugated to the microtubule-disrupting agent monomethyl auristatin E (MMAE), which binds to PSMA-positive cells and induces cytotoxicity. A phase II trial of the drug in 119 mCRPC patients after progression on abiraterone and/or enzalutamide yielded PSA declines of ≥30% in one third of patients,

while CTC counts showed a decline of ≥50% in 78% of patients. Partial responses (PR) and stable disease (SD) were observed in 4/31 and 19/31 evaluable cases, respectively [51].

Given PCa is usually radiosensitive, the most promising data emanate from radioimmunotherapy with use of second-generation anti-PSMA antibodies [52]. The most studied is the J591 anti-PSMA monoclonal antibody, which binds the external domain of PSMA-expressing cells and has been conjugated with ^{90}Y and ^{177}Lu for therapy. Promising early results were reported in at least 10 phase I/II and retrospective studies, with a pooled proportion of patients with >50% PSA of 37% and any PSA decline of 68% [53].

Targeting Survival Pathways

The very high incidence (49%) of gene aberrations in members of the PI3K/AKT/mTOR pathway in metastatic tumors from CRPC patients, including biallelic loss of PTEN, as well as hotspot mutations, amplifications, and activating fusions in PIK3CA, and activating mutations in AKT1 prompted further investigation of this pathway with the aim of elucidating its exact role in PCa progression and designing targeted therapies [54]. PTEN loss has a high clinical relevance as an independent, poor prognostic, and predictive factor in patients receiving abiraterone [55]. Its prognostic value was also found to be concordant between tissue and CTC [56]. At the molecular level, there seems to be a reciprocal interconnection between the AR axis and PI3K/AKT/mTOR pathway, as PTEN loss results in derepression of negative regulators of AR transcriptional activity, while AR pathway inhibition with enzalutamide promotes AKT signaling through attenuation of the negative AKT regulator, PHLPP [57, 58]. AKT inhibition was particularly effective in a genetically engineered mouse model of PTEN-deficient PCa [59]. Another therapeutic strategy which was exploited in the PTEN-deficient CRPC setting involves targeting of the SET-PP2A axis with the PP2A-activating drug OP449 which inhibits growth of enzalutamide-resistant CRPC cells [60]. All these findings supported the clinical development of several PI3K-, AKT-, and mTOR- inhibitors with single or dual inhibitory properties. One approach which has been successfully tested is HER2 inhibition with lapatinib which enhanced the effect of abiraterone in CRPC cells and xenografts [61]. AKT inhibition with AZD5363 significantly delayed the development of enzalutamide-resistant PCa through increased apoptosis and cell cycle arrest *in vivo* [62]. AKT inhibitors were combined with other agents targeting additional pathways, including FGF or MEK, resulting in additive and synergistic activity of the combinations, respectively [63–65]. Third, mTOR inhibition was also tested *in vitro* and *in vivo*, and initial results from small phase II studies of mTOR inhibitors (RAD001, everolimus) with or without AR inhibition (bicalutamide) demonstrated a low to moderate activity [66, 67]. Everolimus was also combined with docetaxel showing encouraging phase I results and revealing a role of PET in

assessment of responses [68]. Another combination of docetaxel with the dual PI3K/mTOR inhibitor NVP-BEZ235 resulted in synergistic cytotoxicity in CRPC cell lines and xenografts [69]. Co-targeting mTOR and AR N-terminal domain (NTD) in PTEN-deleted tumors was proposed as a new strategy to overcome resistance to AR pathway inhibitors (ARPI) [70]. Newer agents with dual mTOR and AR inhibitory properties, such as salinomycin, are under testing [71].

Newer insights into other mediators implicated in CRPC include frequent loss of the promyelocytic leukemia zinc finger (PLZF) tumor suppressor which was found to be deleted in 5–7% of mCRPC patients [72]. Interestingly, PLZF loss contributes to enzalutamide resistance and androgen-independent growth in CRPC cells and xenografts, through different pathways including PTEN/AKT/FOXO3 and MAPK [73]. At the clinical level, its loss of expression correlates with higher Gleason grade and is found in the vast majority of metastases [74].

The study of enzalutamide-resistant xenograft models has also led to recognition of the inhibitor of apoptosis protein (IAP) BIRC6, blockade of which leads to increased apoptosis and was proposed as a new therapeutic strategy [75].

Amplifications, activating mutations, and gene fusions in the kinase BRAF (a common driver in several cancers and typically in melanoma) occur in approximately 1–5% of PCa and may as well be targettable with currently available inhibitors [76].

Targeting DNA Repair

DNA repair is an essential cellular machinery for maintenance of genome integrity, and aberrations in DNA repair genes have been identified as contributors to cancer progression. In CRPC, several studies in single-center [77] and multi-institutional cohorts [54, 78] revealed the presence of DNA repair gene deleterious mutations, most commonly BRCA2 and ATM, in a significant proportion of CRPC patients (20–23%). Notably, the incidence of germline DNA repair mutations was significantly higher among patients with metastatic (11.8%) compared to localized PCa (4.6%) [79].

These observations paved the way for a novel treatment approach, termed synthetic lethality, making use of DNA-damaging agents in DNA repair-defective tumors. Platinum agents (cisplatin, carboplatin, oxaliplatin, satraplatin) have shown modest activity in unselected CRPC patients, but the majority of studies were at phase II level, without demonstrated OS benefit and without correlative assessment for surrogate markers of response [80]. A phase II trial of carboplatin in mCRPC patients with DNA repair defects is currently underway (NCT02311764). An alternative strategy within the same concept of synthetic lethality is based on inhibiting PARP, an enzyme with a key role in recruitment of DNA repair protein complexes at sites of single-strand breaks (SSBs). After successful preclinical testing, olaparib was the first PARP inhibitor tested in mCRPC patients who were previously treated with a taxane (docetaxel or cabazitaxel) or ARPI (abiraterone or enzalutamide) in

the phase II TOPARP trial. A significant response rate of 33% was observed, with 14 out of 16 (88%) responders found to have BRCA2 loss (four patients with biallelic somatic loss and three with germline mutations) or ATM gene aberrations [81]. In an effort to improve responses, classic DNA-damaging agents (cisplatin, temozolomide) were combined with PARP inhibitors; however, increased hematologic toxicity was of significant concern [82, 83].

Interestingly, several lines of evidence support a crosstalk between AR signaling and DNA repair pathways, with many DNA repair proteins (e.g., DNA-PK, PARP, TOP-2B) being involved in regulation of AR transcriptional activity, which in turn results in AR-dependent transcriptional upregulation of several DNA damage sensor and repair proteins [84, 85]. Also, enzalutamide was found to suppress the expression of homologous recombination (HR) repair genes in CRPC cells, thus creating HR deficiency and "BRCAness," proposing a "lead-in" treatment strategy, in which enzalutamide was followed by the PARP inhibitor olaparib [86]. These findings have triggered clinical testing of several combinations of PARP inhibitors with AR pathway inhibitors (NCT02500901, NCT01972217). Although most studies are ongoing, the combination of veliparib and abiraterone resulted in longer mPFS in patients with DNA repair defects compared to abiraterone alone [87]. Likewise, the observation of ATM activation by ARPI-mediated telomere dysfunction provides the rational for development of ATM inhibitors, the use of which was able to enhance the antitumor effect of bicalutamide or enzalutamide in CRPC cells [88].

Targeting Immune Checkpoints

Loss of immune surveillance is common in malignancy, and it may contribute to cancer progression. HLA-DMB expression and CD3+ immune cells are decreased in CRPC metastases compared to primary PCa tumors, supporting their role in CRPC progression [89]. Likewise, loss of IL-33 in a murine PCa model was associated with progression to metastatic disease, whereas reintroduction of IL-33 into metastatic tumors resulted in reduced CTC [90]. B7-H3 (CD276) is another immune checkpoint molecule positively correlated with grade, stage of primary tumors, as well as with development of metastatic CRPC disease [91]. The importance of these findings is accentuated in view of ongoing clinical testing of the monoclonal antibody enoblituzumab (MGA271) in B7H3 expressing treatment-refractory cancers, including CRPC (NCT02381314; NCT02475213). Out of the several tumor vaccines tested in the CRPC setting, sipuleucel-T is the only currently approved autologous active cellular immunotherapy which has shown a 4-month median OS benefit compared to placebo in 512 CRPC patients [15]. A second vaccine that has shown a survival advantage in advanced CRPC is PROSTVAC-VF, a vector-based vaccine designed to activate T cell-mediated immune responses to PSA. In a randomized phase II study, patients in the PROSTVAC-VF arm compared with the placebo arm achieved an 8.5-month improvement in median overall survival (25.1 months vs. 16.6 months) and a 44% reduction in mortality (HR 0.56) [92]. The phase III

PROSPECT trial is completed at this time, and preliminary results are eagerly awaited (NCT01322490).

Another strategy under investigation is use of monoclonal antibodies against programmed cell death 1 (PD-1)/PD-1 ligand 1 (PD-L1) or cytotoxic T lymphocyte-associated antigen 4 (CTLA4), in view of increased tumor mutational load as a result of DNA repair deficiencies, particularly mismatch repair (MMR), which could sensitize to immunotherapy [93]. Although an initial phase III study of ipilimumab (anti-CTLA4) in mCRPC patients progressing after docetaxel did not show OS benefit, interest in checkpoint inhibitors has been invigorated by most recent evidence of increased PD-L1/2 expressing dendritic cells (DC) in the blood of enzalutamide-resistant patients compared to those naïve or responding to treatment and a high frequency of PD-1 positive T cells, implicating PD-1/PD-L1 inhibitors as a potential therapeutic strategy worthy of clinical testing [89]. Early phase II results (NCT02312557) support the activity of pembrolizumab (anti-PD-1) in mCRPC patients progressing on enzalutamide. Three of the first ten patients enrolled had rapid PSA reductions to $\leq$0.2 ng/ml, and two showed partial responses, associated with histological evidence of CD3+, CD8+, and CD163+ leukocyte infiltrates and PD-L1 expression [94]. Establishment and validation of immune response criteria (as opposed to the classical RECIST) as well as exploration and targeting of additional immune checkpoints, including B7-H3,53 LAG-3,54 OX40,55 and 41BB, are currently ongoing and hold promise for the future of CRPC therapeutics [95].

Targeting Epigenetic Regulation

Epigenetic regulation of genes is a common pathway found in many cancer types, including PCa. The Polycomb group (PcG) protein and methyl-lysine reader CBX2 is overexpressed in metastatic CRPC patient-derived xenograft (PDX) models and patients, and it represents a key regulator of proteins involved in mitotic spindle assembly which are significantly downregulated upon CBX2 silencing, including Aurora and Polo-like kinases [96]. Despite the lack of direct CBX2 inhibitors, disrupting the interaction between CBX2 and histone H3K27me3 was suggested as a strategy for inhibiting CBX2 activity and reversing abnormal gene expression programs [96]. Another critical PcG protein with a central regulatory role in modulation of gene expression is EZH2. EZH2 cooperates with N-Myc in transforming the transcriptional program of CRPC during transition to NEPC in *in vivo* CRPC mouse models [97, 98]. Among various targets, EZH2 represses CCN3 which in turn interacts with AR to sequester it in the cytoplasm thereby inhibiting AR action, whereas CCN3 re-expression inhibits growth of CRPC and enzalutamide-resistant cells and xenografts [99]. Gene re-expression strategies with use of EZH2 inhibitors (EPZ-6438, GSK2816126) are currently under phase I/II testing (NCT01897571; NCT02082977). Newer evidence supports a role for the E3 ubiquitin ligase Skp2 in post-translational stabilization of EZH2 in CRPC tumors of PTEN/TP53 null mouse and human tissues, offering another promising therapeutic target [100].

Targeting Epithelial-Mesenchymal Transition and Stemness

Epithelial-mesenchymal transition (EMT) is a normal process during embryogenesis; however, it is also a common pathway used from cancer cells for invasion, metastasis, and treatment resistance. In human PCa cells, ADT-mediated upregulation of AR and AR-V enhances the expression of mesenchymal markers such as fibronectin, ZEB1, and Twist as well as stem cell-related genes such as Nanog, Lin28B, and CD44 [101]. In a feedback loop manner, overexpression of another EMT player, Snail, which is enriched in aggressive primary PCa and metastatic sites, confers resistance to enzalutamide via increased AR signaling [102]. Notably, new evidence from mitochondrial DNA sequencing of bone and visceral metastases of CRPC patients suggests that while partial EMT may occur during dissemination from the primary site, there is a subset of epithelial cells in CRPC bone metastases recognized by nuclear Twist, Slug, and Zeb1 localization which have the potential to seed new metastases, representing a new targetable subpopulation [103]. In TP53- and RB1-deficient *in vitro* and *in vivo* PCa models, the reprogramming transcription factor SOX2 plays a key role in conferring lineage plasticity and acquisition of a basal, AR-independent phenotype which is associated with resistance to enzalutamide [104].

Overall, several members of different signaling pathways, including Notch 1, AKT, Myc, and Ras/Raf/MAPK, cooperate to promote EMT and high self-renewal capacity in CRPC cells and xenografts [105]. Targeting some of these kinases (i.e. with the multi-tyrosine kinase inhibitor, sorafenib) concurrently with AR inhibition in CRPC models has shown promising results [106].

A different proposed strategy to block EMT *in vivo* is suppressing the chromatin-remodeling protein HMGA2, with use of histone deacetylase inhibitors, as HMGA2 regulates stemness and epithelial-mesenchymal plasticity in prostate tumor cells with PI3K/AKT and RAS/MAPK coactivation [107]. Another approach is combining cabazitaxel with enzalutamide, given the ability of cabazitaxel to induce MET and glandular differentiation in a transgenic mouse model of androgen-responsive PCa [108].

The ATP-binding cassette subfamily G member 2 (ABCG2) transporter was recently identified as a potential target within the prostate stem cell (PSC) population, as its presence allows for maintenance of AR-positive, ADT-resistant PSCs, whereas its inhibition results in increased nuclear translocation of AR, with associated increased expression of AR target genes, and luminal differentiation into ADT-sensitive PCa cells [109].

The WNT pathway is another central player, not only in the development of prostate tissues during embryonic/neonatal organogenesis but also in the emergence of CRPC. Mutations in the APC or CTNNB1 (β[beta]-catenin) genes lead to constitutive activation of WNT signaling, similar to those found in colon cancer [110]. Several lines of evidence support a role of Beta-catenin (β-catenin) as a ligand-dependent coactivator of the AR-driven transcription in hormone-naïve PCa, whereas in CRPC AR and WNT/β-catenin signaling pathways stimulate each other to activate specific target genes for promoting androgen-independent

growth [110]. In addition, activation of the WNT/β-catenin signaling pathway has also been linked to PCa stem cells in various studies testing for markers indicative of these subpopulations (CD133+, CD44+) *in vitro* and *in vivo*. Thus, inhibition of the WNT/β-catenin pathway could offer a novel therapeutic opportunity to target CRPC cells and CSCs. One such targeted approach, which is currently in phase I/II development, uses an inhibitor of porcupine, a membrane-bound O-acyltransferase enzyme required for WNT secretion [111].

Targeting Angiogenesis and Hypoxia

Inhibiting angiogenesis through monoclonal antibodies and tyrosine kinase inhibitors as a treatment strategy has played a key role in the management of several different types of cancers. In mCRPC, a phase II trial evaluating bevacizumab, the first monoclonal antibody against vascular endothelial growth factor-A, in men progressing after docetaxel chemotherapy, suggested activity based on PSA responses [112]. Unfortunately, the phase III trial of docetaxel and prednisone with bevacizumab or placebo in men with mCRPC showed no difference in OS between the bevacizumab and placebo arms (22.6 months vs. 21.5 months; HR 0.91) [113]. Moreover, trials evaluating several other antiangiogenic agents, including sunitinib, aflibercept, and lenalidomide, in mCRPC have also failed to demonstrate significant clinical benefit [114]. Likewise, the COMET-1 study which randomized 1028 mCRPC patients who progressed after docetaxel and abiraterone and/or enzalutamide to the oral VEGF receptor/MET inhibitor cabozantinib or prednisone failed to show a significant OS difference (11 months vs. 9.9 months) [115]. An antiangiogenic approach that has provided encouraging *in vivo* results in a CRPC xenograft model and meres clinical testing is the combination of sorafenib and enzalutamide, which was shown to improve the efficacy of enzalutamide through inhibition of the ERK pathway in a synergistic manner [106].

Hypoxia and associated pathways can also promote AR activity in a HIF1α- and HIF2α-dependent fashion via induced expression of the histone demethylase activity-dependent AR coactivator PHF8 [116]. Strategies of co-targeting HIF and AR have revealed a synergistic activity for the combination *in vitro* but remain to be tested *in vivo* [117].

Targeting the Cell Cycle

Among several sequelae promoting cell proliferation and growth, androgens induce cell cycle progression, in part, through upregulation of cyclin D1 (CCND1) expression and subsequent activation of cyclin-dependent kinases 4 and 6 (CDK4/6). This can be exploited for therapeutic targeting, in the presence of

available CDK4/6 inhibitors and also given recent evidence from PCa models. As such, CDK4/6 inhibition was active in CRPC models of AR-V7 or AR mutants or AR loss [118] and is worth further clinical testing. Another important cell cycle regulator, expression of which is upregulated upon castration, is Polo-like kinase 1 (Plk1). Plk1 upregulation after treatment of BRCA1-deficient CRPC cells with the PARP inhibitor olaparib represents a resistance mechanism to the latter that can be overcome with use of Plk1 inhibitors [119], which are already in clinical development.

Targeting Stress Pathways

Upregulation and activation of heat shock proteins (HSP) under conditions of cellular stress is a common survival pathway in various cell types, including PCa. HSPs facilitate binding of DHT to the AR and enhance AR-mediated transcriptional activity. There are several HSP, including HSP90, HSP70, HSP27, and custirsen, targeting of which has been widely studied at the preclinical level [120], with antisense oligonucleotides targeting of HSP27 and clusterin being advanced to late-stage clinical trials [121]. Most recently, the phase III SYNERGY trial which examined the effect of custirsen, a second-generation antisense oligonucleotide that inhibits clusterin production, in combination with docetaxel and prednisone on OS of chemotherapy-naive mCRPC patients failed to meet its primary endpoint [122]. Unfortunately, the AFFINITY trial of custirsen in combination with cabazitaxel/prednisone in patients with previously treated mCRPC also failed to show a survival benefit compared to cabazitaxel/prednisone alone [123].

Targeting the Bone Microenvironment

Bone turnover is regulated by the receptor activator of activated nuclear factor kappa-B (RANK) on osteoclasts and their precursors. RANK is stimulated by RANK-ligand (RANK-L), expressed by osteoblasts and T cells, and upregulated by 1–25-dihydroxivitamin-D, parathyroid hormone (PTH), and parathyroid hormone-related peptide (PTHrP). It is inhibited by the decoy receptor osteoprotegerin (OPG) [124]. Understanding the molecular biology of bone remodeling enabled the design of drugs targeted at this process.

Denosumab (Xgeva) is a humanized monoclonal antibody against RANK-L which functions like OPG to prevent interaction of RANK-L with RANK and prevent osteoclast stimulation. It was studied in both non-metastatic PCa and mCRPC settings. Denosumab significantly increased bone metastasis-free survival by a median of 4.2 months compared with placebo in non-metastatic CRPC patients [125]. In addition, denosumab was better than zoledronic acid

for prevention of skeletal-related events in mCRPC patients with bone metastases, with a median time to first on-study skeletal-related event of 20.7 months as compared to 17.1 months with zoledronic acid [126].

Challenges and Perspectives

In the last few years, remarkable progress was made in unraveling the gene alterations of tumors to inform prognosis and treatment decision-making. However, in the absence of clinically validated biomarkers (intratumoral or circulating) which can direct treatment decisions for patients with PCa, management is based on several clinical factors, including time to progression and tolerability to previous treatments [12]. AR-V7 mRNA assessment in CTC [127] has a great potential to become the first surrogate marker to drive treatment decision-making, particularly with respect to choice and sequencing of AR signaling inhibitors and taxanes.

A non-exhaustive list of targets and drugs undergoing clinical testing in CRPC is presented in Table 29.2. In many cases, despite a strong mechanistic link between the target and the drug, the magnitude of responses may not meet initial expectations, for several reasons including intratumoral target expression heterogeneity, poor or inconsistent bioavailability and concentration of the drug within tumors, the tumor vasculature and stroma, as well as the immune component of the tumor microenvironment [49].

Table 29.2 Selected new drugs under clinical development for treatment of CRPC

Drug	Target	Design	NCT
Galeterone vs. enzalutamide	AR-V7	Phase III (ARMOR3)	NCT02438007
Testosterone + abiraterone or enzalutamide	AR/CYP17	Phase II (RESTORE)	NCT02090114
VT-464	CYP17	Phase I/II	NCT02012920
ODM-204	CYP17/AR	Phase I/II	NCT02344017
ODM-201 vs. placebo	AR	Phase III	NCT02200614
ARN-509 vs. placebo	AR	Phase III (SPARTAN)	NCT01946204
ARN-509 + abiraterone	AR/CYP17	Phase I	NCT02123758
ARN-509 + everolimus	AR/mTOR	Phase I	NCT02106507
EPI-506	AR (NTD)	Phase I/II	NCT02606123
Niclosamide + abiraterone	AR-V7/CYP17	Phase II	NCT02807805
Mifepristone + enzalutamide	GR/AR	Phase I/II	NCT02012296

Table 29.2 (continued)

Drug	Target	Design	NCT
Enzalutamide +/− metformin	AR/mTOR	Phase II (IMPROVE)	NCT02640534
AZD8186 +/− abiraterone	PI3Kβ[beta]/ PI3Kδ[delta]/CYP 17	Phase I	NCT01884285
BI 836845 +/− enzalutamide	IGF-1/IGF-2	Phase Ib/II	NCT02204072
Cabozantinib	c-Met/VEGFR2	Phase III (COMET-1)	NCT01834651
GSK2636771 + enzalutamide	PI3Kβ[beta]/AR	Phase I	NCT02525068
AZF5363 + enzalutamide	AKT/AR	Phase II	NCT02525068
Olaparib + AZD5363	PARP/AKT	Phase I	NCT02338622
Olaparib + abiraterone	PARP/CYP17	Phase II	NCT01972217
Niraparib	PARP	Phase II	NCT02854436
Niraparib + enzalutamide	PARP/AR	Phase I	NCT02500901
Veliparib + abiraterone	PARP/CYP17	Phase II	NCT01576172
Rucaparib	PARP	Phase II	NCT02952534
CC-115 + enzalutamide	DNA-PK/mTOR/AR	Phase I	NCT02833883
Atezolizumab + radium-223	PD-L1	Phase I	NCT02814669
Atezolizumab + sipuleucel-T	PD-L1/PAP	Phase I	NCT03024216
Avelumab	PD-L1	Phase I	NCT01772004
MEDI4736 + olaparib +/− cediranib	PD-L1/PARP/VEGF	Phase I/II	NCT02484404
MGA271 + pembrolizumab	B7-H3/PD-1	Phase I	NCT01391143
MGA271 + ipilimumab	B7-H3/CTLA-4	Phase I	NCT02381314
MOR209/ES414	PSMA/CD3	Phase I	NCT02262910
GSK525762	BET (BRD4)	Phase I/II	NCT01587703
OTX015/MK-8628	BET	Phase I	NCT02698176
ZEN003694	BET	Phase I	NCT02705469
INCB054329	BET	Phase I/II	NCT02431260
Ribociclib + docetaxel	CDK4/6	Phase Ib/II	NCT02494921
Tisotumab vedotin	HuMax-TF-ADC	Phase I/II	NCT02552121
Carfilzomib	Proteasome	Phase II	NCT02047253
MLN8237	AURKA	Phase II	NCT01799278
Rovalpituzumab	DLL3	Phase I/II	NCT02709889
Sandostatin	Somatostatin	Phase II	NCT02631616
EPZ-6438	EZH2	Phase I/II	NCT01897571
GSK2816126	EZH2	Phase I	NCT02082977
CC-486 (oral azacitidine)	DNMT	Phase I	NCT02223052
DCR-MYC	MYC	Phase I	NCT02110563

Conclusively, it is anticipated that in the near future, filling the gaps between the evolving molecular landscape of PCa and the clinical behavior of the disease in its different phases will enable a better identification of the true molecular drivers to inform therapeutic targeting and further improve outcomes.

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Chapter 30
Overview of Prostate Cancer Molecular Classification

Juan-Miguel Mosquera, Brian D. Robinson, and Peyman Tavassoli

Contents

Introduction

Prostate cancer (PCa) is clinically multifocal and heterogeneous disease. About 90% of the PCa are organ confined at the time of diagnosis in North America, but their clinical behaviors are highly variable [1]. Many have an indolent disease, whereas some men face with aggressive disease and subsequently metastases. This makes it difficult to reliably predict PCa prognosis even at low grade or stage. Using the clinical and pathologic information such as PSA, Gleason score, and staging, many nomograms have been developed [2–4]. Nevertheless, none of them by itself is able to effectively predict individual outcome.

J.-M. Mosquera, M.D., M.Sc. • B.D. Robinson, M.D.
Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, New York-Presbyterian Hospital, New York, NY, USA

P. Tavassoli, M.D., Ph.D., F.R.C.P.C. (✉)
BC Cancer Agency Vancouver, University of British Columbia, Vancouver, BC, Canada
e-mail: pe.tavassoli@gmail.com

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*, Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9_30

With advanced technology and application of next-generation sequencing (NGS), it is feasible to identify millions of short-sequence nucleic acid base pairs in parallel with lower cost and shorter turnaround time than previous. This has resulted in enormous upsurge of data including mutational events, gene expression, copy number, and epigenetics changes in PCa. Once the full scope of data is analyzed, we have a better understanding of the development and progression of PCa based on its molecular signature, and shortly, the PCa evolves from a clinically heterogeneous disease with variable outcome to homogeneous subclasses with predicted prognosis. Herein, we review the relevant literature and described the current knowledge of PCa molecular classification.

Molecular Classification of Prostate Cancer

Acinar adenocarcinoma is the conventional histologic variant of prostatic carcinoma. Essentially, most of efforts in PCa research are limited to this variant. The heterogeneity of this histologic pattern has been well known for pathologists resulting in developing the Gleason grading system [3, 5]. Similarly, there is also considerable molecular heterogeneity in PCa; however, accumulating data suggests that specific genomic alterations are often frequent. Genomic and transcriptomic analyses of primary PCa have revealed recurrent somatic mutations, numerous repeated DNA alterations, specifically within the prostatic development regions (chromoplexy), as well as somatic copy number alterations (SCNA), and epigenetic changes. Additionally, alterations in chromatin modification genes, cell cycle regulation, and androgen signaling pathways have been frequently observed [6–9]. These repetitive alterations mainly include carcinogen pathways among variety of cancers, as well as those that are more specific in prostate. Several groups have recently classified prostate cancer based on the aforementioned genetic alterations, notably fusions, mutations, SCNA, and gene expression data [7, 10–13]. There are subtle differences in their approaches and stratifications, but many similarities can be appreciated. Nevertheless, the main goal in all studies is to distinguish indolent from aggressive PCa.

For the first time, The Cancer Genome Atlas (TCGA) network performed a comprehensive study of integrating data from multiple platforms, including exome sequencing, DNA copy number, DNA methylation, and messenger RNA (mRNA) and microRNA (miRNA) expression, to assess the robustness of previously defined PCa classification. This revealed molecular subtypes of PCa, which are defined by frequent specific gene mutations (SPOP, FOXA1), or fusions (ERG, ETV1, ETV5, FLI1) [14]. Regarding advanced PCa, Robinson et al. recently conducted a multi-institutional analysis of the metastatic PCa tissues from patients who had received primary androgen deprivation therapy [15]. Interestingly, they have found many similarities and some differences between primary and treated metastatic disease. Among differences, p53 mutations and alteration in AR signaling were shown to be the most, as these were highly enriched in metastasis compared to primary disease.

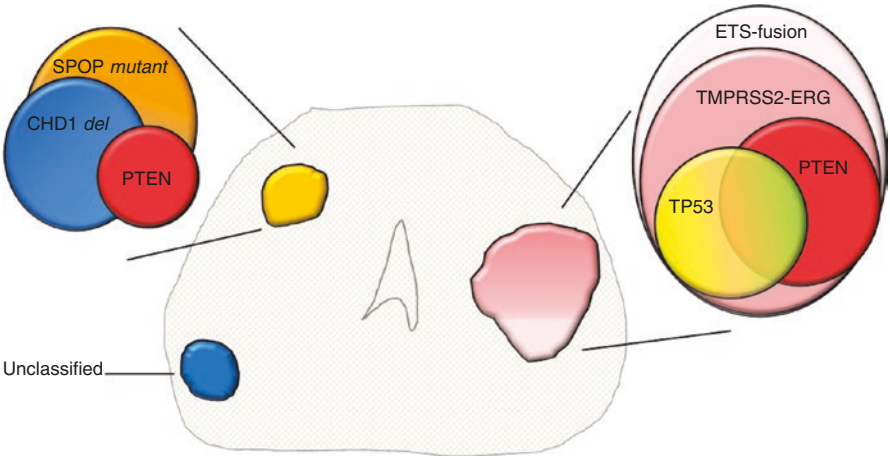

Fig. 30.1 Molecular classification of prostate cancer. Using integrating data from multiple platforms, including exome sequencing and DNA copy number, primary prostate cancer can be classified into three clusters. About 50% of them acquire ETS gene rearrangement, 10% harbor SPOP mutation that is mutually exclusive with ETS fusion, while 25% obtain heterogeneous or occult molecular abnormalities, yet remain to be further classified. Deletion or mutations of TP53 and PTEN are noted in about 25–40% of localized tumors within each cluster

Since about 50% of PCa contain one of the ETS family fusions, molecular classification of primary PCa often starts with ETS-positive and ETS-negative subclasses. In addition, about 10% of PCa harbor SPOP (speckle-type POZ protein) mutation, which exclusively occurs in ETS-negative group. Certain additional alterations, such as PTEN (phosphate and tensin homologue), or TP53 mutations are mainly seen in ETS-positive tumors, while CHD1 (chromodomain helicase DNA-binding protein domain 1) deletion or SPINK1 overexpression is enriched in ETS-negative PCa. Of note, about 25% of tumors harbor heterogeneous or occult molecular abnormalities, which cannot fit into any of the above classes (Fig. 30.1). In the following section, we review the major subclasses of primary PCa with known genetic characterization.

ETS Gene Fusions in PCa

Rearrangement, or gene fusion involving androgen-regulated genes, such as TMPRSS2, and members of ETS transcription factor family, ERG gene in particular, is the most frequent known molecular aberration in prostate cancer. Approximately half of all prostate cancers harbor ETS gene fusions, in which the coding areas of ERG in particular are fused to the 5′ untranslated region of an androgen-regulated gene, mainly TMPRSS2 gene [16–18]. ETS gene fusion has also been detected in high-grade intraepithelial neoplasia (HGPIN) suggesting ETS fusion as an early driver in PCa tumor genesis [19, 20]. The distinction

between ETS-negative and ETS-positive fusion in PCa has been recently supported by the TCGA. Similar to the gene fusion data, integrating gene expression (mRNA) and microRNA expression data of the primary PCa have revealed two different clusters of ETS-positive versus ETS-negative tumors [14].

Accumulating evidence suggests that overexpression of ERG fusion proteins promotes cell migration and invasion [21, 22]. As a result of this fusion, ERG protein is also often overexpressed. It has been shown that the TMPRSS2:ERG fusions detected by FISH are strongly correlated with ERG protein overexpression (>95% sensitivity and specificity) [23, 24]. Therefore, using immunohistochemistry (IHC), ERG overexpression is highly predicting ERG-fused protein. Notably, some of the tumors only overexpress full-length ETS transcripts that are mutually exclusive with ETS fusions [14]. ETS overexpression in these cases could possibly be mediated via epigenetic mechanisms or cryptic translocations that have not been detected by standard means. Other than TMPRSS2:ERG rearrangement, fusion of many other androgen-regulated genes, such as SLC45A3 and NDRG1, with other members of ETS family including ETV1 and FLI1 has been also reported, but these comprise only 5–10% of tumors [17, 25, 26].

Analysis of epigenetic changes in the TCGA data also demonstrated two subclasses of the ETS-positive tumors. About 70% of ETV-positive tumors showed only moderately elevated DNA methylation in a heterogeneous pattern. However, the remaining 30% comprised a distinct hyper-methylated cluster that was almost exclusively associated with ERG fusions [14]. These data demonstrated a potential for further ETS-positive tumor subclassification.

To understand the clinical application of ETS fusions, several studies assessed the effect of TMPRSS2:ERG fusion on prognosis in radical prostatectomy cases. Many found that ETS rearrangement is associated with more aggressive disease while other groups identified an association with more indolent disease. This conflicting data more likely represents differences of patient cohorts (i.e., population-based studies versus retrospective radical prostatectomy group) or variation in diagnostic modalities (i.e., biopsy due to high PSA versus TURP for urinary obstructive symptoms), as well as ultimate measured outcomes (biochemical recurrence versus survivals). In addition, the presence of multifocality and intraprostate heterogeneity of PCa make analyzing data even more complex. In a recent large prospective cohort of 1180 men treated with radical prostatectomy, TMPRSS2:ERG, or ERG overexpression, is associated with tumor stage but does not predict recurrence or mortality among patients [27]. On the other hand, in a PCa cohort diagnosed on TURP samples and received conservative management, there is a significant correlation between ERG rearrangement and adverse outcomes [28, 29]. Lin et al. also showed that ERG fusion is associated with higher Gleason score and increased tumor volume in active surveillance population [30]. Clearly, prospective comprehensive trials correlating natural history, pathological findings, and outcome following prostatectomy will be required to further explore associations between ETS fusion status and outcome.

SPOP-Mutant/CHD1-Deleted PCa

The prostate cancer genome shows relatively low mutation rate in comparison to other epithelial tumors. Less than one mutation per million bases is identified in The Cancer Genome Atlas (TCGA) cohort of primary prostate carcinomas. Among many mutations in PCa, SPOP is the most common point mutations in primary cancers, ranging 6–15% in multiple independent cohorts [31–34]. SPOP mutations have also been reported in HGPIN, and are only seen in ETS-negative tumors. SPOP encodes a Cullin 3-based E3 ubiquitin ligase [35] that has several substrates in the context of the prostate, including the androgen receptor (AR) [36, 37] and steroid receptor coactivator 3 (SRC-3) [38]. Upon SPOP mutation in PCa, ubiquitination of its substrates is demoted leading to enhanced AR signaling and eventually cell proliferation [31, 36]. Gan et al. have recently demonstrated that ERG ubiquitination is also regulated by SPOP [39]. In addition, they showed that ERG fusion proteins evade SPOP-mediated degradation. This might explain the reason for mutual exclusivity of ETS fusion and SPOP mutation in PCa and create a potential novel therapeutic avenue for ETS fusion tumors.

SPOP mutants are significantly associated with CHD1 deletions at 5q21 or 6q21 regions [31]. CHD1 gene controls the transcriptional activity across the genome. It is recurrently deleted in 10–25% of primary and metastatic PCa, and particularly focal homozygous deletions are restricted to ETS-negative tumors [31, 32, 40–42]. The SPOP-mutant/CHD1-deleted subset of PCa have characteristic molecular features, including high levels of DNA methylation, homogeneous gene expression patterns, distinct somatic copy number alterations (SCNA), as well as frequent overexpression of SPINK1 mRNA [14]. The latter is associated with aggressive disease and increased risk of biochemical recurrence [43, 44]. The SPINK1 may act through EGFR pathway; hence, EGFR inhibitors may have therapeutic role in SPINK1-positive PCa [45]. Taken together, SPOP mutations and CHD1 deletions are distinct molecular subclass of PCa and may play an important role in tumor genesis.

Unclassified PCa

The comprehensive integrating data by TCGA showed that about 25% of primary PCa could not fit into one of the aforementioned groups. These tumors are either driven by occult molecular abnormalities or by some of more frequent alterations seen in the first two clusters. It has been shown that a subset of these tumors was enriched for mutations, such as TP53, amplification containing MYC, and deletions of chromosome 6 and 16. The others acquired a high burden of copy number changes or DNA hyper-methylations [14]. Interestingly, this group of PCa is a mix of both good and poor prognosis; hence, enhancing the scale of samples in future studies may reveal a better resolution to stratify this group further.

Common Pathway Alteration in PCa

As mentioned above, ETS fusion and SPOP mutation are the main drivers for prostate cancer initiation. Recruiting either common cancer pathways, including PI3K alteration, or prostate specific pathways, such as androgen signaling modifications, further develops PCa. We will briefly review some of the common well-known molecular changes in PCa.

PI3K Pathway

PI3K (Phosphoinositide 3-Kinase) pathway is one of the most common altered pathways in cancers. Activation of this pathway enhances cell growth and survival, which can be negatively regulated by PTEN. Approximately in 25–70% of PCa, PI3K pathway has been altered either through PI3K overactivation or PTEN inactivation. PTEN, located on chromosome 10q23, is inactivated mainly through deletion in nearly 40%, or mutations in about 10% of PCa. Overactivation of PI3K, yet a less frequent phenomenon in PCa, either occurs due to amplification (25%) or point mutation (5%) of PI3KCA that encodes a catalytic subunit of PI3K.

Inactivation of PTEN is correlated with higher Gleason score, advanced disease, biochemical recurrence, and death [10]. However, adding the PTEN status to combined clinicopathologic data does not provide additional prognostic information.

TP53 (p53)

p53 activates the transcription of genes involved in cell cycle arrest, DNA repair, and apoptosis. Deletion and point mutation at the TP53 locus occur in 25–40% and 5–40% of PCa, respectively [31, 41, 46]. Interestingly, these alterations are not absolutely late events, as they have been shown in 25–30% of clinically localized PCa [6, 31].

Androgen-Androgen Receptor (AR) Signaling

PCa initiation and progression exclusively depend on androgen-AR signaling. Upon androgen-AR binding, they are dragged to AR target genes and recruited coregulators to activate AR target products, e.g., PSA. Alterations in the AR gene itself are mainly limited to tumors following introduction of androgen deprivation therapy (ADT). However, repeated gene alterations have been identified in genes, such as FOXA1 (forkhead box A1) that interact and modulate AR transcription. Recurrent point

mutations in FOXA1 occur in both primary and metastatic PCa. It is noteworthy that tumors with FOXA1 mutations shared mRNA, copy number, and methylation profiles with SPOP-mutant tumors. In addition, some of the SPOP-mutant tumors also possessed FOXA1 mutations suggesting they are present in the same tumor cells [14]. As mentioned previously, SRC-3 is an AR coactivator and a substrate of SPOP. Upon SPOP mutation, the SRC-3 protein level is elevated, leading to increased AR transcriptional activity. Similarly, overexpression of SRC-2, another AR coactivator, has been reported in subset of PCa, mainly due to its gene amplification (NCOA2).

DNA Repair Pathways

Alteration of DNA repair pathway seems to be affected more in advanced and metastatic PCa, but a subset of primary PCa also shows similar disruption [47, 48]. This disruption has been reported ranging from 12% to 19% in different studies [14, 15]. BRCA2, BRCA1, ATM, FANCD2, and RAD51B are among the most common altered DNA repair genes in PCa. The observation in these studies is consistent with a 20% response rate to PARP inhibitors in a group of patients with metastatic castration-resistant prostate cancer (mCRPC) [15, 48]. The underlying mechanism for such alterations varies; homozygous deletions, loss-of-function mutations, and truncating or nonsense mutations have been all described.

Metastatic PCa

Using whole genome sequencing from bone or soft tissue samples, Robinson et al. have recently assessed the genetic alteration of mCRPC from a cohort of 150 samples in comparison to the corresponding primary [15]. Interestingly, they have observed a high frequency of AR pathway alterations, mainly AR amplification and mutation in mCRPC. This indicates that most of those tumors are still dependent on AR signaling for growth. In addition, the overall burden of copy number changes and mutations appeared to be higher in mCRPC compared to primary. Similar to AR, p53 mutation, PI3K, and DNA repair pathways have been enriched in metastatic samples. Nevertheless, despite many additional genetic alterations in advanced PCa, the primary and corresponding metastatic samples appeared to be similar in their subclassification [14].

Variants of PCa

Many histologic variants of prostatic adenocarcinoma have been described, but there are limited publications for their molecular characterization. This is because most of PCa variants often have unknown clinical significance; besides they are

mainly focal and synchronously associated with conventional type. Ductal carcinoma of the prostate is one of the most common variant that is distinct from prostatic acinar adenocarcinoma on both clinical and morphological aspects. They are commonly high-volume tumor with more aggressive clinical course and also frequently seen adjacent acinar type. It has been shown that ERG-positive and PTEN loss status are less frequent among ductal adenocarcinomas and their synchronous acinar tumors compared to matched pure acinar adenocarcinoma cases (10% versus 50%) [49]. Nevertheless, their ERG and PTEN status showed concordance in selected cases of synchronous ductal and acinar adenocarcinoma. This suggests that both variants are clonally related tumors. Sanati et al. have compared the gene expression profiles of 19 cases of ductal and acinar prostatic adenocarcinoma. They did not identify any differences at the global level, but using laser microdissection, they showed that prolactin receptor protein, among a few other genes, is overexpressed in ductal variant [50].

Neuroendocrine differentiated prostatic carcinoma (NEPC) is another distinct variant that becomes recognized more with progression of disease. This is mainly due to epigenetic changes following ADT or novel AR target therapies (e.g., abiraterone). Integration of published genomic profiles has identified recurrent MYC amplification, homozygous PTEN, and RB deletions in NEPC. Using a patient-derived xenograft, Akamatsu et al. found that the placental gene (PEG10) inhibitory mechanism became disrupted during the adaptive response to AR antagonists and subsequently overexpressed in clinical NEPC [51]. SMAD4 mutation has also been suggested as a driver of NEPC transdifferentiation in a single clinical case who developed NEPC upon ADT treatment [52].

Other histologic variants of PCa, such as foamy gland, are less frequent entities, and their clinical significance and molecular characteristic yet remain to be determined.

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B.D. Robinson et al. (eds.), *Precision Molecular Pathology of Prostate Cancer*,
Molecular Pathology Library 15, https://doi.org/10.1007/978-3-319-64096-9