URINARY SYSTEM TUMOR

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Philippe E. Spiess,
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Associate Editors: Yao Zhu,
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URINARY SYSTEM TUMOR
(FIRST EDITION)

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We are pleased to announce that the "AME Research Time Medical Book Series" co-launched by AME Publishing Company, Central South University Press and DXY.cn will be published as scheduled.

Finishing my medical degree after 4 years and 3 months of study, I decided to quit going on to become a doctor only after 3 months of training. After that, I had been muddling through days and nights until I started engaging in medical academic publishing. Even 10 years after graduation, I had not totally lost the affection for being a doctor. Occasionally, that subconscious feeling would inadvertently arise from the bottom of my heart.

In April 2011, Mr. Tiantian Li, the founder of DXY.cn, and I had a business trip to Philadelphia, where we visited the Mütter Museum. As part of The College of Physicians of Philadelphia, the museum was founded in 1858 and has now become an exhibition hall of various diseases, injuries, deformities, as well as ancient medical instruments and the development of biology. It displays more than 20,000 pieces of items including pictures of wounded bodies at sites of battle, remains of conjoined twins, skeletons of dwarfs, and colons with pathological changes. They even exhibited several exclusive collections such as a soap-like female body and the skull of a two-headed child. This museum is widely known as "BIRTHPLACE OF AMERICAN MEDICINE". Entering an auditorium, we were introduced by the narrator that the inauguration ceremony of the Perelman School of Medicine at the University of Pennsylvania would take place there every year. I asked Mr. Li, "If it was at this auditorium that you had the inauguration ceremony, would you give up being a doctor?" "No," he answered.

In May 2013, we attended a meeting of British Medical Journal (BMJ) and afterwards a gala dinner was held to present awards to a number of outstanding medical teams. The event was hosted annually by the Editor-in-Chief of BMJ and a famous BBC host. Surprisingly, during the award presentation, the speeches made by BMJ never mentioned any high impact papers the teams had published in whichever prestigious journals over the past years. Instead, they laid emphasis on the contributions they had made on improving medical services in certain fields, alleviating the suffering of patients, and reducing the medical expenses.

Many friends of mine wondered what AME means.

AME is an acronym of "Academic Made Easy, Excellent and Enthusiastic". On September 3, 2014, I posted three pictures to social media feeds and asked my friends to select their favourite version of the AME promotional leaflet. Unexpectedly we obtained a perfect translation of "AME" from Dr. Yaxing Shen, Department of Thoracic Surgery, Zhongshan Hospital, Shanghai, who wrote: enjoy a grander sight by devoting to academia (in Chinese, it was adapted from the verse of a famous Chinese poem).

AME is a young company with a pure dream. Whilst having a clear focus on research, we have been adhering to the core value "Patients come first". On April 24, 2014, we developed a public account on WeChat (a popular Chinese social media) and named it "Research Time". With a passion for clinical work, scientific research and the stories of science, "Research Time" disseminates cutting-edge breakthroughs in scientific research, provides moment-to-moment coverage of academic activities and shares rarely known behind-the-scene stories. With global vision, together we keep abreast of the advances in clinical research; together we meet and join our hands at the Research Time. We are committed to continue developing the AME platform to aid in the continual forward development and dissemination of medical science.

It is said that how one tastes wine indicates one's personality. We would say how one reads gives a better insight to it. The "AME Research Time Medical Books Series" brings together clinical work, scientific research and humanism. Like making a fine dinner, we hope to cook the most delicate cuisine with all the great tastes and aromas that everyone will enjoy.

Stephen Wang
Founder & CEO,
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As the study of molecular biology continues to develop, we find ourselves increasingly recognizing the biological characteristics and pathogenesis of urinary system tumors, and diagnosis and treatment concepts, methods, and techniques have seen great improvements over the years. Many scholars have come together to compile this book in order to introduce the latest development of urinary system tumors in a comprehensive and systematic manner.

This book mainly discusses three aspects: tumor biology, clinical diagnosis and treatment. It explores the latest developments in urinary system tumors to help clinicians cultivating a scientific and clinical thought process so that they can make accurate diagnoses, as well as formulate suitable treatment strategies. This book discusses topics such as genomics, epigenetics, signalling pathways, and talks about their roles in the development of urinary system tumors. It also covers the latest developments in diagnosis and treatments in this field.

The book includes multiple topics such as the regulating effects of androgen receptors and signal transduction on prostate cancer; the mechanisms of which microRNA, tumor stem cells, and DNA methylation play a role in prostate cancer. In terms of diagnosis, the book elaborates on the function of MRI and PET/CT in diagnosing prostate cancer. In terms of treatment, the book details the current development of chemoradiotherapy and proton beam therapy in prostate cancer, and the treatment of postoperative complications and post-treatment sexual dysfunction. The book also looks into the treatment methods for bladder cancer, kidney cancer, and testicular tumors.

We are very honored to work with AME Publishing Company, and by utilizing an innovative publishing method, we include the latest developments of basic medical sciences and clinical medicine in urinary system tumors and present it in a way that makes it stands out from other materials of similar topic. The book's multi-faceted approach enables clinicians to have a comprehensive and detailed understanding of urinary system tumors. We sincerely hope that readers can benefit from this book, and further their understanding of urinary system tumors, so as to ultimately improve patients' welfare.

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One of the pioneers of modern urologic oncology Doctor Willet F. Whitmore, Jr served as the chairman of the department of urology at the Memorial Sloan Kettering Cancer Center for over 30 years. In addition to his major clinical and surgical abilities and redefining patient care at the time through his scientific contributions, Dr Whitmore was known for his meaningful quotes and insightful reflections. One of his most memorable statements *"Is cure possible? Is cure necessary? Is cure possible only when it is necessary?"*

This embodies a significant dilemma and evolution in oncology from the Halstedian principle of wide tumor resection appreciating adjacent organ resection is often necessary and may curtail significant side effects and complications. Modern surgical oncology has swung the pendulum to a more widespread adoption of minimally invasive surgery often using robotic assisted technology to accomplish these similar goals of tumor eradication with negative surgical margins but doing so in an often less radical manner and with potentially less morbidity and a quicker recovery. Similarly, early medical oncology consisted of systemic agents known for their high potentially lethal toxicity in the glimpse that it may offer cure even if only in rare circumstances. The past decade has been marked by a revolution in medicine most notably in oncology with now a greater fundamental understanding of genetic mutations characterizing various tumor types in large part through the characterization of the Human Genome Atlas. We now can predict treatment response for a host of tumors such as prostate cancer by the specific genetic alterations depicted or the presence of a specific germ line mutation.

Our therapeutic armamentarium for advanced genitourinary malignancies has never been so extensive with targeted therapies, select hormonal ablative therapies, and immune modulatory therapies. These agents are not only improving cancer specific outcomes they are doing so with often an improved side effect profile and durable response that can often be for many years. These truly revolutionary systemic approaches are evolving so rapidly that national and international treatment guidelines are continually being updated offering improved treatment outcomes to patients who only a few years ago had little to no therapeutic options available to them in the setting of advanced disease or often refractory to first and second line agents.

Going back to this fundamental question asked by Dr Whitmore, with our continual struggle of seeking cure and only doing so only when it poses a life threatening risk to our patients, we now have diagnostic and predictive tools at our disposal through personalizing our approach to a given patient based on a clinical and genetic characterization of the patient and their tumor. This 1st edition of *Urinary System Tumor* that I am honored to serve as a Co-Editor for highlights these major advances made in genitourinary oncology, with most of the sections written by international thought leaders on these given topics. There is no question that urologists, oncologists, and healthcare professionals reading this reference book will gain the knowledge and skillset needed in providing the highest quality care to their patients while remaining at the forefront of therapeutic discoveries.

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The field of Urologic Oncology has witnessed tremendous changes. The horizon of scientific knowledge has widened with preventive efforts leading to the earliest detection of cancer and insight into the mechanisms of cancer leading to rational changes into the medical armamentarium. While urological cancer was diagnosed and treated mostly by urologists only this has been replaced by multidisciplinary teams with equal roles for all members. Oncologic Urology with emphasis on Urology has been replaced by Urologic Oncology with emphasis on Oncology. This fundamental change has revolutionized daily practice for uro-oncology patients. Centralization of care is ongoing at a tremendous speed enabling all patients to benefit from standard of care treatment. Surgery as the mainstay of cancer treatment is gradually moving backwards. Organ sparing therapy and image guided therapy are replacing the traditional ablative surgery. On the other hand surgery is considered essential in patients in whom it was thought to be irrelevant. Primary tumor surgery in metastatic patients is a new outlook on advanced cancer treatment.

With immunotherapy swiftly marching to the forefront of cancer treatment, surgery will be used as another treatment modality removing metastases at a previously unheard scale.

A testimony of these changes is to be found in the 1st edition of *Urinary System Tumor*, a compilation of multidisciplinary endeavours in the field of urologic oncology.

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In the New York Times of June 7th, 1924, Joseph Colt Bloodgood, a prominent surgeon practicing in Johns Hopkins, claimed that "*deaths from cancer would be practically eliminated and cures accomplished if persons afflicted sought medical aid immediately upon the discovery of a foreign growth in any part of the body*". Almost a century later, we are still debating the role of early detection and its consequences for patients with urinary system tumors. A pertinent question today is what the best method is to discover these "foreign growths"? The development of novel imaging techniques, and the continuing quest for the development of accurate biomarkers may be an answer to that question, whilst on the other hand the question arises whether or not we want to find these "foreign growths" in early stages in all patients.

There is no question that today we practice medicine in an exciting era where based on well-developed guidelines such as the NCCN and EAU guidelines, we are achieving increasing cure rates for most tumors of the urinary system. And we are doing so in an increasingly minimally invasive fashion. The advent of robotic surgical techniques, early recovery after surgery implementation and organ sparing surgery whenever possible have greatly decreased surgical morbidity in our field. The advent of novel radiotherapy techniques greatly reduces adverse effects of radiation to healthy tissue and in medical oncology, targeted therapies and immunomodulatory agents have drastically changes outcomes and treatment burden for patients with a variety of urinary system tumors.

In spite of this positive evolution, we are still not able to accurately identify the correct treatment for the right patient in many instances. Endeavours such as the international cancer genome atlas, high throughput drug screening by development of xenograft models and micro-arrays and the development of novel biomarkers are tackling this question in the ultimate quest for precision medicine.

The result of our increasing understanding of this devastating disease is that more than ever, patients have higher expectations of their quality of life after surviving cancer. To that end, it is of extreme importance that patients are guided and counseled when they face the consequences of their therapy. One particular example is sexual dysfunction as a result of the treatment of a variety of pelvic and genital tumors. Adequate treatment, exploring alternative expressions of sexuality and realistic expectations are key here. In the 1st edition of *Urinary System Tumor*, a broad overview is given of the full spectrum of urinary oncological care ranging from tumor biology to post-treatment quality of life. The contributions of various internationally renowned experts make it a must have as educational resource for professionals dealing with tumors of the urinary system.

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Urologic cancers comprise a significant proportion of newly diagnosed malignancies in the world, with prostate, bladder and kidney cancers being among the top ten diagnosed in the world. Over the past decade, we have seen a dramatic proliferation of knowledge in understanding mechanisms of cancers, fine tuning of surgical management of disease and development of myriad new systemic therapies. We have come to understand some cancers don't need to be aggressively treated, but rather carefully monitored leading to significant preservation of quality of life. These advancements all come from rigorous basic science and clinical research that collectively propels our field forward promising patients better treatments with fewer side effects. However, we still have a long way to go to make cancer a disease of the past. Death rates for many of urologic malignancies have decreased minimally or remain unchanged and further research and treatment algorithms are needed to battle insidious cancers.

We now have a considerable understanding of the biology of urologic cancers including the genetic, epigenetic, regulation and signaling pathways that are involved in cancer progression. Prostate cancer is the model for the tremendous collaborative research that has led us to our current state of knowledge. This book elegantly summarizes the recent progress made in the understanding of prostate cancer, delving into the mechanisms dictating the androgen receptor biology and genomic regulation, including a chapter devoted to the significance of micro RNA in detecting prostate cancer progression.

We are fortunate to be in a field where we have so much to gain from research in other arenas that help us with diagnosis and management of patients with urologic malignancies. The recent explosion of immune oncology as a means for treating systemic malignancies will help us hone in on its utility for non-muscle invasive bladder cancers and other localized diseases. Advances in diagnostic imaging will help us better detect and manage prostate cancer, particularly patients who are on active surveillance. The book has several chapter devoted to these topics. Surgery remains a vital tool in the management of many cancers and will continue to do so in the foreseeable future. This body of work by internationally renowned authors includes section on surgical treatment of bladder and kidney cancers including the role of cytoreductive surgery in the era of targeted molecular therapy as well as sequence of treatment in locally advanced and metastatic renal cell carcinoma.

No discussion of cancer treatment is complete without inclusion of radiation therapy. There is ample discussion of different modalities of radiation therapy for prostate cancer. In this era it is critical that we not only think about treatment of cancer, but also consider the burden of treatment on the individual patients. Discussions of sexual health and fertility preservation are particularly germane in this regard. This book offers a comprehensive reference for urologist, oncologists and researchers seeking to gain the most up to date information about urologic cancers and provide state of the art care for patients.

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The 20th century saw an unprecedent growth in human medical knowledge. In the 21st century we are witnessing a veritable explosion in the dissemination of such knowledge. Managing this deluge of information and being able to apply it thoughtfully will be the challenge of the next generation.

Urologic oncology has emerged as a significant specialty in medicine as urologic cancers encompass upto 25% of all cancers in the US and two (prostate, bladder) of the top ten cancers worldwide are urologic. Almost all of the urologic cancers are hard to diagnose by clinical exam alone and are dependent on imaging and cellular diagnostics for early identification. Hence urologic oncology is inherently a multi-disciplinary specialty. Advances in laboratory medicine, genomics, radiology, systemic therapy and radiation oncology have significantly impacted our field. Surgical urologic oncology has also observed a dramatic swing from being maximally invasive to minimally invasive to non-invasive. In the case of some cancers such as prostate and small renal masses, we are even choosing not to make the effort to diagnose every case. This evolution in our thinking has occurred with unprecedented rapidity. We are rapidly approaching a point where cancer may be converted to a chronic illness rather than an imminent lethal threat. In fact it has already begun to occur in some urologic cancers.

Synthesizing the massive amount of accumulating data and distilling it into a form that is easily digestible to be applied to patient care is a challenging task. Super computing with machines such as IBM Watson and artificial intelligence are certainly of great aid. However, machine learning is still incapable of interpreting all the nuances of disease manifestation and combining it with natural human intelligence. To that end, books like *Urinary System Tumor* offer a comprehensive compendium of knowledge and collective wisdom gleaned from some of the best experts in the field. They help us appreciate the breadth and scope of information available to the practicing clinician and can then be easily applied into practice. My co-editors and I are thankful to the efforts of these renowned experts who have contributed selflessly to this effort. We are confident that you will benefit enormously from this comprehensive and practical text.

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Table of Contents

Diagnostic Methods of Urinary System Tumor

Treatment of Urinary System Tumor

Surgical Treatment of Urinary System Tumor

Chemotherapy of Urinary System Tumor

Androgen receptor gene mutation, rearrangement, polymorphism

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Abstract: Genetic aberrations of the androgen receptor (AR) caused by mutations, rearrangements, and polymorphisms result in a mutant receptor that has varied functions compared to wild type AR. To date, over 1,000 mutations have been reported in the AR with most of these being associated with androgen insensitivity syndrome (AIS). While mutations of AR associated with prostate cancer occur less often in early stage localized disease, mutations in castration-resistant prostate cancer (CRPC) patients treated with anti-androgens occur more frequently with 10-30% of these patients having some form of mutation in the AR. Resistance to anti-androgen therapy usually results from gain-of-function mutations in the LBD such as is seen with bicalutamide and more recently with enzalutamide (MDV3100). Thus, it is crucial to investigate these new AR mutations arising from drug resistance to anti-androgens and other small molecule pharmacological agents.

Keywords: Androgen receptor (AR); mutations; rearrangements; polymorphisms; androgen insensitivity syndrome (AIS); castration-resistant prostate cancer (CRPC)

Androgen receptor (AR)

The human AR is an important steroid hormone receptor that plays a critical role in male sexual differentiation, development and maintenance of secondary male characteristics and the ignition and maintenance of spermatogenesis (1). AR, as a ligand-activated transcription factor, is activated by binding either of the androgenic hormones testosterone or dihydrotestosterone (2). In humans, AR is a 110 kD protein composed of 919 amino acids that is encoded by the AR gene located on the X chromosome at Xq11-12 (3). The AR contains four domains: (I) the amino terminal activation domain (NTD); (II) the DNA-binding domain (DBD); (III) the hinge region (HR) and (IV), the carboxyl ligand-binding domain (LBD) (4). AR exon 1 encodes the entire N-terminal domain (NTD) (a.a. 1-556) which comprises the bulk of the AR and is the least conserved of the four domains. This variability allows AR to differentially recruit co-regulators conferring androgen specific transactivation. The first 30 amino acids

of the NTD are essential for the amino-carboxyl terminal (N/C) interaction that is required for the appropriate activation of AR (5-10). The NTD region contains the activation function 1 (AF1) element through which AR transactivational activity is predominantly mediated. This distinguishes AR from the other steroid receptors that primarily utilize the AF2 region in the LBD (11). The NTD also contains polyglutamine and polyglycine repeats which are polymorphic. Polyglutamine repeat length has been correlated with PCa risk (11,12) and mutations in polyglutamine repeats have been shown to affect N/C interaction (13).

Exons 2 and 3 encode the two zinc fingers in the AR DNA-binding domain (DBD) which is responsive for binding to the androgen responsive element (ARE) sites (14). The DBD (a.a. 556-624) contains two zinc finger motifs and forms part of the hinge region (15). In addition to mediating binding to AREs, the second zinc finger stabilizes DNA bound AR and facilitates AR dimerization. Adjacent to the DBD is the hinge region (a.a. 625-668). A bipartite

nuclear localization signal (NL1) is present in the DBD and HR (16,17). In addition, the HR contains sites that are important in the phosphorylation, acetylation and degradation of AR. Exons 4-8 encode the short flexible hinge region and LBD which is capable of binding to the ligands and contains activation domain 2 (AF-2) that mediates N/C interaction and has also been shown to interact with a number of co-regulators (18). The LBD (a.a. 669-919) binds to ligands via its ligand binding pocket that is comprised of 12 alpha helices (19,20). In addition, the LBD contains a second nuclear localization signal (NL2) upon androgen binding and a nuclear export signal (NES), in the absence of androgens (17,21).

Role of AR in prostate cancer

Both normal prostate and prostate cancer (PCa) depend on the presence of androgens for growth, and prostate development is dependent on a functional AR. Testosterone, the primary circulating androgen in men is mainly produced by the Leydig cells of the testis (22). In the prostate, testosterone is converted into 5α-dihydrotestosterone (DHT) by the enzyme 5α-reductase so it can bind to the AR and induce growth of the male urogenital structures. AR is the principle mediator of androgen action in the prostate. AR activation by DHT is critical for complete prostate development as men lacking a functional 5α-reductase gene have only a small partial prostate or the prostate is completely undetectable (23).

PCa is also initially dependent on the actions of androgens and functional AR expression and tumors will regress temporarily with castration. AR is expressed in both androgen-dependent (AD) and -independent (AI) PCa and is sustained throughout progression of the disease to hormone refractory PCa (24,25). PCa therapy is focused upon blocking androgen activity and androgen abalation therapy causes atrophy of the prostate epithelium. Treatment of PCa first involves androgen deprivation therapy (ADT) through blocking production of androgens by castration and/or by using anti-androgens such as bicalutamide or enzalutamide (MDV3100). When androgen ablation therapies fail, advanced PCa ultimately progresses to an AI late stage that is refractory to current therapies, also known as castrate resistant prostate cancer (CRPC), and this recurrence results from a reactivation of AR activity.

Decreasing levels of AR protein expression reduces both primary localized PCa and CRPC growth. While ADT is initially successful in most patients (~80%) resulting in tumor regression and AR suppression, these therapies eventually fail and the cancer progresses to a stage where it is unresponsive to blockage of androgens and growth becomes androgen independent. Hormone suppression appears to induce an eventual overexpression or amplification of the AR in CRPC (26). Several mechanisms have been proposed to play a role in this reactivation of AR following ADT including: deregulation (causing over-expression of AR), mutation of AR (gain of function), alternative splicing (causing AR to be constitutively active), co-activator gain of function or loss of co-repressor function, and intracrine androgen synthesis [reviewed in (24)]. Restored AR activity in turn induces an increase in prostate specific antigen (PSA) levels signifying the development of CRPC. Therefore, AR signaling pathways must play critical roles in both AD and CRPC. Unlike AD signaling that depends on actions of androgens to bind AR and activate it, androgen independent pathways do not require androgens, but can be activated by growth factors acting through kinase pathways such as the mitogen-activated protein kinase (MAPK) pathway or the phosphatidylinositol 3-kinase (PI3K) pathway, which phosphorylate and activate AR in the absence of androgens (27). Thus determining how AR drives prostate tumor growth in the absence of androgens is critical for the development of effective therapy for CRPC.

Overview of mutations of the AR

Mutations in the AR can result in defective AR function. Defective AR, including loss-of-function AR alterations and gain-of-function AR alterations, are associated with androgen insensitivity syndrome (AIS), spinal and bulbar muscular atrophy and PCa (28). In the AR gene, four different types of mutations have been detected to generate defective AR: (I) single point mutations resulting in amino acid substitutions or premature stop codons; (II) nucleotide insertions or deletions most often leading to a frame shift and premature rumination; (III) complete or partial gene deletions; and (IV) intronic mutations causing alternative splicing (28,29).

Over 800 different AR mutations have been identified in patients with AIS according to the report from the Androgen Receptor Gene Mutations Database (29). AIS is a recessive genetic disorder of sex development characterized by androgen unresponsiveness which can impair the masculinization of male genitalia in the developing fetus (30,31). AIS consists of three classes based on phenotype:

complete androgen insensitivity syndrome (CAIS), partial androgen insensitivity syndrome (PAIS), and mild androgen insensitivity syndrome (MAIS) (31). The most common mutations of the AR gene in AIS are single point mutations that result in an amino acid substitution. However, insertions/deletions resulting in a reading frameshift, a complete/partial gene deletion, and mRNA alternative splicing have also been identified in patients with AIS syndrome (29,32). Loss-of-function mutations distribute unequally along the length of the exonic regions of the AR. Although exon 1 encodes more than half of the AR protein, the total of exon 1 mutations only represents 25% of all of the mutations in AIS patients (29). More than 70% (89 out of 124) of AIS mutations in exon 1 appear to cause CAIS, and about 18% (22 out of 124) of exon 1 mutations are related with MAIS which is due to single-base substitution (29). In the DBD region, 74 different mutations have been published of which most are single-base substitutions (29). The most common molecular defects of the AR gene associated with AIS are clustered primarily in the LBD region, and the single-base substitution mutation is predominant (29). Interestingly, in 33.3% (25 out of 75) patients with CAIS and 58.7% (37 out of 63) patients with PAIS, no AR mutations have been identified, which challenges the classical assumptions that the AIS phenotype is directly the result of a mutation in the AR (29).

Although AR mutations occur very rarely in the early stages of PCa, approximately 10-30% of patients with CRPC carry AR mutations, especially in PCa patients treated with ADT (33,34). To date, over 150 AR mutations have been identified in PCa tissue, and most of them consist of single-base substitutions due to somatic rather than germline mutations (29). About 45% of the mutations identified in PCa patients occur in the LBD, while 30% occur in exon 1 (29). These gain-of-function mutations allow prostatic epithelial cells to grow in an androgen-refractory manner, suggesting that these mutations in the AR may allow it to bind and be activated by ligands that are normally present in the body (e.g., adrenal androgens) but that do not normally cause substantial activation of the AR (35,36). The presence of multiple mutations within tissues from single PCa patients has been found, which leads to the hypothesis that cancers are based on tissues accumulating mutations in a number of genes in advanced PCa. Interestingly, AR mutations have been identified in PCa patients that are associated with both a loss-of-function and a gain-of-function (i.e., p.R753Q) (29), which further complicates the relationship between genotype and phenotype and suggests the important role of post-translational events in protein generation and carcinogenesis. New mutations in the AR can also lead to resistance to current forms of treatment. For example, mutation in the LBD of AR can lead to resistance of MDV3100 causing AR to become active even in the presence of anti-androgens (37). Thus, it is crucial to investigate these new AR mutations arising from drug resistance to anti-androgens and other small molecule pharmacological agents.

Mutations in the NTD of the AR

The NTD is located in the first exon of the AR and spans amino acids 1-558. It is known to contain the transcriptional regulatory regions or transcription activation units, the ligand-dependent TAU-1 (amino acids 101-370) and the ligand-independent constitutively active TAU-5 (amino acids 360-528) which together are also referred to as AF-1 (8). Mutations in the NTD of the AR account for nearly a third of all mutations in the receptor. In the AR database the NTD is listed as having 42 single-base substitutions in PCa (29). Most of these mutations are thought to occur following androgen ablation therapy (38,39).

Two mutations at amino acids 142 and 221 of the AR which are located in the TAU-1 region of the NTD were discovered from patients who had CRPC (40). The 142 mutation was characterized as a substitution of glycine to valine (G142V), while the 221 mutation was characterized as a substitution of aspartic acid to histidine (D221H). Both of these mutations had an increased response to DHT. In addition, these two mutations had a shorter than normal CAG repeat length than the median of this polymorphic variation in the Chinese population (40,41). Since a reduction of the CAG repeat is associated with increased risk of PCa, it is possible that in addition to the mutations in these two patients, the shortening of the CAG repeat might have also contributed to tumor development and PCa progression.

Another mutation discovered in a primary PCa biopsy is known to affect the N/C interaction of the AR. The L179R somatic substitution mutation results in a receptor that is highly activated and more potent than wild type AR (42,43). Callewaert *et al.* (43) also discovered that the L179R mutation disrupts the N/C interaction and indirectly alters NTD interaction with the coactivator SRC-1. Further characterization of this mutation revealed that this residue is important for TAU-1 function. The 179 lysine is part of the LKDIL motif that is involved in hydrophobicity and the

helical structure of TAU-1. Two alpha helices that surround the lysine at position 179 make up the core of TAU-1, which can act independently of p160 coactivators as an autonomous activation function. These two alpha helices are necessary for full activity of wild type AR and thus the L179R mutation may increase this activity acting as a gain of function mutation (43).

The genetically engineered TRAMP (transgenic adenocarcinoma of the mouse prostate) mouse model of prostate cancer (44) has revealed that hormone ablation selects for AR mutations and these mutations can cause the development of aggressive and metastatic disease (38,45). Most of the mutations (seven out of nine, 78%) identified in this study were localized to the NTD, while the other two were found in the LBD. Two mutations were identified in the most highly conserved region of the NTD that is critical for its structure and recruitment of transcription factors. A229T and E231G exhibit increased basal activity independent of ligand and E231G has a higher responsiveness to coactivators ARA160 and ARA70. Additionally, these two mutations significantly reduced the interaction of AR with the Hsp70-interacting protein (CHIP) which functions as a negative regulator of AR transcriptional activity (38,45). These findings suggest that this evolutionarily conserved AR NTD signature motif plays a role in modulating AR action and support a direct causal relationship between AR-E321G expression and malignant prostate cancer.

More recently, Steinkamp et al. (39) found 26 recurring missense mutations, (mostly in the NTD) that occurred in multiple PCa tumors that had been treated with anti-androgens. Fourteen of the 19 mutations in the NTD localized to four regions: the polyQ tract, the COOH-terminus of Hsp70 interacting Protein (CHIP) interaction domain, the WxxLF motif, and the end of AF5 which is involved in interactions with coactivators (39). Two novel mutations, E255K and W435L were characterized further. E255K (which was found to be mutated in a domain of the AR that interacts with E3 ubiquitin ligase) increased protein stability and nuclear localization in the absence of ligands, while W435L (located within the WxxLF motif) enhanced N-C interaction 50% greater than wild-type AR indicating once again that treatment with anti-androgens selects for gain-of-function AR mutations (39).

Mutations in the DBD and hinge region of the AR

The DBD and hinge region (H) of the AR (located between the NTD and LBD from amino acids 559-670) contain fewer reported mutations associated with PCa than the NTD and LBD regions. As of 2012, there were 10 reported single-base substitutions in the DBDH (7 in the DBD and 3 in the hinge region) (29).

One double mutation in the DBD and LBD of AR has been thoroughly studied for functional relevance (46). Substitutions of two threonines for two alanines (T->A) were found on the same AR transcripts of a mutant AR isolated from CRPC samples. The 575 threonine in the first zinc finger of the DBD of AR and the 877 threonine in the LBD of AR were both replaced by alanine. The authors describe this double mutant as being both "promiscuous" and "unfaithful" as the T877A mutation allows activation by abnormal ligands and the T575A mutation modifies AR transcriptional activity by strengthening AR binding to AR nonspecific promoters (46). Compared with wild-type AR, T575A AR binds preferentially to nonspecific palindromic androgen response elements (AREs) suggesting that the T575A mutation could enrich the binding of AR to non-canonical AR binding elements.

Another mutation in the DBD of AR is a rare inactivating mutation reported in the receptor. C619Y is a somatic substitution (tyrosine for a cysteine at amino acid 619) mutation causing loss of transcriptional activity of AR that was identified in a Caucasian man with stage D1 metastatic PCa (47). This mutation was shown to be transcriptionally inactive and unable to bind DNA in androgen-responsive reporter assays. Treatment of ligand causes C619Y AR to localize abnormally in nuclear aggregates located in both the nucleus and cytoplasm. Additionally, C619Y colocalizes with the coactivator SRC-1 in these aggregates thus demonstrating that interactions between steroid receptors and coactivators may occur in the absence of DNA binding and transcriptional activity (47). While the significance of all known mutations in the DBD has not been fully elucidated, mutations in this region of the AR likely interfere with DNA binding, nuclear export, and coregulator recruitment which would affect AR transactivation (48,49).

Interestingly, a study by Hu et al. (50) identified a novel germline mutation in the DBD of the AR in African American males with familial PCa. This study used genomic DNA from 30 high-risk African American and Caucasian families and identified a germline AR (T559S) substitution in the DBD in three members of an African American family who had a history of early-onset familial PCa (50). This mutation may contribute to disease progression by altering AR-DNA binding affinity and affect AR signaling

in response to androgens or anti-androgen therapy. Since African American men have a higher incidence (70% higher than Caucasian men) and mortality rate (double that of Caucasian men) of PCa than Caucasians and other ethnic groups (51,52), further investigation into the relevance of such mutations in the AR may provide useful information needed to treat these high risk individuals in a personalized manner.

Mutations in the LBD of the AR

To date, of the more than 150 mutations identified in PCa tissue, most occur in the LBD, and a substantial minority occurs in exon 1 (29). The majority of AR mutations identified in the LBD in clinical PCa cluster to four discrete regions: (I) amino acids 670-678, which is located at the boundary of the HR and the LBD; (II) amino acids 701-730, a region which covers the helix 3 which contributes to form the ligand-binding pocket surface, and also the "signature sequence", the highly conserved loop between helices 3 and 4 of nuclear receptors, directly involved in coactivator recruitment; (III) amino acids 741-763, a part of the ligand-binding pocket; (IV) amino acids 872-910, a region which spans both helix 11 and the core domain of AF-2 (53).

Somatic missense mutations in the LBD usually result in decreased specificity of AR to other hormones such as progesterone, estrogens and adrenal steroids, and affect both ligand affinity and coregulator recruitment (54). Importantly, many mutated AR can be activated by anti-androgens, which may be partly responsible for progression to CRPC. Several independent studies revealed that mutations in the LBD affect the ligand pocket and modify the conformational structure of the receptor, which in turn reduces ligand discrimination but does not affect the agonist-induced coactivator recruitment (55-58). Due to the different location and the nature of the substitution, the mutations in the LBD will alter the ligand-induced conformational change of AR, which results in altered ligand binding affinity, N/C-terminal interactions, as well as interactions with coactivators and chaperones (53). However, not all mutations in the LBD reduce ligand specificity by altering the dimensions of the pocket. For example, the H874Y mutant AR is also activated by hydroxyflutamide, oestradiol, and progesterone besides androgens, but the side chain of the residue points away from the pocket and is buried in a cavity between helices 11 and 12, which is formed by ligand induced activation (57).

Different agonists activating the LBD mutant AR may

result in different coactivators binding and regulating different subsets of genes (59). Brooke *et al.* (60) studied the preference of several of the most commonly identified LBD mutants (H874Y, T877A and T877S) for the motifs of LxxLL and phenylalanine-rich motifs like FxxLY and found striking differences in motif utilization dependent upon which ligand was activating the receptor. In the presence of cyproterone acetate, LBD mutants interact with the LxxLL motif while in the presence of hydroxyflutamide the receptors interact with the FxxLY motif. Moreover, the authors demonstrated the mutant AR induced different "patterns" of regulation of a subset of androgen-regulated genes. In the LNCaP cell line which endogenously expresses the well-known T877A mutant AR, the expression of the prostate differentiation factor *KLK2* and cell cycle regulator *CDK2* were induced most strongly by androgen, then hydroxyflutaminde and then cyproterone acetate, while another cell cycle progression associated gene *CDK4* was not regulated by androgen, but by the two anti-androgens. As for the differentiation associated gene *DRG-1*, its expression was highly up-regulated by androgen, and only weakly by hydroxyflutamide (60).

The T877A mutation was the first identified AR mutation in prostate cancer and was initially described in the LNCaP human PCa cell line early in 1990 and was frequently found in flutamide-treated PCa patients (~31%) (61,62). This mutant not only responds to androgens but also to oestrogens, progestins and even the anti-androgens cyproterone acetate and hydroxyflutamide (63). The threonine 877 residue in AR LBD comprises a large portion of the ligand-binding pocket surface, and forms hydrogen bonds with 17β-hydroxyl group of androgen, and the substitutions to the smaller alanine affects the size and conformation of the receptor such that the other ligands can fit into the pocket and activate the receptor (21,38). The AR T877A may drive tumor growth through aberrant activation by the anti-androgen used for treatment.

The L701H mutation, a second AR mutational hot spot, was first identified in a hormone-refractory PCa patient (64). This mutation, in combination with T877A, is also identified in the PCa cell line MDA-PCa 2a, which was established from a bone metastasis of a castrated PCa patient (65,66). Both Leu701 and Thr877 residues are part of the ligand-binding pocket and interact with bound ligand. L701H and the double mutant L701H/T877A are highly responsive to circulating steroids such as glucocorticoids, cortisol and cortisone (67,68). However, the L701H mutation was not well characterized like the T877A until

most recently. Van de Wijngaart *et al.* (69) found that the presence of a hydroxyl group at position 17α is critical for activation of AR L701H. Modeling of the various mutations in the AR LBD structure (such as L701H, L701M, and L701Q) revealed that a unique H-bonding network involving His701 or Gln701, the steroidal 17α-OH group, and the backbone oxygen of Ser778 plays an important role in the cortisol response. Interestingly, the L701H mutants hardly respond to anti-androgens (69). These findings suggest that the L701H mutation does not drive prostate tumor growth upon binding of an anti-androgen used for treatment, which indicates in these cases, tumor growth is dependent on endogenously circulating ligands such as cortisol, a different mechanism of tumor growth observed in T877A mutants.

MDV3100 (enzalutamide) is a novel anti-androgen that was recently approved by the FDA for the treatment of CRPC (70-72). Although MDV3100 has shown significant efficacy in clinical trials, many patients who initially responded favorably develop resistance to this drug, however, the mechanisms driving resistance remain largely unknown. A most recent study by Korpal *et al.* (37) demonstrated that a mutation in the AR LBD, F876L, spontaneously emerges in the majority of MDV3100 resistant clones of LNCaP cell line, which strongly suggests that the emergence of AR F876L mutants may represent a dominant tumor-autonomous mechanism of resistance to MDV3100. Additional studies revealed that the benzamide motif of MDV3100 can extend into the access channel created by the smaller leucine residue, which could potentially prevent the compound from clashing with helix-12 of the AR LBD in the agonistic mode (37). Thus, the F876L mutation may abolish the antagonistic activity of MDV3100 and could potentially allow agonist activity. However, since the clinical relevance has not been identified, further research needs to be done to determine the effect(s) of this mutation. Additionally, since this study was performed in LNCaP cells, it remains to be elucidated whether or not this type of AR mutation is sufficient to convey drug resistance in patients.

AR rearrangements

The recent identification of constitutively active forms of AR, known as AR variants (ARVs), has revealed another important mechanism underlying persistent AR signaling in CRPC (73,74). More than a dozen ARVs containing variable structures have been isolated, but each lacks all or a portion of the ligand binding domain (LBD) (32). The lacking of LBD allows ARVs to be constitutively active in driving AR regulated gene expression and promoting tumor progression even without the presence of androgens (75,76), and leads to their resistance to the current LBD-targeting AR antagonists or other agents that repress androgen biosynthesis. Expression of ARVs is increased in CRPC compared to hormone-naïve metastases and associated with PCa progression and resistance to AR-targeted therapy (75-77).

Recently it was discovered that 22Rv1 cells as well as prostate tissue of some patients with CRPC contained rearrangements of the AR (78). 22Rv1 cells were shown to have increased copy number of AR exons 2b, 3, and CE3 compared with the androgen dependent CWR22Pc cell line which suggested rearrangement of this genomic segment. Further characterization of 22Rv1 cells determined that they contain truncated AR isoforms associated with an intragenic rearrangement of a 35-kb AR genomic segment which contains a cluster of alternative AR exons. Analysis of Genome-Wide Human SNP Array 6.0 (SNP6.0) data from primary PCa patients and metastatic CRPC patients (79,80) revealed that only patients with CRPC had high incidences of rearrangements associated with AR amplification. Additionally, increases in focal copy number between AR exons 2/3 and 3/4 were also observed in patients with CRPC but not in patients with primary androgen-dependent PCa (6/14 in CRPC patients *vs.* 0/44 primary PCa patients). Although the generation of ARVs is due to the aberrant AR splicing or gene rearrangements of the AR gene, it still remains unknown how such aberrant splicing is regulated.

AR polymorphisms

The human AR gene contains two polymorphic $(CAG)_n$ (polyGln/polyQ) and $(GGC)_n$ (polyGly/polyG) repeat sequences with different number in exon 1. The number of polyQ and polyG repeats is 21.6±3.3 (range, 9-31) and 17.4±1.4 (range, 8-21) respectively, in normal men (81). Abnormal length of the polyQ tract has been found to be associated with the pathology in Kennedy's disease (Spinal and bulbar muscular atrophy, SBMA) where the polyQ tract is expanded and varies between 38 and 75 repeat units (82,83). SBMA, as one of the classic trinucleotide repeat expansion diseases, results from a combination of a gain-of-function mechanism in motor neurons and a loss-of-function mechanism in androgen target cells, causing partial loss of AR function in androgen target tissues (28).

Abnormal polyQ lengths have also been associated with race. The average polyQ repeat number differs significantly among African-American (mean: 20.1), Caucasian (22.0), and Asian-American (22.4) populations in the USA (84).

PolyQ tract length has been reported to affect AR activity. Shorter polyQ tract can enhance the critical intramolecular N-C terminal interaction of AR, allowing response to lower androgen concentrations associated with higher levels of specific p160 coactivators (13,85). The relationships between AR polyQ tract and risk of getting certain diseases (including cancers, male infertility, bone and mineral density, Alzheimer's disease, hypertension, muscle and adipose tissue change and personality traits) have been investigate by several different groups (86-90). However the association between polyQ tract length with PCa remains controversial. AR with shorter polyQ tract is associated with increased PCa risk and has been found in the high-risk African-American population (91) whose average polyQ numbers are less than Caucasian's and Asian-American (84). Furthermore, somatic mosaicism of the AR polyQ tract has been found in PCa tumors, which may subsequently lead to the development of PCa (92,93). However, controversial results have been reported by recent publications with larger sample sizes (94,95). In those studies, no association of AR polyQ tract length with PCa risk was found, and the knowledge of AR polyQ tract length provides no clinically useful information to predict PCa risk. Despite failure as a predictor of PCa risk, polyQ tract has been reported to affect progression or treatment response of PCa. Both estradiol and testosterone levels were significantly elevated in men with greater polyQ tract length (94,96).

In order to use experimental tools to test the role of AR polyQ tract length on PCa, Dr. Robins' group developed knock-in mouse strains with human AR alleles containing 12, 21 or 48 CAG repeats (referred to as AR12Q, AR21Q, and AR48Q) (97). All three mouse lines were grossly normal in growth, behavior, fertility, and reproductive tract morphology, with no neurological problems evident in AR48Q, although transactivational differences due to polyQ tract length were detected in expression of AR downstream targets (97). Also, the hAR Q-tract polymorphism mediates *in vivo* tissue androgen sensitivity by impacting negative hypothalamic feedback and trophic androgen effects on target organs (98).

To further investigate the effect of polyQ tract length in oncogenesis, the three mouse strains (AR12Q, AR21Q, and AR48Q) were crossed with a transgenic model of prostate adenocarcinoma (TRAMP) (97). Striking

genotype-dependent differences in PCa initiation and progression were revealed due to the different length of polyQ. Although cancer in the mice with an average human polyQ tract length AR progressed similarly as in wild-type mice, the short polyQ tract AR resulted in significantly earlier tumor development, whereas the long polyQ tract appeared to be protective (97,99). Taken together, those mouse models demonstrate that a functional difference in AR activity within the normal range of polymorphic variation could affect PCa biology. The association between PCa and AR polymorphisms remains unclear, and further investigations with suitable models will provide us more information in the future.

Summary/future perspectives

Currently there are over 1,000 known mutations in the AR and 159 have been reported in PCa tissues (29). While the number of mutations reported continues to rise, the relevance of these mutations in CRPC remains unclear. Mutations in patients with CRPC treated with anti-androgens, such as MDV3100 may provide vital information as to why therapies targeting the LBD of AR eventually relapse or fail. Further investigation is needed to determine the function of mutations derived from prolonged treatment with anti-androgens.

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Footnote

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Androgen receptor epigenetics

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Abstract: The androgen receptor (AR) is a transcription factor that drives the differentiation of prostate epithelium by regulating the expression of several hundred genes. Conversely, AR also plays a central role in prostate cancer (PCa) development, and it continues to be active in tumors that relapse after castration (castration-resistant prostate cancer, CRPC). The transactivation function of AR has been extensively studied, and AR can also function as a transcriptional repressor on a distinct set of genes, but the identity of the AR regulated genes that are critical for PCa remain unclear. Moreover, the extent to which AR acquires new functions during PCa development and progression remains to be determined. Recent studies have highlighted the central role of chromatin structure and histone posttranslational modifications in determining the spectrum of genes regulated by AR and all other transcription factors. While the role of DNA methylation in the epigenetic regulation of gene expression is well established, it is now appreciated that chromatin structure plays a central and dynamic role in the epigenetic regulation of gene expression. The focus of this review is on AR interactions with chromatin and how they regulate AR function in PCa development and progression.

Keywords: Androgen receptor (AR); prostate cancer (PCa); transcription; epigenetics; histone methylation

Introduction

The androgen receptor (AR) is a steroid receptor member of the larger nuclear receptor family. It is comprised of a large N-terminal domain (NTD) that can strongly stimulate transcription, a C-terminal ligand binding domain (LBD) that has a weaker transactivation function, a central DNA binding domain (DBD), and a short hinge region between the DBD and LBD that mediates functions including nuclear translocation. In the absence of androgen, the AR associates with an HSP90 chaperone complex in the cytoplasm. Similarly to other steroid hormone receptors, in response to androgen binding the AR LBD undergoes a conformational change that repositions helix 12 to generate a binding site for coactivator proteins that contain LXXLL-motifs (although this coactivator binding site in the AR LBD binds initially to an LXXLL-like peptide in the AR NTD). The liganded AR then forms a homodimer in the nucleus and binds to regulatory regions of multiple genes

that are critical for prostate differentiation and for its normal function. Significantly, the consistent expression of AR in prostate cancer (PCa), and its continued activity in PCas that relapse after androgen deprivation therapy (castration-resistant prostate cancer, CRPC), indicate that at least a subset of these genes are also critical for PCa development and progression. However, the identity of the AR regulated genes that are critical for PCa remain unclear, and the extent to which AR acquires new functions during PCa development and progression remains to be determined.

AR binding to DNA is mediated by its DNA binding domain through recognition of a particular DNA sequence, referred to as an androgen responsive element (ARE). The AR then recruits multiple additional proteins that can modify chromatin structure and ultimately recruit and activate RNA polymerase II (*Figure 1*). Although this simple model is essentially correct, studies over the past several years have revealed that each step is regulated by
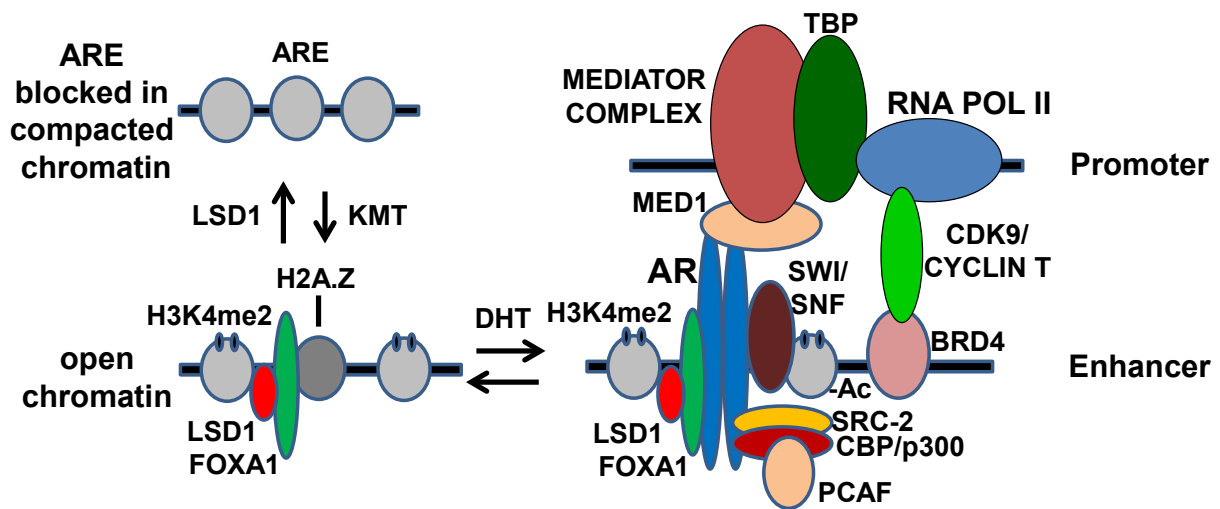
Figure 1 Model outlining steps mediating the progression from compacted chromatin to AR regulated gene expression.

multiple mechanisms involving large numbers of proteins. In particular, the central role of chromatin structure and histone posttranslational modifications in regulating the functions of AR and all other transcription factors is being elucidated. While the role of DNA methylation in the epigenetic regulation of gene expression is well established, it is now appreciated that changes in histone structure play a central and dynamic role in the epigenetic regulation of gene expression. The focus of this review is on the role of histones in the regulation of AR function in PCa development and progression.

Through chromatin immunoprecipitation (ChIP) studies, coupled with massively parallel DNA sequencing (ChIP-seq) methods, it has been found that AR binds to thousands of sites in PCa cells and stimulates the expression of hundreds of genes. The first section below focuses on the role of chromatin structure in regulating AR binding to DNA. The second section then outlines how AR stimulates gene expression, with a focus on how it modulates chromatin structure. The third section describes the role of chromatin modifications in AR function as a transcriptional repressor. The subsequent sections then focus on epigenetic mechanisms that may alter the spectrum of AR regulated genes during PCa development and progression. The final section discusses possible therapeutic implications of AR epigenetics.

Epigenetic regulation of AR binding to chromatin

The AR DNA binding domain can directly mediate AR

binding to AREs on naked DNA, but these sites are not readily available *in vivo* on compacted chromatin, which is tightly wound around nucleosomes. In order for most sites to become available for AR binding, the chromatin must first be "opened", and this is generally achieved by binding of pioneer factors (1). Studies of steroid hormone receptors including ER in breast cancer and AR in PCa have established the central role of FOXA1 as a pioneer factor that binds initially to compacted chromatin and opens it for subsequent transcription factors (2-5). This capability of FOXA proteins is due to their structural similarity to linker histones, which allows them to bind between nucleosomes in compacted chromatin and locally open the chromatin (6,7). ChIP-seq studies in PCa cells have established that FOXA1 is associated with AR at most AR binding sites, and that FOXA1 is present at these sites prior to treatment with androgen to stimulate AR binding. Further studies have shown that these sites are DNAse hypersensitive, which is indicative of a nucleosome free region, prior to androgen treatment and AR binding (8,9).

Comparable results have been obtained for ER in breast cancer cells, and FOXA1 silencing by RNAi in breast cancer cells globally suppresses ER binding and activity (10). Interestingly, while FOXA1 silencing similarly impairs AR binding to a large fraction of sites in PCa cells, many sites are not affected and this also results in AR binding to new sites (11,12). The physiological significance of this observation remains uncertain, but it indicates that FOXA1 is not absolutely required for AR binding at all sites. It is possible that some of these sites may be pioneered by

FOXA2, which is expressed during prostate development (13). AR binding sites are also highly enriched for the GATA2 and OCT1 transcription factors, and GATA2 may have a pioneering function on a subset of genes (4,7,9). Interestingly, FOXA1 mutations have been identified in a subset of advanced PCa (14,15). Therefore, modulation or alteration in FOXA1 is a possible mechanism that could contribute to altering AR function in advanced PCa (see below).

In addition to FOXA1, AR binding sites are enriched for nucleosomes in which lysine 4 on histone 3 has been mono- or dimethylated (H3K4me1 or H3K4me2) (3,8,16). A large body of literature has now established that H3K4me2 (and to a lesser extent H3K4me1) is specifically associated with transcriptional enhancers, while H3K4 trimethylation (H3K4me3) is associated with active promoters. Similarly to FOXA1, nucleosomes with the H3K4me2 mark are present at AR binding sites prior to androgen stimulation, and may contribute to the initial recruitment of FOXA1. However, the precise interplay between H3K4 methylation, FOXA1 binding, and likely other mechanisms in initiating the opening of an AR regulated enhancer remain to be clearly defined. Indeed, it is likely that this enhancer opening is not rigidly controlled, and that a balance between histone methylation, demethylation, and other modifications determines its status. In particular, amongst the many AR recruited proteins is a methyltransferase, SET9, that can methylate H3K4 and may thereby reinforce an open chromatin state (17,18). In contrast, H3K4me1 and H3K4me2 can be demethylated by the histone demethylase LSD1 (lysine specific demethylase 1, KDM1A), and it has been shown that LSD1 overexpression can inactivate AR regulated enhancers (19). Broader roles for LSD1 in AR functions as a transcriptional enhancer and repressor are described in subsequent sections below.

In contrast to H3K4 methylation that is generally associated with active enhancers (H3K4me2) and promoters (H3K4me3), methylation of H3K9 (H3K9me3) and H3K27 (H3K27me3) at promoters are strongly associated with transcriptional repression. Mechanistically, H3K9me3 can mediate interactions with proteins and long noncoding RNA (lncRNA) that localize the gene to transcriptionally inactive nuclear domains (20,21). AR has been found to interact with and stimulate the expression of KDM4B, an enzyme that can demethylate H3K9me3, and KDM4B can coactivate AR transcriptional activity (22). This coactivation may in part reflect H3K9me3 demethylation, but KDM4B also functions through a nonepigenetic mechanism to decrease the ubiquitylation and degradation of AR. In contrast to

H3K9me3, H3K27me3 recruits DNA methyltransferases that methylate DNA, and can thereby mediate long-term gene silencing. H3K27 methylation is mediated by the Polycomb Repressive Complex 2 (PRC2), with EZH2 being the H3K27 methyltransferase in this complex. Previous studies established a strong correlation between increased EZH2 expression and more aggressive PCa (23,24). This correlation may reflect in part the progressive inactivation of AR regulated and AR independent genes that mediate differentiated functions and suppress growth. Indeed, mutations in another enzyme that can demethylate H3K27 (JMJD3, KDM6A) have been found in PCa, which may also contribute to gene inactivation (15). However, EZH2 was recently found to also function as an AR coactivator through a mechanisms that was methyltransferase dependent, but independent of PRC2 and H3K27 methylation (described further below) (25).

Epigenetic mechanisms through which AR stimulates gene expression

AR binds to enhancer sites that are generally distant from the promoter, but AR at these sites interacts with gene promoters by chromatin looping (26,27). A major contributor to this looping is the large multiprotein Mediator complex, a component of which interacts with AR (MED1) while other components interact with and include RNA polymerase II associated TATA binding proteins (28-31). Mutations in a Mediator protein (MED12) have been found in PCa, but the functional significance of these mutations is not clear (14). Additional proteins, as well as lncRNAs, likely contribute to the enhancer-promoter interaction. Interestingly, this looping mechanism has also been implicated in the generation of gene fusions occurring in PCa, including the TMPRSS2:ERG fusion (32,33).

Perhaps the earliest event that can be detected in response to AR binding is loss of a central nucleosome that overlaps the AR binding site, which can be demonstrated by ChIP and by an increase in DNase hypersensitivity, and is also associated with stronger binding of flanking nucleosomes (8,9). Interestingly, this central nucleosome located over the ARE at many AR stimulated enhancers contains the histone variant H2A.Z. This variant is also loaded onto nucleosomes during DNA repair and results in weaker nucleosome binding, which may facilitate the initial binding of AR. The androgen liganded AR then mediates the recruitment of multiple proteins that covalently modify histones and associated proteins, protein complexes that

further unwind chromatin (primarily the SWI/SNF complex), proteins that mediate interaction with the promoter (see above), and ultimately enhance binding and activation of RNA polymerase II.

Many of the initially identified proteins recruited by AR, including the p160 steroid receptor coactivator proteins (SRC-1, 2 and 3), CBP and its close relative p300, and PCAF have lysine acetyltransferase activity and function as histone acetyltransferases (HATs), although it has become clear that they can also acetylate many additional proteins. Acetylation of lysines on histones may reduce their positive charge and thereby weaken their interaction with DNA. Acetylation at some sites may also prevent other modifications that repress gene expression, such as acetylation of H2K27 that would prevent EZH2 mediated trimethylation and gene silencing (see above). A further important function for histone lysine acetylation is the recruitment of proteins that contain bromodomains, which recognize acetyl-lysine residues (34). One such protein is BRD4, which contains two bromodomains and subsequently functions to recruit the CDK9/cyclin T complex (positive transcription elongation factor b, P-TEFb) that phosphorylates RNA polymerase II to drive elongation (35,36). Interestingly, CDK9 can also directly associate with and phosphorylate AR (37). Very recent studies indicate that BRD4 may function primarily on "super enhancers", indicating that the AR-CDK9 interaction may play an important role in mediating interactions between AR bound to the enhancer and the promoter (chromatin looping, see above) for a subset of genes that are less dependent on BRD4.

Changes in histone acetylation (mediated by HATs and histone deacetylases, HDACs) occur rapidly and were initially viewed as the major posttranslational modification mediating the stimulation of transcription in response to hormone stimulation. In contrast, histone methylation on lysines was considered to function over a longer time frame and modulate enhancer availability. However, with the discovery of multiple enzymes that can demethylate histones, it now appears that androgen stimulated methylation of histone and nonhistone proteins also contributes acutely to gene activation. AR recruits and is coactivated by methyltransferases including the arginine methyltransferase CARM1 (PRMT4) (38) and the lysine methytransferase SET9 (17,18). CARM1 methylates arginine 17 on histone 3 (H3R17me2), but also has nonhistone substrates including SRC-3, and the precise basis for its AR coactivation function remains to be determined (39). As noted above, SET9 is recruited by AR to AREs and can methylate H3K4,

and may thereby contribute to maintaining AR regulated enhancers. However, SET9 can also methylate lysines in the AR hinge region, which may enhance the interaction between the AR NTD and LBD. This direct effect on AR may account for SET9 coactivator function, but it will likely have additional substrates that remain to be defined.

As indicated in the previous section, LSD1 was identified initially as a demethylase for H3K4me1 and H3K4me2 (due to its catalytic mechanism, LSD1 can't demethylate trimethylated lysines) (40), and was found to be associated with corepressor complexes (see below). However, it was subsequently found that LSD1 also functions as a critical coactivator for AR, as LSD1 inhibition or silencing by RNAi markedly decreased androgen stimulated expression of multiple AR regulated genes (41,42). Moreover, ChIP studies have demonstrated binding of LSD1 to AR regulated enhancers in these genes. Interestingly, while LSD1 can interact directly with AR, LSD1 binding to these enhancers (similarly to FOXA1 binding) is observed prior to androgen treatment, indicating that other interactions are mediating its recruitment. LSD1 may similarly be a coactivator for a subset of other transcription factors including ER (43,44).

One mechanism that may contribute to LSD1 function as a transcriptional coactivator is demethylation of repressive mono- and dimethylated H3K9 (41). Moreover, histone 3 phosphorylation may mediate a switch in the substrate specificity of LSD1 from H3K4 on AR repressed genes to H3K9 on AR stimulated genes. Specifically, phosphorylation of histone 3 on threonine 11 (H3T11ph) by protein kinase C-related kinase 1 (PRK1, PKN1) was found to enhance the ability of LSD1 to demethylate H3K9me1,2 (45). In contrast, phosphorylation of histone 3 on threonine 6 (H3T6ph) by protein kinase C 1 (PKC1) was found to inhibit LSD1 mediated demethylation of H3K4me1,2 (46). Significantly, both these kinases were found to associate with AR and be recruited by AR to androgen stimulated enhancers, supporting the model that they mediate a switch in LSD1 substrate specificity from H3K4 to H3K9. However, this switch is not absolute as LSD1 mediates some degree of H3K4 demethylation at androgen stimulated genes, and also mediates both H3K4 and H3K9 demethylation at AR repressed genes (see below) (47). Moreover, it is not clear whether alterations in H3K4 and H3K9 mono- or dimethylation at enhancer sites regulate transcription or are instead a consequence of transcriptional activity. In any case, it is likely that further mechanisms contribute to the coactivation function of LSD1 on androgen stimulated genes, which may include

novel histone or nonhisotne substrates.

In addition to LSD1, AR has been reported to recruit the H3K9me3 demethylase JMJD2C (KDM4C, also termed GASC1) to the androgen stimulated PSA gene enhancer (42). Moreover, JHDM2A (KDM3A), an H3K9me1,2 demethylase, has been reported to be recruited to AR stimulated genes and enhance their transcription (48). Therefore, in addition to LSD1, additional histone demethylases may contribute to AR stimulated H3K9 demethylation and transcriptional activity.

Epigenetic mechanisms mediating AR repression of gene expression

In addition to its well established function as a transcriptional activator, AR can also decrease the expression of multiple genes. For a subset of genes this decrease in expression is due to AR binding and interference with other transcription factors including SP1, RUNX2, JUN, and SMAD3 (49-51), or with β-catenin (52-57). The agonist liganded AR also may function more directly as a transcriptional repressor through an epigenetic mechanism by recruiting corepressors that mediate histone deacetylation, including ALIEN, DAX1, HEY, AES, PHB, and SHP, although the role of these corepressors in modulating specific AR regulated genes remains to be determined (58-63).

In contrast to the ligand-dependent DNA binding by steroid receptors, DNA binding by the larger family of nonsteroidal nuclear receptors is ligand-independent and these nuclear receptors generally function as transcriptional repressors in the absence of ligand. This repression is mediated by the corepressors NCoR and SMRT, which are similarly associated with histone deacetylases. NCoR and SNRT contain extended LXXLL-like motifs (CoRNR boxes) that can bind to the unliganded coactivator binding site in the LBD of nuclear receptors, but are displaced after ligand binding. In contrast to other nuclear receptors and steroid receptors, the androgen liganded AR can also associate with NCoR and SMRT (64-67). This interaction is probably through a distinct site on the AR N-terminal domain, and downregulation of NCoR and SMRT can enhance activity of the agonist liganded AR. Significantly, the altered structure of the AR LBD generated by some AR antagonists may enhance NCoR and SMRT binding and contribute to antagonist activity (66-70).

Androgen mediated transcriptional repression also has been linked to histone methylation via AR recruitment of EZH2 and an increase in the EZH2 catalyzed repressive H3K27me3 mark (71,72). Conversely, AR can function directly as a transcriptional repressor through an interaction with LSD1 and histone demethylation (47). As outlined above, LSD1 can function as an AR coactivator, but it has been most extensively characterized as a corepressor that demethylates mono- and dimethylated lysine 4 on histone 3 (40). Consistent with this corepressor function, LSD1 associates with AR on AREs in many AR repressed genes and mediates demethylation of H3K4me1 and H3K4me2 in response to androgen (47). Amongst these genes that are directly repressed by AR in association with LSD1 are the AR gene itself, and genes regulating androgen synthesis (AKR1C3 and HSD17B6), which provides a negative feedback loop to regulate AR signaling. Significantly, a large proportion of other AR repressed genes are in pathways mediating DNA synthesis, which may reflect a physiological function of AR to suppress cell growth and drive differentiation (47).

LSD1 associates tightly with CoREST (REST corepressor 1, RCOR1), which may both stabilize LSD1 and stimulate its H3K4 demethylase activity (73). LSD1 and CoREST, in addition to proteins including HDAC1 and HDAC2, are components of the BHC corepressor complex that is recruited by the transcription factor REST to repress expression of neuronal genes in non-neuronal cells (74). LSD1 also is a component of another multiprotein corepressor complex, the NuRD complex, which similarly contains HDAC1 and HDAC2 (75,76). The JmjC family histone demethylase JARID1B (KDM5B), which can demethylate H3K4me3 (associated with promoters) and thereby generate the H3K4me2 substrate for LSD1, also has been identified as a component of the NuRD complex (77). LSD1 also may associate with additional protein or protein complexes, such as SIRT1 that mediates H4K16 deacetylation, or with lncRNA, to coordinately modify chromatin structure and repress gene expression (78,79). Therefore, AR transcriptional repression that is linked to LSD1 may be driven by additional epigenetic mechanisms mediated by multiple LSD1 associated proteins.

Epigenetic reprogramming of AR during PCa development

Frequent fusions between the strongly AR regulated *TMPRSS2* gene and the Ets family transcription factor *ERG* gene, as well as additional fusions involving TMPRSS2 or other AR regulated genes, have established a genetic mechanism through which AR acquires new functions during PCa development (80). Several genes that may

be directly regulated by ERG have been identified, but the precise mechanisms through which ERG drives PCa development have not been clear (81,82). Recent studies have identified epigenetic mechanisms through which ERG may drive PCa development. One reported downstream functions of ERG in PCa is to increase expression of EZH2, which may then mediate epigenetic gene silencing through H3K27 methylation (82). ERG was also reported to downregulate AR expression and transcriptional activity.

In contrast, studies in the TMPRSS2:ERG fusion positive VCaP human PCa model showed that ERG expression was increasing the number of genes that were stimulated by androgen (83). The most critical ERG dependent AR stimulated gene in VCaP cells was found to be the SOX9 transcription factor. SOX9 regulates ductal morphogenesis in fetal prostate and maintenance of stem/progenitor cells in adult tissues (84-87). In genetically engineering mouse models, SOX9 knockdown can impair PCa development driven by MYC and SV40 T antigen (84), while SOX9 overexpression in prostate on a $Pten^{-/+}$ background results in high grade dysplastic lesions that can progress to invasive PCa (83,88). Mechanistically, ERG appears to be functioning as a pioneer factor by binding to a site 3' of the $SOX9$ gene, with subsequent binding of FOXA1 and opening of an adjacent cryptic AR binding site.

These findings suggested that the oncogenic effects of ERG in prostate specific ERG overexpression mouse models may be mediated through a similar mechanism. Indeed, a recent study showed that ERG expression in mouse prostate, similarly to ERG in human PCa cells, reprograms AR to stimulate the expression of multiple new genes (89). However, SOX9 mRNA is not increased by ERG overexpression in mouse prostate, which may account for the weaker phenotype of transgenic ERG versus SOX9 overexpression in mouse prostate. Consistent with this finding, the ERG and AR binding site identified at the 3' end of the human $SOX9$ gene is not conserved in mouse (83). Interestingly, while ERG does not directly increase SOX9 expression, a recent study suggests that it may indirectly enhance SOX9 activity (90). In any case, these findings taken together indicate that epigenetic reprogramming of AR transcriptional activity contributes to PCa pathogenesis in at least a subset of cases.

Epigenetic reprogramming of AR in advanced CRPC

AR can also acquire new transcriptional activities by epigenetic mechanisms in advanced CRPC. AR in an LNCaP-derived CRPC cell line (LNCaP-abl) was found to stimulate the expression of multiple genes that were not AR regulated in the parental LNCaP cells. The novel AR transcriptional program in LNCaP-abl cells included multiple M-phase cell cycle genes such as CDK1 and UBE2C, which are also overexpressed in CRPC (19). Significantly, this was not just secondary to increased proliferation, as ChIP-seq studies showed that the androgen stimulated expression of these genes in LNCaP-abl cells was associated with increased AR binding to sites linked to these genes. There were also increased levels of H3K4me1,2 at AR binding sites in these genes, indicating that these AR regulated enhancers had been opened by an epigenetic mechanism. Consistent with this conclusion, overexpression of LSD1 could decrease H3K4 methylation and AR binding to these sites.

It is not yet clear whether a pioneer factor or other specific mechanisms are initiating a precise new AR transcriptional program in these cells, versus positive selection leading to the gradual outgrowth of cells that have activated genes mediating proliferation through a variety of mechanisms. However, a recent study uncovered a novel AR coactivator function for EZH2 in CRPC cells that may contribute to AR reprogramming (25). As noted above, EZH2 is a histone methyltransferase associated with the PRC2, and its increased expression is correlated with higher grades and more advanced PCa. EZH2 expression is similarly increased in other cancers, which may reflect progressive silencing of tumor suppressor genes through H3K27 trimethylation. However, this study showed that increased EZH2 in more advanced PCa was not associated with increased H3K27me3. Instead, EZH2 in CRPC cells was found to form a complex with AR that was recruited to genes including CDK1 and UBE2C. Moreover, it functioned as an AR coactivator by a methyltransferase dependent mechanism that was distinct from its ability to methylate H3K27 (25). This coactivator function of EZH2 was dependent on AKT mediated phosphorylation of serine 21 on EZH2. Phosphorylation of this site on EZH2 was shown previously to suppress its ability to methylate H3K27. It is presumed that this AR coactivator function of EZH2 is mediated through methylation of other substrates, which may include AR, but further studies are needed to identify these alternative substrates.

While the above AR reprogramming appears to be dependent on H3K4 methylation and FOXA1, the AR transcriptional program may also be altered by a distinct

mechanism involving downregulation of FOXA1. Recent studies found that FOXA1 downregulation by RNAi caused the expected loss of many AR binding sites, but the unexpected result was a large number of new AR binding sites (11,12). Consistent with their FOXA1 independence, these new AR binding sites were not enriched for H3K4 methylation. However, they appear to be functional enhancers based on production of short enhancer-templated non-coding RNA (eRNA), and AR binding to a subset could stimulate enhancer-promoter looping and gene expression. Interestingly, motif analyses show that these new AR binding sites are enriched for strong consensus AREs, which may be important for FOXA1 independent AR binding. Together these findings indicate that FOXA1, while having an important role as a pioneer factor for AR binding to a large number of genes, may also function to suppress AR binding to another set of genes.

The clinical significance of these findings remains to be determined, but it is intriguing that FOXA1 mutations occur in a subset of PCa and could be driving tumor progression through this AR reprogramming mechanism. Mutations in enzymes mediating H3K4 methylation, MLL2 (KMT2D) and MLL3 (KMT2C) have also been found in PCa, and could possibly function in part by closing some H3K4me2/FOXA1 dependent enhancers and redirecting AR to FOXA1 independent sites (14,15,91). Finally, recent data indicate that AR splice variants lacking the LBD, which are increased in CRPC, may regulate a distinct set of genes that include genes driving cell cycle progression (92). These findings could reflect novel interactions between AR splice variants and EZH2 or FOXA1, but further studies are needed to determine their molecular basis (93,94).

Clinical implications of AR epigenetics

Current efforts to ablate AR focus on reducing androgen synthesis and developing direct AR antagonists. However, these approaches are not selective and instead broadly suppress AR stimulated regulated genes, many of which do not mediate tumor growth and some of which may suppress tumor growth. Moreover, these therapies may also enhance the expression of AR repressed genes. Data outlined in this review show that the spectrum of genes regulated by AR is not hard-wired, and that epigenetic modifications can have a profound effect on the genes AR stimulates or represses. Therefore, as an alternative approach, it may be possible to develop agents that can epigenetically modify the spectrum of genes the AR regulates, and possibly thereby enhance

its differentiation functions. Such approaches could be of particular value for PCa prevention or for treatment of early disease. However, a better understanding of the epigenetic mechanisms regulating AR functions may be needed, and in particular it is not clear whether genes mediating specific functions or pathways are controlled by distinct epigenetic mechanisms. One possible approach to address this question may be to further characterize AR function in other tissues where AR clearly regulates distinct repertoires of genes, and determine the epigenetic basis AR functions in these tissues.

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Footnote

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Androgen receptor genomic regulation

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Abstract: The transcriptional activity of the androgen receptor (AR) is not only critical for the normal development and function of the prostate but also pivotal to the onset and progression of prostate cancer (PCa). The studies of AR transcriptional regulation were previously limited to a handful of AR-target genes. Owing to the development of various high-throughput genomic technologies, significant advances have been made in recent years. Here we discuss the discoveries of genome-wide androgen-regulated genes in PCa cell lines, animal models and tissues using expression microarray and sequencing, the mapping of genomic landscapes of AR using Combining Chromatin Immunoprecipitation (ChIP)-on-chip and ChIP-seq assays, the interplay of transcriptional cofactors in defining AR binding profiles, and the genomic regulation and AR reprogramming in advanced PCa.

Keywords: Androgen receptor (AR); prostate cancer (PCa); AR transcriptional regulation; AR-target genes; Combining Chromatin Immunoprecipitation (ChIP)-on-chip; ChIP-seq assays

Introduction

The androgen receptor (AR) is a member of hormonal transcription factors. The expression of AR protein and its activation by male hormone androgen are fundamental to prostate development during pubertal and malignant transformation during later ages. These biological/pathological processes are determined by critical regulation of downstream molecules/pathways by the AR. AR is a DNA-binding protein that regulates a wide-range of target genes through directly binding to cis-regulatory elements. In the absence of androgen, the AR is sequestered in the cytoplasm by the chaperone super-complex including heat shock proteins (Hsp) 90, 70 and 56 (1). Once bound by androgen, AR undergoes conformational changes to dissociate from Hsp complex, becomes phosphorylated and translocates into the nucleus. For decades, understanding of AR-mediated transcriptional regulation was largely built upon the analysis of a handful of androgen-induced genes, one prototype of which is PSA. AR has been shown to form homodimers which preferentially bind DNA that contains androgen-responsive elements (AREs) (2,3). This binding activity and thus AR-mediated transcriptional regulation are tightly controlled by a large cohort of AR co-factors. Despite of these successes, very few AR target genes have been identified and characterized until recent advances in high-throughput genomic technologies. The advent of DNA microarrays at the beginning of this century and the emergence of massively parallel next-generation sequencing have rapidly transformed this field. Taking advantage of these approaches, a burst of studies have in recent years very carefully examined AR transcriptional regulation at the genome scale. Here we review these studies to provide up-to-date understanding of genome-wide androgen-regulated genes and the genomic landscapes of AR and its regulation during prostate cancer (PCa) progression.

Genome-wide analysis of androgen-responsive genes

Androgen-responsive genes or androgen-regulated

genes are defined as genes whose expression levels are significantly altered by androgen treatment. The products of these genes are essential in the biological processes responsible for prostatic development, function and disease. Therefore, identification and characterization of androgen-responsive genes can potentially lead to the discovery of novel biomarkers and approaches for PCa diagnosis and treatment. For decades very few androgen-responsive genes have been identified, mostly limited to KLK3 (PSA), KLK2, and NKX3-1 (4). However, the completion of the human genome project and the advent of microarray technologies at the beginning of this century have enabled parallel analysis of the expression of thousands of genes at a time. Consequently, an unprecedented list of androgen-regulated genes have been recently identified and characterized through expression microarray analysis of PCa cells and tissues.

High-throughput profiling of androgen-regulated genes using PCa cell lines

Androgen-sensitive PCa cell lines such as LNCaP have been critically useful to identify the downstream genes/molecules that are regulated by androgen. Pioneered by Sanger sequencing of cDNA or EST libraries, researchers began the endeavors of genome-wide analysis of androgen-responsive genes approximately a decade ago (5). Through serial analysis of gene expression (SAGE), androgen-regulated genes were greatly expanded from a handful to approximately 100 of them (6-8). These EST-based assays, though have certainly made significant contributions to the field, were labor-intensive and cost-inefficient for the purpose of expression profiling. Rather, following the characterization of all human genes, EST clones were printed onto glass slides to generate cDNA microarray, which was then used for expression profiling in an efficient and affordable manner. The use of microarray has extensively facilitated the identification and the analysis of androgen-regulated gene expression under various biological/pathological conditions. For example, through microarray analysis of LNCaP cells before and after androgen stimulation, studies have revealed more than 500 genes that were differentially regulated by androgen (9,10). Further analysis revealed that approximately 300 genes were up-regulated while another 300 were down-regulated by androgen (11).

To pinpoint potential direct targets of AR, studies have examined global gene expression changes following androgen stimulation over a time-course. For example, Massie *et al*. identified more than 3,000 genes with altered expression in response to androgens by assessing 27 time points between 0 to 24 h following 1 nM R1881 stimulation in LNCaP cells. Out of these androgen-regulated genes, approximately 550 genes responded to R1881 within 5 hours and are likely to be directly regulated by AR (12). Despite some differences regarding androgen-regulated genes derived from various datasets, plausibly due to differences in array platforms and experimental conditions, a core set has been repeatedly identified. Based on a review from Dehm and Tindall, about 1.5-4.3% of genes expressed in LNCaP cells are androgen regulated (13). Through analysis of PubMed literatures, Androgen Responsive Gene Database (ARGDB) showed that at least 1,785 human, 583 rat, and 993 mouse genes have been reported as androgen-regulated (14). By comparing 9 representative studies of gene expression in androgen-treated LNCaP cells, we found that more than 1,000 genes have been reported in at least 2 independent studies, among which a core set of over 200 genes have been shown to be androgen-regulated in more than 4 independent studies (*Tables 1,2*).

Genome-wide analysis of androgen-regulated genes in animal models

In addition to cell lines, animal models have been utilized to study androgen response *in vivo*. Although the structure of rodent prostate differs considerably to that of human, androgens are nonetheless critical for rodent prostate cell differentiation, proliferation, and overall prostate development (15). Human and rodent species are thought to share a variety of fundamental biochemical and functional pathways that are regulated by AR signaling. Using both subtractive hybridization and microarray approaches, Jiang *et al*. reported the identification of more than 100 androgen-responsive genes in the rat ventral prostate (16). Using the Dunning rat R3327 model system, Pfundt *et al*. identified several sets of prostatic androgen-responsive genes that are alternatively regulated in androgen-dependent and androgen-independent prostate tumors (17). Through microarray analysis of gene expression profile changes in the mouse prostate following castration and hormone replacement, Wang *et al*. identified a number of androgen-responsive genes, a majority of which are also regulated by androgen in cell line models. In particular, the authors reported sixty-five genes as androgen down-regulated (i.e., up-regulated upon castration and repressed by hormone replacement) (18), among which 72% have potential AREs, suggesting a direct transcriptional suppressor role of the AR on these genes. It

Table 1 Microarray profiling of androgen-regulated genes

	Technology	Overlap with Massie's dataset (%)	Massize et al. 2011	Rajan et al. 2011	Ngan et al. 2009	Li et al. 2008	Velasco et al. 2004	Nelson et al. 2002	Deprimo et al. 2002	Segawa et al. 2001	Xu et al. 2001
Massie et al. 2011	Illumina bead array	100.0	3,050								
Rajan et al. 2011	Exon array	68.2	373	547							
Ngan et al. 2009	ABI microarray	56.7	349	222	616						
Li et al. 2008	RNA-seq	47.7	327	181	134	685					
Velasco et al. 2004	Oligonucliotide array	28.0	186	54	58	69	665				
Nelson et al. 2002	cDNA array	49.5	52	45	43	48	23	105			
Deprimo et al. 2002	cDNA array	51.4	225	122	105	144	48	32	438		
Segawa et al. 2001	Oligonucliotide array	27.4	144	61	56	63	124	27	48	525	
Xu et al. 2001	SAGE	21.1	12	6	5	14	11	5	7	6	57

*Datasets, in which half of identified genes were common with another study, are highlighted. *Massie's dataset, 3,319 probes correspond to 3,050 unique gene symbols.

Table 2 Common androgen-regulated genes from 9 microarry studies listed in *Table 1*

Number of studies (observing number of common androgen-regulated genes)	Symbol of regulated genes
6 [43]	DHCR24, LIFR, NDRG1, DBI, MMP16, CTBP1, FKBP5, KLK3, APPBP2, DDC, ALDH1A3, KRT8, ELL2, HERC3,TPD52, SEC24D, CDK8, BCHE, ABHD2, IDI1, DNAJB9, MERTK, SORD, ABCC4, ODC1, PIK3R3, PTPRM, KLK2, GATA2, BARD1, TMPRSS2, SGK, IQGAP2, FN1
5 [59]	SMARCD3, DPYSL2, TSC22D1, MAPK6, ACSL3, SEPP1, ATAD2, ANKH, PEA15, GHR, PLA2G2A, FOLH1, NKX3-1, ORM1, CALU, UGT2B15, PPAP2A, PRKD1, BAMBI, SNX25, PPP1CB, OPRK1, PKIB, NCAPD3, MPHOSPH9, SLC35F2, LCP1, TBRG1, TMEPAI, CAMKK2, RAB27A, ABCC1, HMGCS1, DNM1L, CENPN, LONRF1, ST7, PGM3, SPHAR, TXNIP, COLEC12, MTMR9, ATP2B1, LMAN1, CXCR7, B2M, MYC, PURA, CALD1, ADD3, ZBTB16, PDIA5, UBE2G1
4 [122]	KCNMA1, DDEF2, PTPRR, SLC15A2, LRRN1, SASH1, ACAD8, SLC39A7, NAP1L3, HOMER2, ADAMTS1, MANEA, RHOU, SERPINI1, BTG1, THYN1, HS3ST1, NR4A1, SMAD1, PTPN21, WIPI1, PPM1K, CBLL1, AKAP12, SPDEF, AZGP1, SEC61G, DEGS1, ABHD3, SYTL2, KRT18, PECI, MID1, BCAP29, SOCS2, SPCS3, CEBPD, LRRFIP2, WDR41, WWC1, NEDD4L, ARMET, PGC, KCNN2, SMAD7, SERP1, MAF, IDH1, FDFT1, SQLE, PPFIBP1, PCTP, UBE2J1,GARNL3, TIMP2, KDELR2, HIBADH, TRIB1, MAP2K4, KCTD3, TRPS1, ERN1, MLPH, CYFIP2, MAP7D1, TWIST1, TRIM36, KCTD9, SELENBP1, STK17B, SI, UTX, SSBP2, TARBP1, VGLL4, ABLIM1, STK39, ST6GALNAC1, ANGPT2,AFF3, PIK3IP1, C9orf91, KLF4, LDLR, MKLN1, SMS, VEGFA, SESN1, RAB4A, PIK3R1, BTD, NFKBIA, SCAP, IL1R1, SAT1, ARF4, NDFIP2, SLC7A2, INPP4B, CEBPG, MBOAT2, PAK2, IMPDH2, TMEM87B, PICALM, MYH1, PBX1, NET1, GRB1, LRIG1, FUT8, ZCCHC6, ARFGAP3, NFKB1, ERGIC2, ATP1B1, HOXB13, C1orf21, SLC44A1, TULP4, LAMC1, VCL

Table 2 (*continued*)

Number of studies (observing number of common androgen-regulated genes)	Symbol of regulated genes
3 [243]	PGM2, CREB3L2, CXCR4, RLN2, PELI2, GDF15, GRB10, ID3, NIPSNAP3A, SERPINB5, CLGN, TMEM39A, PLDN, ARHGAP18, KIAA0247, FAM105A, GMPPB, ABCG1, SDCBP, GLUD2, SLC16A6, NUP93, OCLN, LOC400451, NFIB, SEC24B, LRRC16, RAB3B, ALDH4A1, TIPARP, SLITRK3, CPEB3, PART1, SLC43A1, GNMT, KLF5, CDK2AP2, TNFAIP8, GPR177, SLC25A20, SIPA1L2, C5orf30, TNFRSF10B, EXTL2, ST5, OSR2, NUPL1, SLC12A2, TMEM144, SMPD2, MAPRE2, C14orf4, ADORA2B, VAPA, ANXA9, MRPS18A, TMEM140, STEAP4, LRIG3, C8orf32, MATN2, GPR126, CAMK2B, PEX10, CADPS2, TMEM79, VLDLR, TBC1D8, ZNF462, PRKCH, ANTXR1, IMPDH1, FZD3, INTS10, GFM1, KLK4, ENC1, MANBA, STK3, TGM3, TPM1, SALL2, MAK, CABLES1, NFAT5, HPGD, PAK1IP1, GLI3, TMEFF2, PPAPDC1B, GPR160, TNFAIP3, NANS, CBLN2, FXYD3, INSIG1, KCNS3, HEBP2, PPM1D, CREB3L4, LOC81691, MCCC2, EIF2C4, SEMA6A, EAF2, AGR2, STCH, PMS2, GRAMD1C, DNAJC10, THRAP3, SORL1, PSCD3, C5orf13, C4orf18, PCSK6, RBM6, AP2S1, CA12, C14orf24, GSR, FAM3C, PFKFB2, MAPKAPK3, PACS1, FZD5, ERBB2, USP33, HIST1H2AC, SLC26A2, ATP2B4, ID2, DDX21, C21orf33, ICAM3, JUP, SLC41A1, GTF2E2, NINJ1, IFRD2, CHPT1, GLRX2, BCL9, DSC2, SSR3, SLC38A2, TMED5, BRP44, FRAP1, ACPP, CAMK2N1, SRP19, LASP1, CUL1, CLDN12, CDYL2, GRIK1, ENPP5, ZBTB1, PSAT1, TBC1D1, ENDOD1, IGF1R, CAPRIN2, SHROOM3, HMGCL, ASNS, FRK, CHD4, PRAME, RELN, C1orf116, GOLGA4, RAB7A, H1F, ELOVL5, ZIC2, POLR1E, TRAM1, SC4MOL, ATP1A1, RALGPS1, SEC63, PSMD8, LRBA, LUZP2, RCN1, SREBF2, WWP1, GFPT1, PLCB4, PYGB, HIST2H4B, ARPC1B, SNRK, SSR1, F5, GLUDP5, OBFC2A, ERRFI1, SEC61B, PBX2, CAPZB, KIAA247, PACSIN2, BIK, FASN, ST3GAL1, ARNT, HIST1H3B, DSG2, JAG1, DNAJC3, ELOVL7, ZBTB43, CDCA7L, MTHFD2, EPRS, CTH, SEMA3B, CSNK1A1, CHKA, TBC1D4, SS18, SESN3, HIST1H3H, UROD, HERPUD1, CAPRIN1, MB, REEP5, RHOB, XPA, EPHX1, SRPRB, MRPS27, PCCA, SLC45A3, C8orf76, BMPR1B, TLL1, SNAI2, AMD1
2 [722]	PHLDB1, PNMA1, PKNOX2, KLHL13, TMEM34, C9orf78, CYP39A1, STYK1, APIP, MALT1, SEC23B, HOXC13, COTL1, GSC, GADD45G, CERK, NOSTRIN, ZNF365, PCOLCE, TMEM118, PCYT2, NUDT9, IRX5, OPTN, PCDH11Y, HGD, KLHL1, GPR137B, COL9A2, SMAD3, GFPT2, RTN4R, MYLK, C6orf81, ROR2, MAOA, CORO2A, HOXC9, GREB1, EMP2, ALG2, ARPM1, ABCA1, SLC26A3, BBS4, KIAA1324, DUSP5, COL18A1, SLC12A6, EFHD1, PPAP2C, C14orf132, HK2, G6PD, THRB, C19orf48, CPNE3, CDC42EP3, RPS27A, DGAT2, STRBP, FGD4, HCFC2, SFRS5, TNFRSF21, TSC2, RASSF5, ACAA1, SEC11C, ARSG, MYNN, GLDC, ZBTB10, TTC18, SUSD4, SAT, MAPT, FBXW2, LRRC31, NPPC, MESP1, JMJD1C, SEC14L2, IL1RN, IRX3, IFIT2, C9orf61, PKIA, NAB1, APCDD1, PRAC, PPM1M, SOX9, TM4SF1, TM7SF3, TTN, MAP3K8, LOX, FGF13, C13orf1, TRIM45, COL5A2, ARHGEF17, C6orf168, DIO1, GCG, C14orf143, ChGn, RGS10, HYPE, TRIM48, CASC1, MCFD2, NEK1, ORM2, ENO2, ITPR1, EDN2, CDC42EP2, FBXO38, TCFL5, CD83, KLF13, GALNT7, FLJ20254, PODXL, ASRGL1, CTNNA2, MTP18, APC, CSMD1, LAMA1, FZD4, PER3, DLX1, SEPT6, AUTS2, MMP10, C10orf33, PHLDB2, ARHGEF10, LIN7B, F8, MGC14376, S100A11, EFHC2, DFNA5, PDE4A, SMAD6, GALNT10, ORC5L, ADCY1, ARNT2, C10orf47, DHRS2, FSTL1, MMP2, GPR98, BTG2, TUT1, PFKP, C1orf85, FAM134B, RDH10, HIST1H4J, TRIB3, COPS7A, EFNB1, C11orf2, C19orf10, ABR, DUSP4, UFM1, EIF1, RFX3, CRELD2, GCLM, GMPPA, SNX19, SEC14L1, ID1, PIK3CB, CDC42BPA, LRRC8A, MYO5C, SARS, EML3, PRDX4, ATF7, KIAA196, NFIL3, GCA, GLG1, XPC, ZNF350, SORBS2, GLYATL2, LMO4, SKAP2, HIST1H4I, CCT4, GRHL1, RPN1, CPD, HIST1H4L, HIST1H3E, TARSL2, TNFRSF1A, PHGDH, AGER, PARP4, SLC35B1, IGFBP2, DNAJC1, NR1D2, RNF43, PHF12, SETBP1, MAST4, CCDC14, SPTLC3, PHYH, LAMA3, RALB, TESK1, EBP, TDP1, HIST1H4B, CITED2, NLGN4X, C9orf72, HACL1, CBX4, ADCYAP1, AGRN, TLE3, SPRY1, CDON, NETO1, SKIV2L2, ZHX2, LRRC49, SMARCA4, TIMM23, PPP2R2A, PLEKHH1, THBS1, ABCC5, SNAPC5, MOCOS, SLC3A2, RBM5, NCL, HIST1H3C, CLCN3, CANT1, C6orf66, GPAM, BRE, THRA, SNAPC2, PEG3, CRIP1, ARG2, JARID1A, LRCH1, C3orf58, EDG7, HIST1H3D, C1orf118, SIM2, HIST1H4H, PUF6, SH3RF1, GPR63, MICAL1, SLC27A2, DDO, SEPT11, SDF2L1, IGFBP3, PSMA6, TERF1, EIF3I, CYB5A, NCOA7, HIST1H1C, ABCA5, DNAJB14, WRB, HIST1H4A, HIST1H3J, MT2A, ROR1, ADAM2, FAM13A1, NR1D1, SDHA, UGT2B28, HYAL1, DNASE2B, MYB, ANKRD37, XRCC2, SLC44A4, KIAA1712, COX5A,

Table 2 (continued)

Table 2 (*continued*)

Number of studies (observing number of common androgen-regulated genes)	Symbol of regulated genes
2 [722]	SMARCC2, HIST2H4, CD4, GTF2E1, SFPQ, RGS16, DIAPH2, PSMA1, BMPR1A, C6orf192, RALY, DGKH, HTR2C, CACNB3, CCDC141, RBBP9, PLEKHB1, MRPL33, FAS, CNKSR3, MRPL12, MGAT1, GOLPH3, CLK2, HSD17B11, RANP1, B4GALT1, SELS, PDXK, PRMT2, COX17, PRKAA1, MTF1, FLOT2, MAP4K1, CDKN2D, HPR, ZFHX4, TSPYL2, EIF2S3, SMPD1, VIM, LDHA, PDK2, MPZL1, ADAM9,IFNGR2, CAP1, PKP4, GCN5L2, CD97, RBX1, HIST1H3G, POLR3E, RBL2, RCAN3, FCHSD2, PFKM, VARS2, H2AFZ, MST1, ELMOD2, KDELR3, MGMT, SLITRK6, ACHE, KRT6A, HIST1H4K, CDC2, CLK1, GLYATL1, ALS2CR13, L1CAM, RGS2, PTMA, KIAA1324L, GIMAP2, SFRS2, CLSTN1, HSP9B1, EPHA3, PTPN1, PCDH1, CYP1A2, MGAT2, NUCB2, NUBP1, HIST1H2BG, NFIX, AKAP1, CCDC53, ERBB3, FIGNL1, AGPAT2, TP53INP1, RNF144B, CLU,RRM2, COG5, NCBP2, EFNB2, NLGN1, NAV1, POLR1D, LRRC59, TXNDC9, MAP2, NOS3, FBL, GSTM3, XBP1, BPHL, OTUD7B, HIST4H4, CAP2, DSEL, SLC29A2, RBMX, HIPK2, GPER, EIF4A2, CLDN8, NDUFA9, ZNF77, RRAGD, STC2, AMT, DIS3L, CTSH, TMEM2, AHNAK2, VTA1, CRISPLD2, NXPH1, CEP57, HIST1H3F, ZNF133, TFF3, SUOX, S1A11, NPC1, INPP5A, PITPNB, TRAP1, BTG3, APP, KLHL24, SPOCK1, KCNJ2, MUM1L1, TACC2, PDHA1, TKT, PAK1, CYCS, GYS1, SLC33A1, TMCC3, SLC16A14, RAB3IP, UGT2B17, HIST1H4E, GOT1, C3orf25, FADS1, EPS8, HSD11B2, RIOK1, DDB2, HECTD2, KIAA431, QDPR, MAPK12, RIN2, HYOU1, TPCN1, CDH15, DDX39, C12orf44, PTMS, PIP4K2B, RGL1, ABI2, PLD1, WDR6, ZCCHC9, NEBL, CBFA2T2, CARS, NEK6, IL1RL2, PFKL, CHAF1A, FTH1, CTSB, NFRKB, SEC13, WDR91, ITGB1, ZNF177, EPS15, CBX1, RRAS, SURF4, DEPDC6, C7orf41, ANAPC1, PTPN14, RBM3, ZKSCAN1, CRAT, ZWINT, DERL2, SLC7A11, SLC31A2, SPATA2, TXNDC12, PTPN9, CREM, PTGER4, SSFA2, TEAD4, EEF1A2, ME2, CGNL1, D4S234E, RABIF, EFNA5, PALM, FLJ45803, PPP3CB, TLN2, C1orf26, GADD45A, AASS, FSCN1, KRT6C, TRIM52, HIST1H4D, FAM15A, TCEB1, ARL6IP5, IARS, SRP54, ACVR1, C5orf3, STIP1, HSPE1, RALGPS2, GUSB, COPS3, PHLDA2, COL16A1, ST3GAL4, PHF8, KRT19, SBDS, GABRB3, NR2C1, TGIF1, ANKRD13A, YARS, ADAM17, CTNND1, PPP1R11, CPS1, AGPAT5, WDR68, PPIC, ETFB, MXD1, RAB1A, C2orf3, ADPGK, IDE, LPIN1, FAM111B, GALNT1, SNAPC3, ARCN1, SEC24A, SGK3, NAT1, ME1, SRRM2, EDEM3, LARP5, HYAL3, RFX5, HLTF, ZBTB34, SQRDL, ARL1, PROM2, NRP1, FAM129A, RAB3GAP1, CRLS1, CYP2U1, ATF3, CALML4, TTLL6, KLHL23, HLA-E, YEATS2,ARID3A, KIF5B, HSD17B4, ELF3, RAP1GAP, MEF2A, PODXL2, RAI14, NPAT, ARHGEF2, ARF1, ECOP, PSCD1, HSPA4L, CRY1, MAP2K6, SNAPC4, CDK6, SNAP25, RAG1AP1, NAT6, IQWD1, WARS, PPP2CB, CYC1, KYNU, CYBASC3, STARD3NL, LIPA, CDH26, ACO1, C1orf128, CD46, C17orf48, PNPLA8, ILF3, ADRM1, PEX6, MCEE, TXNDC16, IQCB1, ATF4, TBX15, COASY, VIPR2, SEL1L, EIF4B, TAX1BP1, OGT, RARA, ANKRD16, PML, TLE1, PRIM1, ACADSB, IGF2, ETFDH, SNAP23, MXI1, GSTK1, YIPF1, GALK2, HES6, SAP18, LIMCH1, MMAA, CCNG1, ACAT2, TROVE2, ELOVL6, MDK, KHDRBS3, COL14A1, ADK, HIST1H4F, CRYZ, UBAP2L, SLC25A33, PAPSS1, TRIM32, CYP51A1, COPG, MGAT4A, PPAPDC2, SNIP, RAVER2, PPFIBP2, SRGAP3, ZNF137, RPL13A, LY6E, SCNN1A, C16orf61, PLD3, UPP1, SC5DL, SMARCA2, UBP1, LGALS8, DDR1, KLF10, NME3, HIST1H3A, PMM2, ANXA4, ATIC, PRKCA, MAL2, RPS6KA3, RAB8A

was highlighted that these androgen-repressed genes may potentially inhibit prostate tumorigenesis. Furthermore, one of these androgen-repressed genes, H-Cad was experimentally evaluated and shown to function as a potential tumor suppressor in human PCa.

Androgen-regulated molecular pathways and cellular functions

Alongside the discovery of a comprehensive list of androgen-responsive genes, the downstream molecular pathways and cellular processes that are controlled by androgen became apparent. Initially, more than fifty androgen-regulated genes were identified by SAGE in LNCaP cells (6). Functional annotation revealed that a majority of them are involved in the regulation of transcription and energy metabolism. In addition, a significant number of genes regulating cell cycle, signal transduction and cellular protein trafficking were found to be induced by androgen, supporting the role of androgens

in promoting survival and growth of prostatic epithelial cells. Subsequently, a microarray study by Deprimo *et al*. analyzed the gene expression program induced by R1881 in LNCaP cells and found that a significant portion of androgen-regulated genes are related to the production of seminal fluid (9). In accordance, Nelson *et al*. also found that androgen-responsive genes contribute to metabolism, protein trafficking, cell proliferation and differentiation (19). Study by Massie *et al*. highlighted that calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2) is overexpressed in PCa and acts as an important effector of AR signaling in controlling anabolic metabolism, cell proliferation and cell migration/invasion of PCa cells (12). In summary, a remarkable fraction of the genes induced by androgen appeared to be related to (I) production of modification of secretary proteins, protein folding, trafficking, and secretion (6,8,9); (II) cell-cycle, metabolism and biosynthetic pathways (6,12,20,21); (III) regulation of transcription factors such as GATA2, PDEF, ETV1, CREB3L4, HOXB13 and NKX3.1.

Androgen-regulated genes play important roles in PCa

Not only is androgen signaling indispensable to prostate development and function, it is also a key driver of PCa initiation and progression. Understanding of androgen-regulated genes/pathways is thus a first step towards the development of novel biomarkers and therapeutic targets of PCa. Accompanying the identification of a large set of androgen-regulated genes using cell line and animal models, studies have attempted to characterize the expression and function of some of these target genes in PCa. For example, Velasco *et al*. demonstrated that FKBP51, one of androgen-regulated genes identified by an oligonucleotide array study in LNCaP cells, was expressed significantly higher in prostate tumor samples relative to benign prostatic hyperplasia samples through tissue microarray immunohistochemistry (IHC) staining. FKBP51 may be as a potential diagnostic marker for PCa (10). Numerous studies have also comparatively analyzed the expression profile of androgen-regulated genes in model systems and in clinical specimens derived from PCa patients at different developmental stages or with varying treatment histories. For instance, Holzbeierlein *et al*. and Mostaghel *et al*. determined genes that were differentially expressed in human PCa specimens following androgen deprivation therapy (ADT) (22,23). More than 20% of these genes also showed significant expression changes in LNCaP cells after

36 h of hormone-deprivation (22). On the other hand, there were also many that were not down-regulated after short-term castration in LNCaP cells, indicating that androgen deprivation may be insufficient to completely suppress androgen activity, which may contribute to PCa cell survival in a low androgen environment (23). In addition, for the most part, these two human PCa tissue profiling studies revealed very few common genes (less than 5%), suggesting that androgen-responsive genes may vary considerably among individuals.

Besides analyzing androgen-responsive genes, a significant research area has been to determine genes that are differentially expressed in castration-resistant prostate cancer (CRPC) and thus may be responsible for the development of castration resistance. AR signaling pathways may differ between androgen-naïve and castration-resistant PCa. For example, Wang *et al*. reported that M-phase cell-cycle genes including UBE2C were upregulated by AR specifically in castration-resistant LNCaP-abl cells but not in androgen-sensitive LNCaP cells. Tissue microarray analysis further demonstrated that UBE2C protein overexpression correlates with the occurrence and progression of PCa (21). Interestingly, a recent study revealed that this increased expression of UBE2C may be due to induction by constitutively active AR splice variants, AR-V7 and ARV567ES, that were found in CRPC cells (24).

Besides regulation of downstream gene expression, groundbreaking studies have recently revealed an innovative mechanism of AR in promoting PCa through inducing chromosomal translocations. Back in 2005, Tomlins *et al*. made the seminal discovery of genomic translocations that juxtapose the androgen-responsive TMPRSS2 gene promoter with the oncogenic transcription factor ERG, leading to outlier profile of ERG expression in a subset of localized as well as metastatic human PCa (25). Further studies from this and other groups have in the subsequent years characterized a set of recurrent chromosomal translocations in PCa, a majority of which involve an androgen-responsive promoter and an oncogene (26-30). Among these, TMPRSS2-ERG gene fusions were the most frequently identified and have been shown to occur in 40-80% of PCa (31,32). Very interestingly, studies have later shown that these PCa-specific gene fusions were indeed generated by AR-induced chromosomal proximity, cellular stress, double-stand break, and erroneous DNA repair (33-35). Although the exact mechanisms and functions of these gene fusions in PCa are yet to be fully characterized, they undoubtedly represent an important pathway by which

androgen regulates PCa.

New-generation targets of androgen

In addition to regulating conventional genes, AR's transcriptional activities may also be manifested by mRNA alternative splicing events. In a study using exon array analyses of the LNCaP transcriptome, Rajan *et al*. were able to simultaneously identify global androgen-dependent gene expression and alternative mRNA isoform expression (36). Among more than 1,000 putative androgen-regulated alternative events, several validated events were derived from alternative promoter selection and direct binding by the AR, while others may be due to indirect effects of androgen. In addition, the AR itself may be alternatively spliced to expressed AR variants (37,38). Some of these AR variants have been shown up-regulated in aggressive PCa and may be associated with castration resistance (24,39,40). It is believed that these AR variants are able to turn on an alternative AR program thereby regulating distinct set of genes, which are important research areas currently under intensive investigation. Recent development of RNA-seq technology has further revolutionized the studies of whole transcriptomes, providing potentially unlimited measure of all transcripts and splicing variants that are expressed in a cell type. The Fu's group pioneered in using RNA-seq to screen androgen-responsive transcripts in PCa cells. With a sequencing depth of ~10 million of 36-nt sequence tags per sample, they identified ~700 androgen-regulated genes in LNCaP cells and a large fraction of tags corresponding to alternative exons (41).

In the past few years, evidence has accumulated that non-coding RNAs (ncRNAs), like coding genes, may play similarly important roles in regulating cellular functions. The ncRNAs have been shown highly abundant and functionally important in a number of essential cellular processes (42-44). Abnormal expression patterns of ncRNAs have been detected in a variety of human diseases including PCa. In addition, some ncRNAs have been shown to have prostatic-specific expression pattern. These includes microRNAs (miRNAs) (45), small nucleolar RNA (snoRNA) (46), and long non-coding RNAs (lncRNA) (47). For instance, Ribas *et al*. reported 16 miRNAs that were induced by androgen in both LNCaP and LAPC4 cells, and provided evidence that elevated expression of miR-21 promotes enhanced androgen-dependent tumor growth and castration resistance *in vivo* (48). Most recently, by genome-wide RNA profiling of LNCaP cells across 9 time points from 0 to 48 h following 10 nM DHT treatment, more than a hundred miRNAs were found to respond to androgen, among which 3 miRNAs (miR-19a, miR-27a and miR-133b) were found to be induced by androgen. These miRNAs were found to be directly regulated by AR and play essential roles in regulating cell viability (49). Further, these miRNAs may regulate the expression of a large set of mRNAs, which were previously found to respond to androgen.

Some efforts have also been made recently to identify AR-regulated small ncRNAs in PCa cells. Louro *et al*. detected approximately 40 intronic antisense ncRNAs that were up-regulated by androgen in LNCaP cells (50). Functional ARE motif has been reported at the upstream region of some of these ncRNA such as that mapped to MYO5 locus and Combining Chromatin Immunoprecipitation (ChIP)-PCR confirmed androgen-activated AR binding to this loci. These intronic transcripts may be involved in transcriptional regulation similar to miRNAs, but their biological functions remain undetermined.

Through transcriptomic analysis of PCa specimens, several prostate-specific lncRNA have been reported, including PCA3 (51), PCGEM1 (52), ANRIL (53) and PCAT-1 (54). Although an increasing number of new lncRNA has been identified in the last few years, a majority of these lncRNA has not been functionally characterized, which will be important lines for future investigation. Interestingly, an androgen-responsive lncRNA CTBP1-AS, located in the AS region of C-terminal binding protein 1 (CTBP1), was recently reported. It was shown promote AR transcriptional activity through suppression of CTBP1 (55). This provocative study suggests an innovative basic regulatory pathway involving an antisense lncRNA counteracting the activity of its corresponding coding gene.

Genome-wide location analysis of AR in PCa

AR regulates target genes through binding to cis-regulatory elements

Androgen regulates downstream genes by acting as a ligand of the hormonal transcription factor, the AR. Once liganded, AR translocates into the nucleus, where it homodimerizes and binds directly to DNA through the ARE. The consensus AR-binding motif (i.e., canonical AREs, AGAACAnnnTGTTCT) consists of two hexameric half-sites (5'-AGAACA-3') often arranged as inverted repeats with 3bp of separating nucleotide. AR recognizes and interacts with AREs through its DNA-binding domain
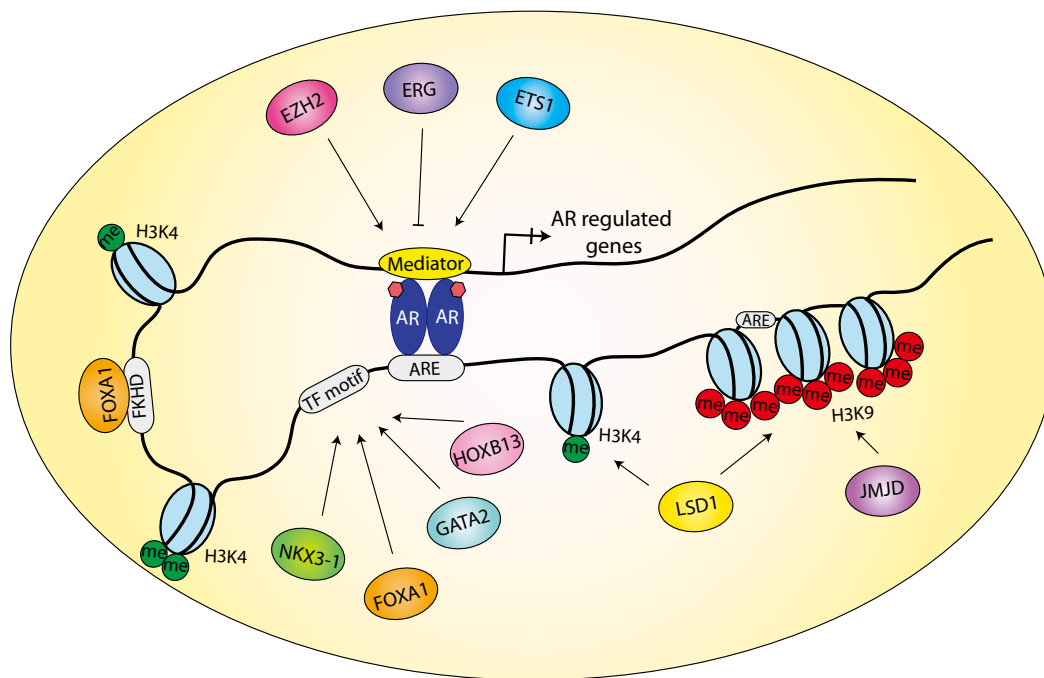
Figure 1 The cooperative regulatory network that controls AR chromatin binding and prostate gene expression. The genomic AR binding and expression of AR-responsive genes are tightly regulated by various mechanisms including histone modifications, pioneer factors, transcription factors, epigenetic modifiers and other AR coregulators, as well as long-range chromatin interactions and loopings.

(DBD). In addition to ARE, the selective binding of AR across the genome is tightly regulated by a collection of transcription co-factors and/or pioneering factors. Accumulating evidence suggests that AR primarily binds distal enhancers that can be several kb to over 100 kb away from the promoter regions of coding genes. These AR-bound enhancers have been shown to interact with the promoters through chromatin looping (56,57) (*Figure 1*). AR recruits the translation initiation complex and regulates transcription through interaction with as many as over 150 co-regulators, some of which are co-activators while others are co-repressors. In order to fully understand AR-mediated transcriptional regulation, in the past decade researchers have put forth significant efforts to determine the DNA binding patterns and the genomic landscapes of AR and its cofactors, taking advantage of modern technologies.

DNA microarray analysis of AR binding sites

Immediately following the completion of the human genome project, DNA microarrays were invented to contain oligonucleotides complementing to selected regions of the genome or tiling through the entire genome. For example,

promoter microarrays contain oligonulceotides that span through the promoters of all annotated genes. ChIP with DNA microarrays, termed ChIP-on-chip, researchers begun to determine whether AR binds to any of the regions represented on the DNA microarrays. Using ENCODE tiling arrays Takayama *et al.* identified 10 AR binding sites and subsequently validated AR recruitment to and regulation of these genes (58). In a separate study, Massie *et al.* used proximal promoter microarrays to identify 1,532 genomic sites that were occupied by AR. Motif analysis revealed that half ARE and ETS motifs were enriched within these regions, which led to the discovery of cooperated regulation of target genes by AR and ETS-family transcription factor ETS1 (59). Using custom oligonucleotide tiling arrays covering approximately 104 kb genomic regions centered on the TSS of 548 pre-selected potential androgen-regulated genes, another report demonstrated that a large proportion of AR binding sites are not within the proximal promoter regions, rather they are often more than 10 kb away from the TSS of androgen-responsive genes (60). In addition, many androgen-responsive genes were found to harbor two or more AR binding events within their regulatory regions. Using Affimetrix microarrays tiling

through the chromosomes 21 and 22, Wang *et al.* identified 90 ARBS in the LNCaP cells, most of which were also far away from the promoter of androgen-responsive genes (61). Similarly using tiling arrays, another group examined AR binding to chromosomes 19 and 20 in C4-2B cells (62). They reported that there were no androgen-responsive genes at the vicinity of a majority of these ARBS, indicating that they may not be functional in terms of transcriptional regulation. However, H3 acetylation at these loci could be used to define the subset of functional ARBS associated with androgen-responsive genes (62).

Although above studies have analyzed AR binding events at selected regions, it was not only very recently has genome-wide mapping of ARBS become possible. Using whole-genome tiling arrays, Wang *et al.* pioneered in mapping global ARBS in PCa cells (21). They identified approximately 8,000 and 6,000 ARBS genome-wide in LNCaP and LNCaP abl cells, respectively. Comparatively analysis revealed a set of abl-specific AR binding events that lead to upregulated expression of genes involved in cell cycle, one of which is UBE2C. This study revealed that a distinct AR transcriptional program is associated with CRPC. This rapid expansion of ARBS from tens to thousands has also greatly advanced the understanding of AR-chromatin interaction. Although AR is thought to have high affinity binding with canonical full AREs, a majority of the ARBS were found to bind primarily half AREs (59-61). For example, while 78% of ARBS identified in LNCaP cells were found to harbor at least one half ARE, only 10% contained a full ARE (61). Similar results were observed in an independent study which showed that 79% and 27% of ARBS respectively contained half ARE and full ARE (59). Likewise, Chen *et al.* reported that 4% and 46% of the ARBS had the canonical ARE and half ARE, respectively, in an independent PCa cell line 22Rv1 (63). Moreover, the majority of these non-canonical half ARE ARBS may be functional. *In vitro* oligonucleotide pull-down assay and ChIP analysis confirmed that AR is able to bind to these 'half-sites' (59), while reporter assays verified that they mediate significant androgen-induced luciferase activity (61). Therefore, like full AREs half AREs are able to recruit AR to regulate transcription. However, it remains to be determined what fraction of these AR binding events is functional and what the determinants are.

High-resolution mapping of AR genomic landscape

Although ChIP-on-chip assays are potentially able to provide genome-wide protein location provided genome-wide tiling arrays were utilized, such approaches are usually very costly and time consuming. It was not until very recently that genome-wide mapping of protein-DNA interaction became easily feasible for regular labs due to the development of ChIP-seq, which couples ChIP with massively parallel next-generation sequencing (64,65). Unlike ChIP-on-chip, ChIP-seq is in the most part able to provide protein occupancy across unlimited genome with much increased sensitivity and accuracy. ChIP-seq of AR in PCa cells was first carried out in the PC3 cell line that expresses ectopic AR (66). This study suggested that as many as 800 genes may contain an AR binding site within their cis-regulatory elements and are responsive to androgen treatment. Unlike in LNCaP cells, ectopic AR appears to directly induce genes of the growth-inhibition response program in PC3 cells, being consistent with its reported role in this particular condition.

At the mean time, we become the first to map genomic landscape of endogenous AR in PCa cells using ChIP-seq (67). We identified more than 37,000 and 13,000 ARBS respectively in the androgen-sensitive LNCaP and VCaP cells, covering nearly all ARBS that have been identified previously using various technologies. Approximately 60% of the ARBS found in the VCaP cells overlapped with those identified in LNCaP. The fewer number of ARBS identified in VCaP cells is likely due to technical issues as ChIP-PCR was able to detect a number of ARBS that were only detected in LNCaP by ChIP-seq. Being consistence with previous results, ChIP-seq data also demonstrated that a majority of AR binds distal enhancers and intragenic regions, with less than 10% binding to promoters. De novo motif search of all ChIP-seq ARBS revealed a consensus sequence highly resembling the canonical ARE.

Recently, multiple groups have reported thousands of AR binding events in PCa cells such as LNCaP and VCaP (12,68-72). Significant overlap of ARBS between these cell lines have been observed and AR was consistently found to primarily bind distal enhancers that can be more than 50 kb away of any coding genes. However, Massie *et al.* reported that genes located within 25 kb of an AR binding event were the most significantly enriched for androgen-regulated expression (12). Moreover, AR ChIP-seq has also begun to be carried out in breast cancer cell lines such as MDA-MB-453 cells with the intention to examine the role of AR in breast cancer (68,72).

In summary, combinatorial efforts using ChIP-on-chip

and ChIP-seq have resulted in high-resolution mapping of the genomic landscape of AR in a variety of AR binding sites (*Table 3*). It will be of great interest for future studies to determine how AR binding profile changes during development and diseases. In addition, future technological advances, such as ChIP-exo, may be able to determine base-pair level mapping of AR binding sites, which may further enhance our understanding of AR transcriptional regulation (73,74). Most studies have thus far unanimously found that AR binds to distal enhancers, suggesting a model wherein chromatin-looping brings the AR-bound enhancers to the proximity of a target promoter, thereby regulating transcription (21,56,61) (*Figure 1*). Recently, Hi-C and chromatin interaction analysis with paired-end tag sequencing (ChIA-PET) have been successfully used to uncover three-dimensional organization of the genome and global long-range chromatin interactions (75,76). Yet, it remains largely undetermined where these chromatin loopings occur. In addition, as AR primarily binds to enhancers which can be tens or hundreds of kb away from a target gene, it has been challenging to pinpoint the target gene of a particular binding event. Although 3C-based assays are useful in demonstrating chromatin looping, functional assays are missing to decisively show the regulation *in vivo*. This, however, has become increasingly plausible with the development of genome editing tools including transcription activator-like effector nucleases (TALENs) and clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas-based RNA-guided DNA endonucleases (77-79). We thus believe that genome-wide mapping of AR binding events is just a first step towards the elucidation of AR transcriptional regulation and intriguing results will soon emerging in the near future.

Genomic regulation of AR binding profile

Pioneering factors define AR binding profile and prostate gene expression

Functional AR binding sites were not only determined by sequence motifs but also chromatin accessibility. Single-gene based approaches have already demonstrated the importance of chromatin-opening transcription factors such as FOXA1 in regulating AR binding to target genes such as PSA (80). FOXA1 is able to directly bind to the chromatin to open up the local nucleosomal domain (81). In prostate cells, FOXA1 protein has been shown physically interact with the AR protein and plays critical roles in regulating the

transcription of prostate genes such as PSA (80). Following recent mapping of genome-wide ARBS, the mechanisms underlying AR recruitment to genomic loci have also become increasingly explicit. Studies from the laboratories of Dr. Myles Brown and others have established a model wherein the interactions between epigenetic modifications, pioneering factors, and AR define prostate-specific AR binding profile (61,82-84). Histone modifications such as histone H3 Lys4 mono- and di-methylation (H3K4me1 and H3K4me2) exhibit distinct genomic landscapes between prostate and breast cells and are thought to guide cell type-specific recruitment of FOXA1. The binding of FOXA1 induces chromatin accessibility, which subsequently enables AR binding to these pre-selected sites. Other active histone marks such as histone acetylation have also been associated with AR binding and androgen-induced expression (62). By contrast, AR is much less likely to bind chromatin regions marked by repressive histone modifications, such as H3K9me1 and H3K9me2 (84). Once AR binds target promoters and enhancers, they form DNA loops that coordinately assemble a multi-protein transcription complex (*Figure 1*) (85-87).

Counter-intuitively, recent studies showed that FOXA1 knockdown in PCa cells did not result in global inhibition of AR binding (70,71). Rather it resulted in an overall increase in AR binding events accompanied by a significant redistribution. This suggests that while FOXA1 mediates AR-binding to some genomic regions, it primarily exhibits an inhibitory role on a majority of other potential AR binding sites. It appears that FOXA1 defines a prostate-specific AR binding profile by restricting/facilitating AR binding to specific sites (e.g., those containing both ARE and FKHD motifs), while inhibiting/reducing its binding to other sites (e.g., those containing only ARE motifs) (*Figure 1*). The detailed mechanism as to how FOXA1 regulates AR binding profile, however, remains largely unclear and entails careful investigation.

Besides FOXA1, several other transcription factors such as GATA2 and HOXB13 may have similar pioneering cofactor effects on AR-chromatin binding and transcriptional regulation. Similar as FOXA1, GATA family transcription factors have also been shown to open compact chromatin through their conserved zinc finger domains (81,88). In addition, GATA2 expression is also essential for AR-mediated transcription of prostate genes such as PSA and TMPRSS2 (61,89). Likewise, HOXB13 is a member of the homeodomain family of sequence-specific transcription factors. Mouse studies have shown that HOXB13 play an

Table 3 Global investigation of AR binding in prostate cancer genome

Study	Samples	Method	Results
Takayama *et al.* 2007	LNCaP	ChIP-on-chip	(I) Identified 10 ARBSs from 30 Mb human genomic DNA; (II) most of the ARBSs were located within intronic regions or gene upstream regions at least 10 kb apart from the transcriptional start sites; (III) all of the ARBSs contain canonical ARE sequences
Wang *et al.* 2007	LNCaP	ChIP-on-chip	(I) Identified 90 ARBS from chr21 and chr22; (II) most of ARBS are far from the promoters of ARG; (III) 78% of ARBS contain half ARE, 10% have a canonical ARE; (IV) noncanonical AREs mediate AR-dependent transcription; (V) collaborating factors may assist AR in binding to noncanonical AREs
Massie *et al.* 2007	LNCaP	ChIP-on-chip	(I) Identified 1,532 ARBS; (II) 410 (26.8%) of the 1,532 AR promoter-binding sites containing canonical AREs; (III) AR 'half-site' occurred in 1,212 (79.2%) of the AR candidate promoter sequence; (IV) *In vitro* oligonucleotide pull-down assay showing that the AR can bind directly to these 6-bp 'half-sites'; (V) ChIP analysis showed androgen-dependent recruitment of the AR to 'half-sites' at a level similar to that of the KLK2 promoter
Bolton *et al.* 2007	HPr-1AR	ChIP-on-chip	(I) Identified 524 AR binding regions; (II) 67% of our AREs resided within ~50 kb of the TSS of 84% of our ARGs; (III) most ARGs were associated with two or more AREs; (IV) AREs appeared typically to be composite elements, containing AR binding sequences adjacent to binding motifs for other transcriptional regulators
Jia *et al.* 2008	C4-2b	ChIP-on-chip	(I) Identified 62 highly reproducible ARORs, 32 (52%) of them were also marked by AcH3, (chromosome 19 and 20); (II) analysis of the AROR sequences, identified binding sites for AR transcriptional coregulators FoxA1, CEBPβ, NFI and GATA2
Wang *et al.* 2009	LNCaP, Abl + DHT	ChIP-on-chip	(I) Identified 8,708 from LNCaP, 6,353 from abl; (II) the level AR occupancy at target sites is greater in LNCaP cells than in abl cells; (III) greater occupancy of AR binding near abl-specific AR upregulated cell-cycle genes and M-phase genes in abl cells than in LNCaP cells; (IV) greater levels of AR binding are correlated with higher expression of target cellcycle and M-phase genes in abl; (V) CDC20, UBE2C, CDK1, and ANAPC10 are preferentially AR-occupied, highly express in abl as compared with LNCaP in the presence of DHT and have significant AR occupancy in the absence of hormone only in abl and not in LNCaP; (VI) 3C-PCR revealed significantly greater interaction between these two putative enhancers (−32.8 K, +41.6 k) and the UBE2C promoter in abl cells than in LNCaP cells in the absence of hormone
Chen *et al.* 2010	CWR22Rv1, 10 nM DHT 2 hr	ChIP-on-chip	(I) A total of 1,225 and 2,021 AR-binding sites (FDR ≤0.05) were identified in R1 and Rv1; (II) in Rv1 cells, only 4% (86/2,021) of the sites had the canonical ARE and 35% (700/2,021) had the AR half-site motif; (III) in R1 cells, 6% (76/1,225) of the sites had the canonical ARE and 46% (568/1,225) had the AR half-site motif; (IV) canonical ARE is not required for AR binding in the majority of the genes examined, and that the half-site is sufficient for AR function
Lin *et al.* 2009	PC3-AR	ChIP-seq	(I) revealed 6,629 ARBS; (II) 22.4% of ARBS can be mapped to within 2 kb of the transcription start site; (III) a total of 859 genes are changed in expression levels in response to androgen treatments, containing AR binding sites; (IV) most significantly enriched GO term of up-regulated genes in PC3-AR cells is negative regulation of biological process; (V) the GO terms enriched by the down regulated genes include GO terms involved in transport and cellular localizations, and in general metabolic process
Yu *et al.* 2010	LNCaP, VCaP,10 nM R1881 16 h	ChIP-seq	(I) Identified 37,193 ARBS in LNCaP cells, 12,965 ARBS in VCaP cells; (II) canonical ARE motif was the most enriched; (III) the binding sites containing full ARE motifs had significantly higher enrichment peaks than those with half ARE motifs; (IV) approximately 40% of all AR binding sites contained at least one ARE motif and about 29% contained an ETS motif

Table 3 (*continued*)

Table 3 (*continued*)

Study	Samples	Method	Results
Massie *et al*. 2011	LNCaP, VCaP,1 nM R1881, 4 h	ChIP-seq	(I) Identified 11,053 AR binding sites in LNCaP cells and 51,811 androgen-dependent AR binding sites in VCaP cells; (II) over 90% of the LNCaP AR binding sites were found in the VCaP cells; (III) we identified 15,761 androgen-dependent RNAP II regions in LNCaP cells using ChIP-seq, 1,283 of which overlapped with androgen-stimulated AR binding sites
Sahu *et al*. 2011	LNCaP-1F5, 100 nM DHT, 2 h	ChIP-seq	(I) Identified 17,022 ARBs (LNCaP-1F5 siFoxA1) as opposed to 6,215 ARBs (LNCaP-1F5 parental); (II) 43% of the parental cell ARBs (2,698 sites) were lost upon FoxA1 depletion. Importantly, 13,505 completely new ARBs were found in siFoxA1 cells; (III) in VCaP cells, close to 32,000 new ARBs were found in siFoxA1 cells, and around 6,000 ARBs were lost upon FoxA1 depletion; (IV) the ARBs in parental LNCaP-1F5 cells exhibited 87% overlap with those in parental VCaP cells; (V) the ARBs independent of FoxA1 contained a top-scoring ARE; (VI) top-scoring cis-element for ARBs that required FoxA1 pioneering, containing an ARE half-site and a partial FoxA1 motif
Wang *et al*. 2011	LNCaP +/– DHT	ChIP-seq	(I) Identified 3,115 ARBS (65% co-incident with H3K4me1, 54% of co-occupied by FoxA1) in LNCaP cells; (II) approximately 60% of the original AR binding events were 'expectedly' lost in response to FOXA1 RNAi, 40% of AR binding events as the 'conserved' AR program. A massive gain of 10,869 new AR binding loci, referred to as the 'gained' AR program; (III) FoxA1 may facilitate AR binding to its original binding program, but transrepress AR from binding to other genomic regions that lack FoxA1-binding sites in the gained program
Ni *et al*. 2011	MDA-MB-453, 10 nM DHT, 4 h	ChIP-seq	(I) Identified 2,406 ARBS, the majority of the binding sites are cell-specific; (II) predominantly at distal intergenic and intronic regions; (III) A significant overlap (37%) between the AR and FOXA1 cistromes in MDA-MB-453 breast cancer cells; (IV) motif enrichment analysis of the AR cistrome revealed ARE motifs and FKHD motifs; (V)
Robinson *et al*. 2011	MDA-MB-453, LNCaP	ChIP-seq	(I) Identified 22,439 ARBs in MDA-MB-453 cells, 26,847 ARBs in LNCaP cells; (II) the Forkhead motif was also enriched at the centre of the AR binding regions, implying potential cooperativity between AR and Forkhead proteins in mediating AR binding; (III) ARBs to be a near complete subset of the FoxA1 binding regions, with 98.1% of all AR binding events over-lapping with a FoxA1 binding region; (IV) all AR binding events in molecular apocrine breast cancer cells may be mediated by FoxA1; (V) FoxA1 and AR in the LNCaP prostate cell line also have a high level of concordance (82%) while FoxA1 and ER in MCF7 breast cancer cell only overlap by 52%
Andreu-Vieyra *et al*. 2011	LNCaP, 4 h with 10 nM DHT	ChIP-seq	(I) Identified 4,357 ARBS; (II) about 20% of the AR-occupied regions were associated with AcH3; (III) nucleosome-depleted regions (NDRs) are present in TMPRSS2 and KLK2 enhancer modules in the absence of ligand, or after long-term androgen withdrawal. This NDRs percentage increases after short-term treatment with DHT; (IV) GATA-2 is important for NDR maintenance at theTPMRSS2 enhancer in the absence of hormone
Tan *et al*. 2012	LNCaP, 100 nM DHT, 2 h	ChIP-seq	(I) Identified 18,117 and 75,296 AR binding sites (ARBS) before and after DHT treatment in LNCaP cells; (II) a canonical ARE motif that was significantly enriched in the ARBS from LNCaP cells treated with DHT; (III) approximately 41% of the ARBS contain full AREs, whereas 19% harbor half AREs; a large fraction of the ARBS (40%) lacked either motif; (IV) AR resided mainly at distal regions of known genes; (V) NKX3-1 is colocalized with AR near androgen-regulated genes in prostate cancer cells
Xu *et al*. 2012	abl, EtOH	ChIP-seq	(I) LNCaP abl AR chromatin binding was enriched at the center of EZH2 solo peaks but not at ensemble peaks; (II) a robust physical interaction between EZH2 and AR in abl cells; (III) EZH2 solo peaks in abl cells significantly overlapped with AR global binding, and EZH2- and AR-activated genes also overlapped significantly in abl cells

Table 3 (*continued*)

Table 3 (*continued*)

Study	Samples	Method	Results
Decke *et al*. 2012	LNCaP,abl, EtOH; LNCaP 10 nM; DHT 4 hr; abl, 10 nM DHT 16 h	ChIP-seq	(I) Identified 7,135 AR binding sites with statistically increased binding in LNCaP DHT+ cells as androgen-dependent occupied regions (AD-ORs); (II) the 896 sites with statistically increased binding in C4-2B DHT cells as androgen-independent occupied regions (AI-ORs); (III) whereas the vast majority of AD-ORs are located at intergenic and intronic regions in line with previous findings, 54% of AI-ORs are at promoters, exons and tRNA genes; (IV) motif analysis showed that both canonical ARE and FoxA1 motifs are not enriched at AI-ORs; (V) AI-ORs are preferentially located at genomic loci with constitutively open chromatin structures; (VI) AI-ORs possess AR-dependent enhancer activity in CRPC cells (Knockdown of AR resulted in a decrease of basal enhancer activity at 9 out of 10 AI-ORs in C4-2B cells; DHT significantly inhibited enhancer activity at AI-ORs in C4-2B cell); (VII) AI-ORs directly interact with AI-upregulated genes, which are required for CRPC growth; (VIII) AI-upregulated genes are enriched for cell cycle functions and overexpressed in CRPC tumors
Chng *et al*. 2012	VCaP cells at 0, 2 and 18 h after 100 nM DHT stimulation	ChIP-seq	(I) AR, ERG ChIPseq in VCaP cells at 0, 2 and 18 h after 100 nM DHT; (II) the substantial overlap of the AR and ERG cistromes suggests transcriptional collaboration between them; (III) HDACs (HDAC1,HDAC2,HDAC3) and EZH2 function together with ERG and AR to attenuate androgen-dependent transcription
Sharma *et al*. 2013	12 tissue samples, LNCaP, VCaP, 22RV1	ChIP-seq	(I) Identified AR-occupied regions in 12 tissue samples and 3 cell lines; (II) a unique AR transcriptional program exists in PC tissue; (III) divergent transcriptional complexes are present at ARBS *in vitro* and *in vivo*; (IV) a clinically relevant signature is identified from PC tissue

essential role in prostate development (90). Through analysis of several candidate genes, HOXB13 has been shown a multifaceted regulator of AR-chromatin interaction, similar as FOXA1 (91). Specifically, HOXB13 was found to inhibit transcription of genes regulated by AR binding to ARE, but enhances AR binding to cis-regulatory regions containing HOX elements juxtaposed to AREs, thereby inducing corresponding gene expression. How GATA2 and HOXB13 regulate genome-wide AR binding profile, however, are yet to be carefully examined.

Co-factor regulation of AR transcriptional activity

AR transcriptional activity is tightly controlled. Over the years, a significant number of cofactor proteins have been identified that regulate AR signaling by remodeling the chromatin structure or affecting the recruitment of RNA polymerase II to the promoters of target genes. These include but are not limited to p160 family of transcription factors, CBP/P300, HDAC, CARM1 and LSD1 (92). In addition, PDEF, a prostate epithelial-specific ETS transcription factor, was reported as a co-regulator of AR, leading to enhanced transcription of PSA gene (93). Using

ChIP-Seq, genome-wide location analyses have recently enabled more accurate characterization of cofactor co-occupancy at subset of target genomes (59,61,67,83,91,94). For instance, motif analysis revealed that ETS motif and AR half-sites co-occur in approximately 70% of AR-bound promoter regions. This led to further experimentation showing that ETS1 protein physically interacts with AR and enhances AR transactivation (59). Similarly, through motif analyses of AR binding sites found in VCaP cells we reported ETS motif as the 2nd most enriched motif, only ranked after the AREs (67). ChIP-seq analysis of ERG showed that it indeed co-occupied a majority of the ARBS in VCaP cells. Mechanistic studies revealed that ERG protein physically interacts with AR but surprisingly inhibits AR transcriptional activity, thus acting as a co-repressor. This role of ERG in inhibiting AR-mediated prostate gene expression has later been independently validated by multiple studies (95,96).

In addition to ETS family cofactors, genome-wide location studies have yielded the discovery or validation of a number of other AR cofactors at the genome scale. For instance, OCT and GATA motifs were found most enriched in AR binding sites found in chromosome 21 and

22 through ChIP-on-chip assay (61). GATA2 and OCT1, together with AR, form a regulatory hierarchy that governs androgen-dependent gene expression. In a separate study, analysis of ChIP-seq data discovered that 92% of the NKX3-1 binding sites overlapped with the ARBS across the PCa genome (94). NKX3-1 is a homeobox transcription factor that contributes to prostate tumor progression. This study showed that NKX3-1 and AR directly regulate each other through a feed-forward loop. Moreover, NKX3-1 collaborates with AR and FOXA1 to mediate gene expression in advanced and recurrent prostate carcinomas.

Collectively, these ChIP-seq studies have been valuable in identifying novel AR cofactors and in revealing the cooperative regulatory network that controls AR chromatin binding and prostate gene expression (*Figure 1*). These AR collaborating cofactors often exhibit some common characteristics such as: (I) they often physically interact with AR; (II) they frequently co-occupy the genome with the AR; (III) they regulate the transcription of AR target genes; (IV) they might directly regulate the expression of AR itself (e.g., ERG, GATA2) or might themselves be a target of androgen or AR (e.g., GATA2, NKX3-1); (V) they usually play critical roles in prostate development (e.g., ERG, FOXA1, HOXB13, and NKX3-1). These results suggest a complex network of transcription cofactors that altogether tightly controls AR-chromatin interaction and androgen-mediated gene expression. Alterations to this regulatory network might result in the disruption of AR signaling and consequently lead to PCa.

AR as a transcriptional repressor

Although a majority of studies of AR signaling and genomic regulation have focused on androgen-induced genes, microarray analyses of androgen response have consistently revealed genes that are down-regulated by androgen. In fact, AR itself was found to be repressed by androgen in VCaP cells (97). Very few studies, however, have examined androgen-repressed genes such as c-MET (98), SOX2 (99), and DDC (100). Androgen-repressed genes as a while have not been carefully investigated in the past likely due to the difficulty in determining distal AR-binding enhancers on these genes. With the use of ChIP-seq technology to map AR binding across the entire genome, AR-repressed genes have lately become the focus of multiple studies. For example, an AR binding site was found within the intragenic region of the AR gene itself (97). AR binding to this site represses AR gene expression via recruitment of LSD1 and

demethylation of H3K4me1 and me2.

Recently, we have systematically examined AR binding on the regulatory elements of androgen-induced and—repressed genes in LNCP cells (101). We report that AR can act as a transcriptional repressor to directly inhibit gene expression. This repression is mediated by AR binding to AREs and facilitated by EZH2-mediated repressive chromatin remodeling. EZH2 thus cooperates with AR in transcriptional repression of target genes. Through meta-analysis of microarray datasets profiling androgen-treated PCa cells, we have nominated a number of robust AR-repressed genes (57). These genes may have important cellular functions and their repression by androgen may be critical for prostate physiology and disease. They are important genes for further examination which may lead to the development of novel biomarkers and therapeutic targets of PCa.

AR genomic regulation in CRPC

Altered AR transcription program in CRPC

AR is a key driver of PCa progression. The expression and transcriptional activity of AR remain required and sufficient to CRPC growth. Numerous studies have attempted to understand the mechanisms underlying AR activity in an androgen-depleted milieu. Through ChIP-on-chip comparison of AR binding in LNCaP and abl cells, Wang *et al.* has demonstrated that AR acquired new binding sites and regulates a distinct transcriptional program that is responsible for CRPC cell growth (21). Similarly, Decker *et al.* investigated genome-wide AR binding in LNCaP and C4-2B cells under the androgen-deprived conditions to understand how AR functions in CRPC (102). Like Wang *et al.* study, this study also revealed that AR persistently occupied a set of genomic regions in the absence of androgen in CRPC cells that were void of AR binding in LNCaP cells. Interestingly, these androgen-independent ARBS have constitutively open chromatin structure, often locate at promoter regions, lack ARE motif, and are independent of FOXA1. These data suggested that androgen deprivation may result in a dramatic alteration of genome-wide AR binding profiling and that nonconventional AR binding sites may be acquired (21). It will be very interesting to determine in future studies how this oncogenic AR program is regulated and may be targeted for therapy.

In addition to androgen deprivation, recent studies

show that FOXA1 knockdown may also trigger a distinct AR binding profile resulting in dramatic alteration of the androgen response pathway (71). AR was again shown to bind new genomic loci, which contribute to gene expression that enhanced cell growth and established an appropriate microenvironment in CRPC. Interestingly, transcriptomic studies have recently discovered recurrent FOXA1 gene mutations in PCa, suggesting that the wildtype FOXA1 may be beneficial whilst the mutants are more tumorigenic and thus colonially selected (103,104). Being consistent with this perception, we have recently reported that FOXA1 possesses an AR-independent and even-opposing role in inhibiting cell motility and tumor metastasis, a functionality that was significantly impaired by the FOXA1 (105). However, it remains a challenge to dissect out the potentially tumor suppressive role of FOXA1 in the context of altering AR binding profile and its downstream transcriptional activity.

To determine AR binding profile in human prostate tissues and during PCa progression, Sharma *et al*. mapped the genomic landscape of AR in 12 human PCa tissue samples (2 benign, 3 untreated localized tumors, 2 treatment-responding cancer, 5 CRPC), and 3 cell lines (LNCaP, VCaP and 22RV1) (106). Thousands of ARBS were identified in CRPC tissues, which, surprisingly, have little overlap with the ARBS identified in PCa cell line. ARBS identified in CRPC tissue significantly overlapped with E2F, MYC and STAT binding sites, while ARBS found in PCa cell lines showed no such enrichment. In addition, many genes adjacent to the ARBS found in CRPC showed androgen regulation in xenografts, but, surprisingly, not in cultured LNCaP cells. This study suggests again that AR may be reprogrammed during PCa progression and that the AR transcriptional programs may differ significantly not only between disease stages but also among CRPC tissues. It will be critical for future studies to investigate the regulation or evolution of oncogenic AR transcriptional programs in human PCa between or within individual patients.

AR reprogramming by oncogenic transcription factors

It remains puzzling how AR transcriptional activity became reprogrammed in CRPC. Several studies have recently begun to address this important research paradigm. AR overexpression, which occurs in about 30% of CRPC, may allow AR to acquire new binding sites (107). FOXA1 knockdown has been shown to reprogram AR to activate an oncogenic transcriptional program (71). LSD1, in addition to being recruited to AR intragenic region to suppress AR expression, has also been shown to globally inhibit many other androgen-repressed genes through similar mechanism (97). Androgen deprivation, conversely, decreased AR and LSD1 recruitment to target genes, thereby restoring the expression of a subset of androgen-repressed genes that contribute to increased androgen synthesis, DNA replication, and proliferation in CRPC.

Recently, we have reported that the polycomb group protein EZH2 cooperates with AR to mediate its transcriptional repression of target genes (101). For example, we showed that AR occupies the distal enhancer of NOV, an AR-repressed gene, and communicates with the NOV promoter through DNA looping (57). This AR activation recruits EZH2, which subsequently catalyzes histone H3 lysine 27 tri-methylation around the NOV promoter, resulting in the suppression of NOV gene transcription. Very interestingly, another study has demonstrated similar AR and EZH2 cooperation, however, on androgen-induced genes (108). Xu *et al*. found that, in CRPC cells, EZH2 acts as a coactivator for AR through phosphorylation at Ser21, which is mediated by the PI3K-Akt pathway. EZH2 thus cooperates with AR to induce a set of genes, which are significantly overexpressed in CRPC cells. It is important to note that EZH2 is among the most highly expressed gene in metastatic PCa (109). Therefore, AR transcriptional program may be altered by oncogenic transcription factors that become abundantly expressed in CRPC. Many of these regulations are yet to be identified and such studies may lead to important discovery with significant clinical impacts.

Future directions

In summary, significant advances have been made in the last decade regarding genomic regulation of AR. Global androgen-responsive genes have been carefully examined in various cell line systems, animal models, and clinical specimens. Genome-wide AR binding profile in PCa cells have been comprehensively mapped by several independent research labs in various systems. These successes will form a solid foundation for potentially ground-breaking discoveries in the years to come. The identification and characterization of non-conventional targets of androgen, such as miRNAs and lncRNAs, are still in their infancy. Although studies to date have mapped the basal AR binding profiles in PCa cells, a lot remain to be learned regarding the

transcriptional regulatory network that determines precise AR binding events at each developmental and disease stages. The precise mechanisms by which AR pioneering factors and coregulators control AR transcriptional program are yet to be delineated. It will be exceedingly important for future studies to determine AR transcriptional reprogramming in CRPC and how this is regulated by various oncogenic factors. Such mechanistic studies will be essential for strategic disruption of AR signaling in CRPC and may dramatically improve patient care.

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Footnote

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Decoding the androgen receptor splice variants

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Abstract: In the past five years, multiple structurally and functionally distinct androgen receptor (AR) splice variants have been decoded and characterized. The mature transcripts for the majority of the fully decoded AR splice variants contain a transcribed "intronic" sequence, capable of encoding a short variant-specific peptide to replace the AR ligand-binding domain (LBD). Functionally, AR splice variants represent a diverse group of molecules often demonstrating cell context-specific genomic functions that may or may not be coupled with the functions of the canonical full-length AR (AR-FL). However, the full spectrum of their functional diversity and the underlying mechanistic basis remains very poorly characterized. In clinical specimens derived from men treated with a variety of hormone therapy regimens, AR splice variants are almost always expressed at detectable, yet lower levels when compared to that of AR-FL. In spite of the collective *in vitro* data supporting the putative role of AR splice variants in therapeutic resistance to hormone therapies, the extent to which AR splice variants mediate resistance to each individual regimen is not known and awaits thorough investigations in a clinically relevant setting using specimens from men undergoing treatments. Among the AR splice variants, AR-V7 is more abundantly and frequently expressed in castration-resistant prostate cancer (CRPC) and remains the most important variant identified so far. The relative importance of different AR molecules, including AR-FL, should be functionally dissected in the setting of castration-resistant prostate cancer, particularly in tumors resistant to more potent inhibitors of AR-FL recently approved by the FDA. In this review, we will focus on the discovery and characterization of AR splice variants, their putative functions and roles in mediating constitutively active AR signaling, and key areas of investigation that are necessary to establish their clinical relevance.

Keywords: Androgen receptor (AR); AR Splice Variants; AR signaling; full-length AR (AR-FL); prostate cancer; castration-resistant prostate cancer (CRPC)

Introduction

Prostate cancer is a common cause of cancer-related death in aging males in the Western world. Because hormone therapy is the standard of care for men with metastatic prostate cancer, most men succumb to the disease following developing resistance to at least one of the hormone therapy regimens. Castration-resistant prostate cancer (CRPC) is a term used to describe prostate cancers that relapse following first-line hormone therapy (1). It is known that CRPC is not completely refractory to further hormonal manipulation, and AR signaling remains as a pivotal driver for disease progression despite castrate levels of androgens (2,3). Sustained AR signaling may be mediated by a number of mechanisms, including AR gene amplification and overexpression (4-6), intra-tumoral androgen synthesis (7), overexpression of AR coactivators (8), aberrant kinase pathway activation (9-11), AR mutation (12), and constitutively active AR splice variants (13).

Novel therapies have been recently developed to treat CRPC patients by targeting overexpressed AR and intra-tumoral androgen synthesis. Abiraterone acetate, designed to inhibit CYP17A1, was approved on November 2011 for treating metastatic CRPC previously treated with

A

AR: Xq11-12

B

AR-Vs	Alternative names	Transcriptional activity	Transcripts	Proteins

AR-FL — Ligand-stimulated — **AR-FL:MTLG**DNLPEQAAFWRHLHIFWDHVVKK stop

AR-45 — Conditional — **AR45:** start MILWLHS**LETARDHVLPIDYY---FHTQ stop**

AR-23 — Ligand-stimulated — **AR23: KVFFKRAAE**EIPEERDSGNSCSELSTLVFVLP**GKQKYLCA---FHTQ stop**

AR-V1 — AR4 — Conditional — **AR-V1:MTLG**AVVVSERILRVFGVSEWQP stop

AR-V2 — Unknown — **AR-V2:MTLG**AVVVSERILRVFGVSEWQP stop

AR-V3 — AR1/2/2b — Constitutive — **AR-V3:RAAE**GFFRMNKLKESSDTNPKPYCMAAPMGLTENNRNRKKSYRETNLKAVSWPLNHT stop

AR-V4 — AR1/2/3/2b, AR5 — Constitutive — **AR-V4:MTLG**GFFRMNKLKESSDTNPKPYCMAAPMGLTENNRNRKKSYRETNLKAVSWPLNHT stop

AR-V5 — Unknown — **AR-V5:MTLG**D stop

AR-V6 — Unknown — **AR-V6:MTLG**AGSRVS stop

AR-V7 — AR3 — Constitutive — **AR-V7:MTLG**EKFRVGNCKHLKMTRP stop

AR-V8 — Unknown — **AR-V8:MTLG**GFDNLCELSS stop

AR-V9 — Conditional — **AR-V9:MTLG**DNLPEQAAFWRHLHIFWDHVVKK stop

AR-V10 — Unknown — **AR-V10:MT**PSSGTNSVFLPHRDVVRTGCRSNSGYHSCSCEYHDYCFL stop

AR-V11 — Unknown — **AR-V11:MTLG**GKILFFLFLLLPLSPFSLIF stop (EXON RUNON)

AR-V12 — AR^v567es — Constitutive — **AR-V12:KALP**DCERAASVHF Stop

AR-V13 — Inactive — **AR-V13:LFSI**NHT Stop

AR-V14 — Unknown — **AR-V14:SVQP**ITPDAMYL Stop

AR-8 — Inactive — **AR8: YSGPYGDMR**NTRRKRLWKLIIRSINSCICSPRETEVPVRQQK stop

Figure 1 Decoding the androgen receptor splice variant transcripts. (A) AR gene structure with canonical and cryptic exon splice junctions marked according to GRCh37/hg19 human genome sequences (not drawn to scale); (B) Nomenclature, functional annotation, exon compositions, and variant-specific mRNA (color matched to *Figure 1A*) and peptide sequences (in gray).

docetaxol (14), with expanded indication approved on December 2012 to include patients who did not receive prior docetaxol (15). Enzalutamide, a more potent anti-androgen, was approved on August 2012 for post-docetaxol metastatic CRPC (16). The successful clinical development of these two new agents (14-16) underscores the importance of understanding the mechanism of sustained AR signaling in CRPC. In this light, most AR splice variants identified so far do not contain the intended therapeutic target, the AR ligand-binding domain (LBD), for any of the existing hormone therapy regimens including the two new agents. In this review, we will discuss discovery and characterization of the structural and functional diversity of AR splice variants for which the key features have been documented in the literature (key features of the 18 AR splice variants are summarized in *Figure 1*), their potential roles in mediating constitutively active AR signaling, and key areas of investigation to establish them as a mechanism of CRPC, particularly in the setting of resistance to abiraterone and enzalutamide.

The canonical AR-FL

In a normal male genome, there is only one copy of the AR gene located on Xq11-12. The AR gene is considered the most important gene in prostate cancer. The AR-FL cDNA was first cloned in 1988 (17). Structurally AR-FL resembles other nuclear receptors, containing a highly conserved DNA binding domain (DBD) encoded by Exon 2/3, a ligand binding domain (LBD) encoded by Exon 4-8 at C-terminus

with lower sequence homology, a poorly conserved N-terminal domain (NTD) encoded by Exon 1, as well as a hinge region encoded by Exon 3/4 (18). Unique to AR, it has a long NTD domain (~538 amino acids) harboring two transactivating regions, termed transcription activation unit 1 (TAU1) and 5 (TAU5), that are indispensible for AR activation (19).

Earlier reports of AR splice variants: AR45 and AR23

In 2005, a NTD-truncated AR isoform with a deduced molecular weight around 45 kDa (so called AR45) was discovered by 5' rapid amplification of cDNA ends (5' RACE) from human placenta RNA (20). AR45 contains an intact DBD, hinge region, LBD, and a novel seven amino-acid long N-terminal peptide encoded by the novel exon 1b located ~22.1 kb downstream of AR exon 1 (*Figure 1*). The AR45 mRNA was found mainly in heart but also detected in skeletal muscle, uterus, prostate, breast, and lung (20). Exogeneously expressed AR45 did not stimulate the transactivation of androgen response element (ARE)-luciferase reporter in the presence of ligand. AR45 was proposed as a dominant negative AR that suppresses the function of AR-FL (20). In 2007, another AR splice variant, named AR23, was identified from a CRPC bone metastasis specimen (21). AR23 resulted from aberrant splicing of a 69 bp intron 2 sequence (corresponding to 23-amino acid residues), leading to in-frame insertion of a 23-amino acid sequence between two zinc fingers of the DBD (*Figure 1*). The genomic function of AR23 was not established because it does not translocate to the nucleus upon ligand binding, though cytoplasmic AR23 was partially active in androgen-responsive promoter reporter assays (21).

AR Splice variants lacking LBD due to splicing of cryptic exons

AR splice variants drew more attention since 2008 primarily due to the discovery of a number of variants that lack LBD. Such variants have the potential to mediate constitutively active AR function, on the basis of earlier *in vitro* studies on AR deletion constructs generated in the laboratory (22). In 2008, Dehm *et al.* performed 3' RACE with primers anchored at exon 1 and identified a new exon (termed exon 2b) 37 kb downstream of exon 2, in the CWR22Rv1 cell line that demonstrated ligand-independent AR activity (23). Splicing of exon 2b yielded two novel C-terminally

truncated AR variants, AR1/2/2b and AR1/2/3/2b (23). Due to the presence of stop codons in exon 2b, LBD was replaced by the variant-specific 11-aa peptide encoded by exon 2b. Both variants demonstrated constitutively active AR function by *in vitro* luciferase reporter assays. AR1/2/2b also lacks the second zinc finger of the DBD, while AR1/2/3/2b retains the entire DBD. Because the transcript structure of AR1/2/3/2b was explained by a duplicated DNA sequence unique to CWR22Rv1 cells (24), this variant was thought to be specific to this cell line. In contrast, AR1/2/2b was more commonly found in other PCa cell lines, including VCaP, LNCaP, and LAPC4, as well as PCa xenografts (23).

In 2009, Hu *et al.* reported the identification of more AR cryptic exons in both cell lines and clinical specimens (25). Using a strategy combining exhaustive analysis of expressed sequence tags mapped to the human AR locus and experimental cloning to determine the precise splice junctions, Hu *et al.* identified three cryptic exons named CE1, CE2, and CE3 in intron 3, and CE4, identical to exon 2b (23) discovered by Dehm *et al.* (25). Splicing of the cryptic exons generated seven AR splice variants (named AR-V1 to AR-V7) (*Figure 1*), all lacking LBD due to stop codons present in the transcribed "intronic" sequences (i.e., cryptic exons). Among these, AR-V1 and AR-V7 were readily detectable in clinical prostate cancer specimens, with ~20-fold higher levels detected in CRPC specimens compared to hormone naïve prostate tumors. Importantly, a variant-specific antibody was developed for AR-V7, and used to detect the translated product of AR-V7 in prostate cancer cell lines and xenografts. Both PSA reporter assays and expression microarray analysis confirmed that AR-V7 was constitutively active in driving expression of canonical androgen-responsive genes (e.g., KLK3, KLK2, and NKX3.1) in an androgen-independent manner (25).

Guo *et al.* reported the discovery of LBD-truncated variants AR3, AR4, and AR5 using 3' RACE in 2009 (26). AR3, AR4, and AR5 contained coding sequences identical to those in AR-V7, AR-V1, and AR-V4, respectively. A variant-specific polyclonal antibody was also developed for AR3 (AR-V7), and used to detect protein expression in both hormone naïve and CRPC specimens. In addition, knockdown of AR3 in CWR22Rv1/CWR-R1 cells revealed a set of 117 genes that were preferentially regulated by AR3 (26). This study also reported the cloning of multiple additional variants that were not further characterized.

AR splice variants discovered by other approaches

Combining 3' RACE with next generation sequencing, Watson *et al.* not only confirmed the known AR-V1 and AR-V7, but also found 4 more AR splice variants named AR-V8 to AR-V11 (27) (*Figure 1*). This experiment was carried out in the VCaP cells, a prostate cancer cell line derived from vertebral metastatic lesion of a CRPC patient (27,28) that was shown to express the AR-V7 protein (25). These four new AR variants show splicing junctions between exon 3 and different regions of intron 3, with the predicted AR variant proteins truncated after AR DBD with 10-39 amino acid extension before the stop codon (*Figure 1*). Using VCaP xenograft in SCID mice, Watson *et al.* found that AR-Vs (AR-V7 and AR-V1) and AR-FL were upregulated by castration in both mRNA and protein levels, while re-administration of testosterone suppressed the expression of both AR-FL and AR-V7 in VCaP cells. Similar regulation of AR-FL and AR-Vs by androgens was also demonstrated in LuCaP35 xenografts with modest variation. However, only AR-V7, but not AR-V1, conferred gain-of-function on accelerating the LNCaP xenograft growth in castrated mice and colony formation in soft-agar assay (27).

More recently, Hu *et al.* employed a modified RNA amplification method, termed selective linear amplification of sense RNA (SLASR), for unbiased detection of transcribed AR sequences using arrayed 60-mer probes tiled across the human *AR* gene locus, directly in clinical CRPC specimens (29). This study provided a snapshot of the expression peaks along genomic sequences downstream of AR exon 3 and identified 3 new variants named AR-V12 to AR-V14 (*Figure 1*). Importantly, this study revealed expression peaks within intron 3 as well as sequences further downstream of exon 8 (named exon 9). These previously unappreciated expressed sequences have the potential to participate in AR splicing. One example is AR-V12 (*Figure 1*), which has the same open reading frame with AR^{v567es} (see below) but contained untranslated sequences mapped to exon 9.

AR^{v567es} and AR8

Sun *et al.* investigated the AR isoforms in a panel of 25 LuCaP prostate cancer xenografts (30). With a primer set anchored on exons 2 and 8 in RT-PCR, a short AR transcript spanning exon 2 to 8 was discovered. Sequencing revealed a novel AR variant arising from skipping of exons 5 to 7 while retaining the full sequence of exons 1 to 4 and exon 8. This new variant is named AR^{v567es} (30). This exon combination (1/2/3/4/8) shifts the open reading frame (ORF) of AR^{v567es} to an early stop codon just after the first 29 nucleotides of exon 8 (*Figure 1*). AR^{v567es} is unique in that it retains the full hinge domain encoded by part of exons 3 and 4. The AR hinge domain contained important sequences for AR localization and activity (31). Similar to AR-V7, AR^{v567es} activates androgen-responsive genes (such as *KLK3*, *TMPRSS2*, and *NKX3.1*) in a hormone-independent manner when ectopically expressed in LNCaP cells (30). The coding sequence for AR^{v567es} is identical to AR-V12, which is encoded by a transcript containing exons 1/2/3/4/8/9 as later reported in Hu *et al.* (29) (*Figure 1*). However, more in-depth studies of AR^{v567es} have been hampered by lack of a variant-specific antibody, as well as lack of suitable sequences to target for variant-specific knockdown that is an important tool to determine protein translation and function.

In CWR-R1 cells, Yang *et al.* identified a membrane-associated AR variant, named AR8 by RACE (32). Using an alternative splicing acceptor site 186 bp upstream of exon 3, the deduced protein of AR8 (Exon1-3'-3-3b/CE3) contained 33 unique amino acids after the NTD domain. Higher expression level of AR8 was detected in castration-resistant cell lines (C4-2, C4-2B, CWR22Rv1). This C-terminal truncated AR-V has no DBD or LBD and no transactivating function in ARE-luciferace reporter assay. Possibly due to palmitoylation of two cysteine residues within its unique C-terminal sequence, this protein was found mainly in plasma membrane when overexpressed in COS-1 cells or PCa cells (LNCaP and CWR-R1). Membrane-bound AR8 complexes with AR-FL and EGFR and may serve as a mediator in Src-induced AR activation (32).

Nuclear localization of AR splice variants

A prerequisite for AR to exert its genomic function is to enter the nucleus. Upon androgen binding to LBD, AR-FL exposes a nuclear localization signal (NLS) within C-terminal end (CTE) of DBD and hinge region to interact with importin proteins for translocation through the nuclear pore complex (33). A canonical bipartite nuclear localization signal (NLS) was mapped at the junction of DBD and the hinge region (amino acid 617-RKCYEAGMTLG--ARKLKK-633) (34). With the exception of AR-12/AR^{v567es}, other AR isoforms may have variable capability in nuclear import due to loss of NLS. Evidence provided by

immunofluorescent staining supports constitutive nuclear localization of AR-V7/AR3 and AR-12/AR^{v567es} in the absence of androgens, while AR-V1, AR-V9, and AR-V13 are mainly cytoplasmic (25,27,29,30), possibly due to lack of basic amino acids characteristic of the bipartite nuclear localization sequence (NLS) (35). Interestingly, genomic functions of AR-Vs do not always parallel to their localization. For example, AR-V1 and AR-V9 showed ligand-independent activity in LNCaP cells but not in PC-3 cells (29). Such variants were termed "conditionally active" variants (29), to differentiate them from constitutively active variants (see below), because their functions are conditional on the cellular context. To further understand the nuclear transport of AR-V7 and its relation with AR-V transcriptional function, Chan *et al.* showed that part of its unique sequence at C-terminus (aa 628-EKFRVGNCKHLKMTRP-643) resembles the truncated bipartite AR NLS. Mutation of amino acid residues K629 and R631 to alanine in AR-V7 shifted its expression from predominantly nuclear to a mixed nuclear/cytoplasmic pattern; while alanine mutation at K636 or K639 had no effect on nuclear localization of AR-V7 (36).

Diverse and cell-specific functions of AR splice variants

Among the AR-Vs listed in *Figure 1*, AR-V7 (also named AR3) and AR^{v567es} have received more attention due to their unequivocal constitutively active nuclear functions (25,26,30). Both AR-Vs activate transcription of canonical AR-FL target genes when overexpressed in cell lines with or without activated AR-FL. Other AR-Vs may be conditionally active, i.e., their transcriptional activities are cell type-specific (37). For example, AR-V1 and AR-V9 demonstrated transcriptional activity when introduced in AR-FL positive LNCaP cells but not in the AR-FL negative PC-3 cells (37). It is possible that the conditional activity of AR-V1 and AR-V9 may require nuclear localization that was not readily detected by immunofluorescence. Previous studies showed androgen receptor (AR) deletion mutants that retain a partially truncated LBD did not have constitutive activity (22,35,38). Hu *et al.* demonstrated examples of inactive AR splice variants that retain a partially truncated LBD, including AR-V13 and AR-V14 (37) (*Figure 1*).

Expression levels of AR-V7 are dramatically increased after suppression of AR-FL signaling by androgen depletion, AR-FL knockdown, or treatment with enzalutamide in VCaP cells and LNCaP95 cells but not in

LNCaP and CWR22Rv1 cells, suggesting that in addition to cell-context specific functions of AR splice variants, the regulation of AR variant levels may also depend on a specific cellular context (39).

Molecular origin of AR splice variants

In clinical specimens, AR splice variants coexist with AR-FL, and the expression levels of individual AR variants almost always constitute a small fraction of the expression level of AR-FL (25,27). In addition, AR splice variants are also expressed in benign prostate epithelium (25,30), again at a much lower level relative to AR-FL. AR-FL is often overexpressed in CRPC due to AR gene amplification (40,41) or other genomic changes (42). In addition, elevated AR expression in CRPC may involve AR self regulation. Cai *et al.* showed that lysine-specific demethylation-1 (LSD-1) was recruited to AREs in intron 2 of the AR gene and acts as a repressor when AR-FL was activated. This recruitment was abolished when androgen was depleted (43). It is therefore possible that expression of AR splice variants are generally coupled with the transcriptional output from the AR gene locus (44). Supporting this possibility, AR variant levels were downregulated by testosterone replacement in castrated mice in parallel with a decrease of AR-FL in VCaP and LuCaP35 xenografts (27). In cell line models (VCaP and LNCaP95) with higher levels of AR-FL and low levels of AR variants, suppression of AR-FL signaling by enzalutamide resulted in an unequivocal increase of AR-V7, and a relatively moderate increase of AR-FL (39). In addition, increased expression of AR-FL, AR-V7 and AR^{v567es} was also observed in castration-resistant LuCaP xenograft (LuCaP23CR and LuCaP35CR) when AR-FL signaling was inhibited by abiraterone (45). Thus, although AR-V expression may not strictly parallel that of AR-FL, and the magnitude of AR-V mRNA increase is generally greater than that of AR-FL in CRPC and in experimental models (39), AR-V expression is strongly coupled with AR-FL expression.

In some cell line and xenograft models (e.g., CWR22 and LuCaP86.2), AR intragenic rearrangement or deletions may be responsible for high AR variant expression. In CWR22Rv1 cells, an intragenic copy number increase occurred in an approximately 35-kb AR genomic segment between introns 2 and 3, with the rearranged segment flanked by long interspersed nuclear element (5'-LINE-1 and 3'-LINE-1) (46). To further investigate the association of focal imbalance of the AR gene and AR variant expression,

Li *et al.* demonstrated a complex pattern of focal copy number imbalance with or without AR gene amplification. In LuCaP 86.2 xenograft cells, an 8579-bp deletion of AR exons 5, 6, and 7 may be responsible for the high level of AR^{v567es} variant expression (47). The extent to which similar genomic alterations contribute to the generation of AR splice variants in clinical specimens remains unclear.

Detection of AR splice variants in clinical specimens

The majority of AR-Vs listed in *Figure 1* can be detected in prostate cancer tissue specimens by RT-PCR (25-27, 32,37). Alternatively spliced transcripts containing premature stop codons may be degraded through the nonsense-mediated decay (NMD) mechanism (48). Therefore it is also critical to demonstrate protein expression by detecting the corresponding variant protein product in order to draw functional relevance. Variant-specific antibodies have been reported for AR-V7 (AR3) (25,26), AR8 (49), and AR1/2/2b (50). In all these efforts, the variant-specific c-terminal peptides were used (*Figure 1*) as antigens. Among these, AR-V7/AR3 remains the only AR splice variant with a proven protein product that can be detected in clinical specimens using variant-specific antibodies (25,26,39), including a monoclonal antibody to AR-V7 (39). An alternative approach to detect the potential existence of LBD-truncated AR variants is to combine data obtained using antibodies recognizing AR-NTD and AR-LBD, respectively. For example, Zhang *et al.* showed a wide distribution of the AR-NTD/LBD ratio in clinical CRPC specimens (51). Higher ratios of NTD/LBD were detected in more aggressive tumors. However, this approach is based on the assumption that excess AR-NTD detected in CRPC specimens originated from the expression of AR-Vs.

Genomic functions of AR splice variants

A key question in relation to the genomic functions of AR splice variants is whether they active the same transcriptional programs directed by AR-FL. Hu *et al.* showed data suggesting that endogenously induced AR variants are not sufficient to "rescue" the suppressed AR-FL, when a set of canonical AR-FL target genes are evaluated (39). Instead, increased expression of AR variant paralleled the increased expression of cell cycle genes, and forced expression of both AR-V7 and AR^{v567ES} induced the same set of cell cycle genes in both the presence of absence

of canonical AR-FL signaling (39). Li *et al.* performed gene expression profiling in rearrangement-driven AR-V positive cells following specific knockdown of the AR-FL and AR-Vs to differentiate genes activated by the two different receptor molecules (52). AR-V-dependent cell cycle genes were found to demonstrate a biphasic response. They were induced at low AR-V levels but repressed when higher AR-Vs were expressed in the cells. This observation mirrors the canonical biphasic androgen-stimulated (i.e., AR-FL-mediated) growth response observed in cell line models. The findings suggest that AR-V expression reactivates and restores the AR-FL transcriptional programs, rather than by targeting a unique set of genes. These seemingly opposing findings may be explained by cell-context differences as well as the different methodologies used in the studies. More in-depth analysis will address cell-context specific genomic functions mediated by the AR splice variants.

Future directions and priorities

In spite of intense interest in the putative role of AR splice variants in CRPC and the years that have elapsed since their discovery and characterization, the field is still in infancy and investigations encompassing the full spectrum of mechanistic characterization and clinical translation are still at a nascent stage. Successful clinical development of abiraterone and enzalutamide (14-16), both intended to target the AR LBD (which is missing in AR-Vs), is directly driven by laboratory mechanistic studies establishing intra-tumoral androgens and AR protein overexpression as the key molecular determinants of CRPC (2). Thus we envision that mechanistic studies dissecting the genomic functions of different AR molecules will facilitate efforts in developing new therapies to overcome resistance to abiraterone and enzalutamide. Given the expanded clinical use of abiraterone and enzalutamide, there is an urgent need to dissect the various putative mechanisms of resistance to these new, more potent inhibitors of AR-FL signaling. Although AR splice variants provide a biologically plausible explanation for therapeutic resistance, the concept has not been validated in clinical specimens due to the recent approval of the two new agents, and consequently lack of sufficient number of relevant specimens collected from treated patients. Nevertheless, the discovery of AR splice variants has already stimulated efforts to develop novel agents that target all AR molecules to overcome resistance (53-64). Further conceptual advances in the field will provide a sustained impetus for such efforts.

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Footnote

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Androgen receptor-mediated non-genomic regulation of prostate cancer cell proliferation

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Abstract: Androgen receptor (AR)-mediated signaling is necessary for prostate cancer cell proliferation and an important target for therapeutic drug development. Canonically, AR signals through a genomic or transcriptional pathway, involving the translocation of androgen-bound AR to the nucleus, its binding to cognate androgen response elements on promoter, with ensuing modulation of target gene expression, leading to cell proliferation. However, prostate cancer cells can show dose-dependent proliferation responses to androgen within minutes, without the need for genomic AR signaling. This proliferation response known as the non-genomic AR signaling is mediated by cytoplasmic AR, which facilitates the activation of kinase-signaling cascades, including the Ras-Raf-1, phosphatidyl-inositol 3-kinase (PI3K)/Akt and protein kinase C (PKC), which in turn converge on mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) activation, leading to cell proliferation. Further, since activated ERK may also phosphorylate AR and its coactivators, the non-genomic AR signaling may enhance AR genomic activity. Non-genomic AR signaling may occur in an ERK-independent manner, via activation of mammalian target of rapamycin (mTOR) pathway, or modulation of intracellular Ca^{2+} concentration through plasma membrane G protein-coupled receptors (GPCRs). These data suggest that therapeutic strategies aimed at preventing AR nuclear translocation and genomic AR signaling alone may not completely abrogate AR signaling. Thus, elucidation of mechanisms that underlie non-genomic AR signaling may identify potential mechanisms of resistance to current anti-androgens and help developing novel therapies that abolish all AR signaling in prostate cancer.

Keywords: Androgen receptor (AR); ERK MAP kinases; cell proliferation; non-genomic signaling; prostate cancer

Introduction

Androgen receptor (AR) is a classic steroid hormone receptor that is critical for prostate cancer development and progression. In its unbound conformation, AR is located primarily in the cytoplasm in complex with heat shock proteins, cytoskeletal proteins, and other chaperones (1-5). These proteins also enable modulation of AR conformation for efficient ligand binding (6,7). When androgen binds AR, AR forms a homodimer, undergoes a conformational change, and interacts with additional proteins that facilitate its nuclear translocation (8-10). Once in the nucleus, AR binds to the androgen response elements (AREs) on promoter/enhancer regions, recruits coregulators, and forms the transcriptional machinery for AR-regulated gene expression (10). This AR-signaling pathway, known as the genomic pathway, relies on AR nuclear translocation and AR-DNA binding for cell proliferation. The genomic pathway is thought to occur over several hours and is characterized by increased expression of specific AR-regulated genes (*Figure 1*).

However, studies have shown a rapid and reversible AR signaling that occurs within minutes and results in regulation of prostate cancer cell proliferation (11-13). This
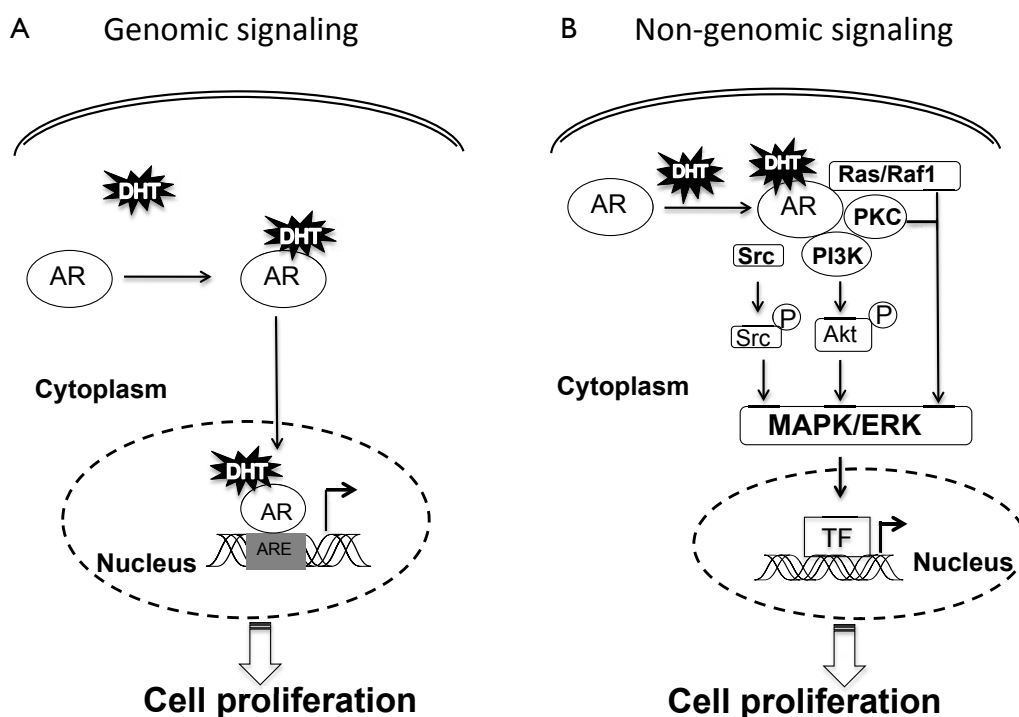
Figure 1 Genomic and non-genomic AR signaling in prostate cancer cells. (A) Genomic AR signaling. After binding with the activated form of androgen, 5α-dihydrotestosterone (DHT), AR undergoes a conformational change and translocates to nucleus. In the nucleus, AR binds to the androgen response elements (AREs) on promoter/enhancer regions, recruits coregulators, and forms the transcriptional machinery for AR-regulated gene expression; (B) Non-genomic signaling. Activated AR in the cytoplasm can interact with several signaling molecules including the phosphatidyl-inositol 3-kinase (PI3K)/Akt, Src, Ras-Raf-1, and protein kinase C (PKC), which in turn converge on mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) activation, leading to cell proliferation.

AR-signaling pathway, known as the non-genomic pathway, requires neither AR nuclear translocation nor AR-DNA binding. Instead, cytoplasmic AR signaling may function through mitogen-activated protein kinase (MAPK) signaling cascades, converging on extracellular signal-regulated kinase (ERK) activation (14,15). Treatment of AR-positive prostate cancer cells with 5α-dihydrotestosterone (DHT) leads to increased ERK-1/2 phosphorylation within 5 minutes in a dosage-dependent manner (13) (*Figure 1*).

While non-genomic AR signaling has thus far been shown to primarily require MAPK/ERK activation, cell signaling can also occur without ERK activation. Non-ERK pathways involve activation of mammalian target of rapamycin (mTOR) via the phosphatidyl-inositol 3-kinase (PI3K)/Akt pathway or involvement of plasma membrane, G protein coupled receptors (GPCRs) and the sex hormone binding globulin receptor (SHBGR) that modulate intracellular Ca^{2+} concentration and cyclic adenosine monophosphate (cAMP) levels, respectively (16,17).

In addition, non-genomic AR signaling may be mediated by a membrane-bound AR that can regulate intracellular Ca^{2+} concentration and membrane ion channels (18,19). Studies with bovine serum albumin (BSA)-bound DHT, a compound that is unable to penetrate the plasma membrane, show a dose-dependent suppression of the PI3K and MAPK pathways (20). These data indicate that non-genomic AR signaling may suppress proliferation via membrane-bound AR (21) or activate proliferation via cytoplasmic AR.

Finally, recent data indicates that the non-genomic AR signaling may regulate genomic AR signaling and that the non-genomic and genomic AR signaling may work together to coordinate gene regulation in prostate cancer cells. In this manuscript, we provide a comprehensive review of non-genomic AR signaling with an emphasis on the established role of MAPK/ERK in prostate cancer cell proliferation. Clinically, understanding of these non-genomic AR signaling pathways is important, as they may represent potential mechanisms of resistance to AR antagonists.

AR antagonists including casodex and flutamide have no effect on non-genomic AR signaling, as evidenced by ERK phosphorylation in the presence of these drugs (13). Thus, elucidation of non-genomic AR signaling pathways may enable development of novel agents to inhibit all forms of AR signaling in prostate cancer.

ERK-1/2 mediated non-genomic AR signaling

The MAPK/ERK signaling cascade is important in regulating diverse biological functions including cell survival, motility, and proliferation, which are essential to prostate carcinogenesis (22). Aberrant activation of kinases in this pathway is frequently reported in human cancer (23-25). Studies of DHT-responsiveness in prostate cancer cells in our lab and others show evidence of ERK-1/2 phosphorylation within 1-2 minutes of DHT treatment and peak levels of ERK-1/2 phosphorylation within 5-10 minutes. Activated ERK-1/2 then translocates to the nucleus and directly interacts with and phosphorylates transcription factors (TFs), such as nuclear ETS domain-containing Elk1 (26-28). Elk1 transcriptionally regulates immediate early genes (IEGs) such as c-*fos* (26,29), which coordinately regulates the expression of several genes involved in cell proliferation (26,27). This response is AR-dependent as no effect was observed in AR-negative PC-3 prostate cancer cells (13). Thus, while ERK phosphorylation occurs within minutes and serves as a measurable response of non-genomic activation, the molecular processes involved in cell proliferation occur over several hours and days.

DHT-induced ERK activation in prostate cancer cells may be mediated via multiple pathways, including the PI3K/Akt, Ras-Raf, and protein kinase C (PKC) pathways. Extensive evidence suggests AR associates with plasma membrane lipid rafts that facilitate AR activation of these pathways (30). AR activation of the PI3K/Akt pathway involves direct interaction of AR with the p85α regulatory subunit of PI3K (31), while the activation of the Ras-Raf pathway may involve the sequential activation of Ras, Raf and MEK kinases and may be dependent on the formation of an AR-Src complex (29,32). Importantly, Src or scaffolding proteins like proline-, glutamic acid-, and leucine-rich protein-1 (PELP1) may modulate the interaction of AR with Akt (31,33). PKC activation of ERK may involve modulation of intracellular Ca^{2+} concentration (13). Furthermore, ERK and Src are calcium-dependent kinase cascades suggesting AR could directly regulate them via mobilization of intracellular Ca^{2+} levels (32,34). Each of

these AR signaling pathways can result in ERK activation and represent redundancy that ensures a proliferative response to DHT. Further, crosstalk between AR-mediated signaling cascades suggests a complex network of signals that converge on ERK phosphorylation. Each of these pathways is individually discussed in detail below.

PI3K/Akt/PTEN pathway

Ligand binding induces AR to directly interact with the p85α regulatory subunit of PI3K, resulting in the activation of the PI3K/Akt pathway (31). PI3K phosphorylates Akt (also known as PKB), a subfamily of serine-threonine protein kinases. Akt expression is frequently observed to be elevated in human prostate, ovarian, and breast cancers (35-37). The PI3K/Akt pathway activates the MAPK/ERK cascade and is regulated by phosphatase and tensin homolog (PTEN). PTEN, a protein phosphatase that dephosphorylates phosphatidyl-inositol (3,4,5)-triphosphate (PIP3) thereby inhibiting PI3K induced activation of Akt (38,39), is one of the most commonly lost tumor suppressors in prostate cancer (40-42). PTEN loss of function often results in constitutively active Akt and may result in chronic activation of the proliferative genes.

The PI3K/Akt pathway activates mTOR and forkhead box protein O1 (FOXO1) and the MAPK/ERK cascade. Kinase inhibitors and dominant negative mutants of PI3K disrupt DHT-mediated activation of ERK and have supported a central role for the PI3K/Akt pathway in non-genomic AR signaling (13). Further, DHT-mediated activation of PI3K/Akt is AR-dependent (31,32,43).

Src pathway

Several studies have also implied the importance of Src in AR activation of kinase signaling cascades (17,32,43). In its inactivated conformation, interaction of the Src homology 2 (SH2) and Src homology 3 (SH3) domains causes autoinihibition of Src. AR interacts with the SH3 domain of Src relieving its autoinhibition and resulting in Src activation of the adaptor protein, Shc, a known upstream regulator of the MAPK pathway (44-46). AR-Src complexes may be noted in immunoprecipitation assays resulting in activation of Shc (29,43). Inhibition of the Src/MAPK pathway decreases DHT-induced ERK-1/2 phosphorylation (47).

In addition to Src-mediated direct activation of the ERK1/2 signaling cascade, Src may also activate the expression of receptors such as the insulin-like growth

54

Liao et al. Non-genomic regulation of prostate cancer by AR

factor 1 receptor (IGF-1R) (47). Activated AR can also directly regulate IGF-1 gene expression as the IGF-1 promoter contains two AREs (48). However, data from quantitative RT-PCR studies shows expression of IGF-1R may be independent of AR-DNA binding. While the exact mechanism is not clearly elucidated, induction of IGF-1R expression appears to depend on Src/MAPK activation. Inhibition of the Src/MAPK pathway decreases IGF-1R expression and decreases ERK1/2 phosphorylation (47). IGF-1 signaling has been shown to promote prostate cell proliferation, migration, and tumor angiogenesis, resulting in prostate carcinogenesis and cancer progression (49).

Further, IGF-1 signaling can subsequently activate the PI3K/Akt pathway in prostate cancer cells (49). Increased IGF-1R binding of IGF-1 results in activation of the PI3K/Akt pathway, which can then regulate the action of proteins like mTOR and FOXO1. These processes activate multiple pathways including the Src/MAPK pathway early, subsequently IGF-1 pathway and later the PI3K/Akt pathway being temporally activated, ensuring a robust proliferation response to DHT.

Ras-Raf pathway

The Ras-Raf pathway is comprised of the Ras family of small GTPases and their downstream interaction of Raf kinase proteins. The Ras-Raf pathway is part of the larger Ras-Raf-MEK-MAPK-ERK signaling cascade that ultimately results in phosphorylation of the kinases ERK-1/2 (25,50,51). Dominant negative constructs of Raf-1 abrogated the DHT-induced ERK-1/2 but also reduced basal activity, which may have been present from residual hormone in the culture medium (13).

PKC pathway

Studies with PKC inhibitors indicate that AR utilizes PKC as a mediator of MAPK/ERK pathway activation (13,17,52). PKC kinase activity is regulated by both modulation of intracellular Ca^{2+} concentrations and diacylglycerol (DAG) binding to PKC itself (53). Mechanisms of non-genomic AR-mediated regulation of Ca^{2+} concentration appear to be cell-type dependent (11,54). Ca^{2+} could be released via internal stores and/or through influx from extracellular space. Interestingly, these mechanisms may not be blocked by AR antagonists (54). The etiology for cell type differences may indicate a role for cell type-specific AR cofactors (54). These findings also hint at the association

of cytoplasmic AR or membrane-bound AR with plasma membrane receptors such as GPCRs or ion channels that may modulate intracellular Ca^{2+} ion concentration resulting in PKC activation.

Plasma membrane lipid rafts

AR in the plasma membrane may mediate DHT-induced activation of the PI3K/Akt, Ras-Raf, and PKC pathways (30). Non-cytoplasmic AR may be localized to the membrane and/or specialized liquid-ordered micro-domains within the lipid bilayer of the plasma membrane that are enriched with sphingolipids, caveolins, Src family kinases, G proteins and signaling mediators called "lipid rafts" (55,56). Several observations support the existence of and role for non-cytoplasmic AR in mediating non-genomic AR signaling. First, AR has been detected in the membrane and in lipid rafts. Cell membrane binding sites for androgens have been identified in several different cell types including rat osteoblasts (57), rat vascular cells (58), murine RAW 264.7 and IC-21 macrophages (19,59), murine splenic T lymphocytes (18), human prostate cancer cells (20,60), as well as in human prostate carcinoma cells (61). In PC3-AR cells, both AR and EGFR are found within plasma membrane lipid rafts (62). Co-localization of AR with caveolin-1 was found within lipid rafts of human aortic endothelial cells in response to testosterone treatment (33). Studies, using DHT-BSA, a large plasma membrane-impenetrable compound, showed binding of DHT to the membrane (18,19,63). Secondly, AR has been detected in complexes with multiple members of the lipid rafts. In caveolin-rich lipid rafts, a direct interaction was noted between caveolin-1 and a conserved nine-amino acid motif in the ligand-binding domain (LBD) of AR (30,64). AR forms a DHT-sensitive complex with the serine-threonine kinase Akt1 in caveolin-negative lipid rafts and mediates a non-genomic signal independently of Src and PI3K to activate Akt (65). Thirdly, androgens induce a rapid rise in intracellular Ca^{2+} concentration that can be blocked by pertussis toxin or by phospholipase C (PLC) inhibitor indicating a membrane-binding site for androgens that may be in close association with a GPCR (57,59,66). Finally, DHT-induced signaling cascades namely the Ras-Raf (67,68), MAPK/ERK (69,70), adenylyl cyclase (71), and PI3K(72), pathways have been shown to be enriched in lipid rafts. While specific membrane receptors, GPCR30 and membrane progesterone receptor (mPR), have been identified for estrogen and progesterone,
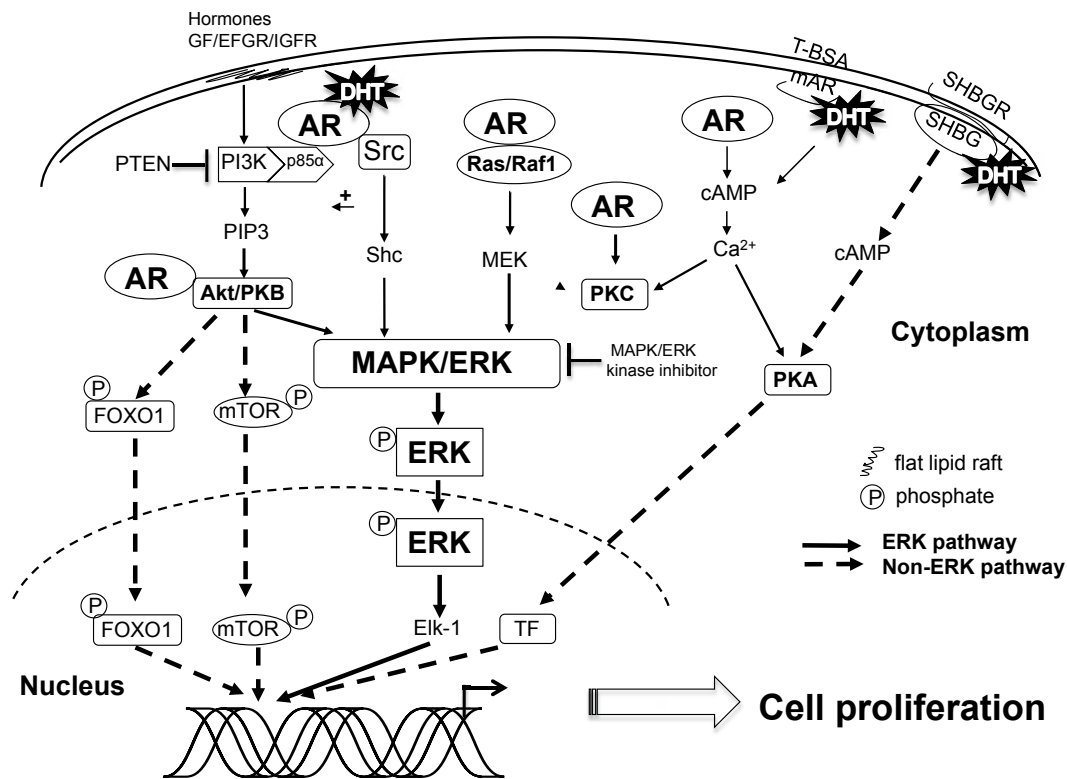
Figure 2 ERK and non-ERK mediated non-genomic AR signaling. ERK mediated non-genomic AR pathways are highlighted in solid line arrows. (I) AR interacts directly with the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K) and activates Akt pathway. (II) AR interacts with Src resulting in Src activation of the adaptor protein, Shc, a known upstream regulator of the MAPK pathway. (III) AR interacts with Ras-Raf leading to sequential activation of Ras, Raf1 and MEK kinase converging on the phosphrylation of ERK. (IV) AR also utilizes PKC as a mediator of MAPK/ERK pathway activation. PKC kinase activity can be regulated by intracellular Ca^{2+} concentrations. Intracellular Ca^{2+} concentration may be modulated through plasma membrane G protein-coupled receptors (GPCRs), the sex hormone binding globulin receptor (SHBGR) and by a membrane-bound AR via up-regulation of cyclic adenosine monophosphate (cAMP) levels. Activated MAPK/ERK translocates to the nucleus, directly interacts with and phosphorylates transcription factors (TFs), such as Elk1, which coordinately regulates the expression of several genes involved in cell proliferation. Non-ERK mediated non-genomic ARpathways are highlighted in dash line arrows and include (V) PI3K/Akt/mTOR or (VI) forkhead box protein O1 (FOXO1) pathway activation. Akt activation may result in FOXO1 phosphorylation resulting in its retention in the cytoplasm and subsequent degradation. In addition, AR interacts with FOXO1 and impairs FOXO1 DNA binding ability and its ability to mediate pro-apoptotic pathways. (VII) Non-ERK signaling can also occur through activation of kinases such as protein kinase A (PKA), whose activation is regulated by intracellular Ca^{2+} concentration.

respectively (73,74), to date, a membrane AR has not yet been purified or cloned. A clear understanding of the functional importance of non-cytoplasmic AR-mediated (membrane-bound or lipid raft) non-genomic signaling is lacking.

NonERK-mediated non-genomic AR signaling

Non-genomic AR signaling may occur without ERK participation through either PI3K/Akt/mTOR pathway activation or changes in intracellular Ca^{2+} concentration

that result in activation of kinases such as PKA. For example, AR interaction with its p85α PI3K regulatory subunit may induce Akt-mediated phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2) to generate the second messenger PIP3 (75,76), which then activates downstream signaling pathways important in cell proliferation (77). Alternatively, AR-directed Akt activation may result in FOXO1 phosphorylation resulting in its retention in the cytoplasm and subsequent degradation (78,79). In addition, liganded AR physically interacts with FOXO1 and impairs FOXO1-DNA binding ability and its

Crosstalk between AR genomic and non-genomic pathways
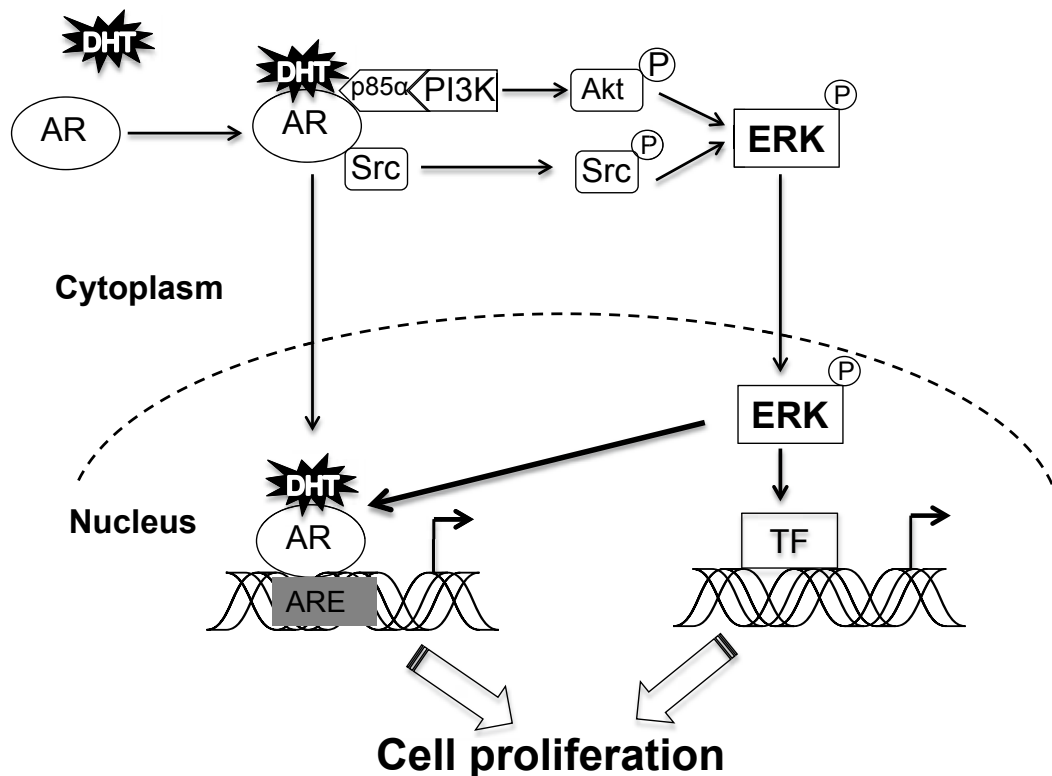
Figure 3 Crosstalk between genomic and non-genomic AR pathways. Non-genomic AR signaling may regulate genomic AR signaling. ERK has been shown to enhance AR transcriptional activity through the direct phosphorylation of AR and its coregulators. The non-genomic and genomic AR signaling may work together to coordinate gene regulation in prostate cancer cells.

ability to mediate pro-apoptotic pathways. Finally, Increased intracellular Ca^{2+} concentration and increased level of cAMP induced by GPCR or SHBGR also activate protein kinase A (PKA) (67,68), which then enhances prostate cancer cell proliferation (17,69).

Critically, each of these pathways is also capable of activating ERK1/2. This suggests that a number of signaling cascades may be activated in tandem with the MAPK/ ERK pathway to induce proliferation (*Figure 2*). Crosstalk between these pathways further amplifies the signal and ensures that the cell responds to androgenic stimulation.

Crosstalk between genomic and non-genomic pathway

ERK has been shown to enhance AR transcriptional activity through the direct phosphorylation of AR and its coregulators (13). This autocrine loop could present a

non-genomic mechanism to control AR transcriptional activity and could be important in cell adaptation to low androgen environments.

Non-genomic AR signaling mediated by induction of cAMP and PKA activation may involve SHBGR (80,81). DHT-SHBG also enhances AR transcriptional activity via phosphorylation of AR and AR coregulators facilitating their binding to AR (17). Thus, PKA can enhance prostate cancer cell proliferation and AR transcriptional activity even at very low levels of androgen (17,67,82). Thus, some of the non-genomic AR actions mediated by second messenger activation may influence the AR genomic responses (54) (*Figure 3*).

Implications in prostate cancer

Second-generation anti-androgens including MDV3100 and ARN-509 competitively target the activation of

AR, its nuclear translocation and its genomic activity (68,69). However, non-genomic AR signaling that functions through cytoplasmic AR may still be active in MDV3100 treated prostate cancer cells. Prior studies have indicated that DHT-mediated non-genomic activation of ERK-1/2 in prostate cells are insensitive to anti-androgens specifically hydroxyflutamide and casodex (13). Thorough evaluation of the non-genomic AR axis is mandatory in assessing the effect of drugs targeting AR signaling in prostate cancer (13).

Conclusions

The existence of rapid, non-genomic AR signaling is incontrovertible. AR non-genomic regulation functions through the activation of intertwined complex signaling cascades resulting in expression of proliferative genes and responses. Non-genomic AR signaling may act to modulate genomic AR signaling and enable a coordinated, sustained and vigorous response to androgenic stimuli. Non-genomic AR signaling may represent a potential mechanism of resistance to anti-androgens.

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Footnote

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Liao et al. Non-genomic regulation of prostate cancer by AR

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Novel non-AR therapeutic targets in castrate resistant prostate cancer

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Abstract: Castrate resistant prostate cancer (CRPC) remains a disease with significant morbidity and mortality. The recent approval of abiraterone and enzalutamide highlight the improvements which can be made targeting the androgen receptor (AR) axis. Nonetheless, resistance inevitably develops and there is continued interest in targeting alternate pathways which cause disease resistance and progression. Here, we review non-AR targets in CRPC, with an emphasis on novel agents now in development. This includes therapeutics which target the tumour microenvironment, the bone metastatic environment, microtubules, cellular energetics, angiogenesis, the stress response, survival proteins, intracellular signal transduction, DNA damage repair and dendritic cells. Understanding the hallmarks of prostate cancer resistance in CRPC has led to the identification and development of these new targets. We review the molecular rationale, as well at the clinical experience for each of these different classes of agents which are in clinical development.

Keywords: Androgen receptor (AR); non-AR therapeutic targets; castrate resistant prostate cancer (CRPC); abiraterone

Introduction

Since the initial experiences of Charles Huggins treating with advanced prostate cancer, it has been clear that the prostate cancer does not uniformly and completely regress as a result of androgen ablation(1,2). Subsequently, there have been decades of research to identify and characterize pathways and targets which allow prostate cancer to progress independently of androgens. More recently, it has become clearer that the androgen receptor (AR) remains a principal target even in castrate resistant prostate cancer (CRPC). This is highlighted by the clinical success of potent AR antagonists and steroidogenesis inhibitors in men with CRPC (3-5). The continued usefulness of PSA as a prognostic marker in CRPC also highlights how the AR axis remains a principal target (6). However, despite these recent improvements, CRPC remains a lethal disease and the search for new and improved treatments continues.

In recent years, an increased understanding of the molecular biology of CRPC has led to a proliferation of novel targeted therapeutics in clinical evaluation. *Table 1* lists some of the non-AR targets which are in clinical evaluation in CRPC. These novel targets emerge as CRPC develops more genetic and epigenetic alterations over time (7). In addition, tumours acquire resistance through alternative pathways as a result of the selective pressure of current treatments. Accordingly, the potent AR-targeting agents abiraterone and enzalutamide will likely lead to cancers that survive and proliferate through activation of alternate pathways.

Along with the excitement of the number of new and emerging non-AR therapeutics in the CRPC clinical space also comes some hesitation as to their eventually utility given the large number of recent disappointing phase III trial results in CRPC. This includes almost all targeted therapies in combination with docetaxel (*Table 2*). This highlights the difficulties in the generalizability of pre-clinical models and the need for well-designed and planned

Table 1 Selected approved and experimental therapeutics agents currently in clinical evaluation in CRPC which do not target the AR (Source: clinicaltrials.gov)

	Stress response pathways	Proliferative signal transduction targets	Immune escape	Critical cellular proliferative components	Tumour microenvironment
Targets	Clusterin; Hsp90; Bcl-2; Hsp27	PI3K; Akt; mTOR; Mu-opoid receptor; eIF4E; IGF-IR; Her-2	Dendritic cells; CTLA-4; PD-1	Microtubules; PARP1; SERCA pump	Osteoclasts; IL-11Ra; RANK-L; FAP; Endoglin; alpha V integrin; VEGF/FGFR; Neurotransmitters; Somatostatin receptor
Approved therapeutics			Sipuleucel-T	Docetaxel; Cabazitaxel	Denosumab; Radium-223
Experimental therapeutics	OGX-011; OGX-427	BEZ235; BKM120; AZD5363; MK2206; AZD8186; Naltrexone; ISIS 183750; Everolimus; Temsirolimus; Linsitinib; Lapatanib	Ipilimumab; BPX-201; BMS-936558; Pidilizumab	Tesetaxel; Patupilone; Ixabepilone; G-202	Sibrotuzumab; TRC-105; EMD 525797; BMTP-11; Dovitinib; Bevacizumab; Pazopanib; Phenelzine; Pasireotide

PI3K, phosphatidylinositol triphosphate kinase; CTLA4, cytotoxic T-lymphocyte antigen 4; PD1, programmed cell death protein 1; AMPK, adenosine monophosphate-activated protein kinase; VEGF, vascular endothelial growth factor; mTOR, mammalian target of rapamycin; PARP, poly-ADP ribose polymerase; IL-11Ra, interleukin-11 receptor alpha; SERCA, sarcoplasmic/endoplasmic reticulum calcium adenosine triphosphatase.

Table 2 Summary of selected Phase III clinical trials of combination therapy with docetaxel in CRPC. Adapted from Wissing *et al.* (8)

Docetaxel + prednisone with	Mechanism of action	Median OS
Estramustine	Alkylating agent	17.5 *vs.* 15.6 months
High-dose calcitriol	Vitamin D	17.8 *vs.* 20.2 months
Bevacizumab	Angiogenesis inhibitor	22.6 *vs.* 21.5 months
Risedronate	Bisphosphonate	19.2 *vs.* 18.4 months
Atrasentan	Endothelin A receptor antagonist	18 months (Phase III)/ 17.6 months (Phase II)
GVAX	Immunotherapy	13 months in both arms (predicted) 16 months in both arms (predicted)
Zibotentan	Endothelin A receptor antagonist	24.5 *vs.* 22.5 months
Aflibercept	VEGF-inhibitor	No significant improvement
Lenalidomide	Anti-angiogenesis, antineoplastic, immune modulation	N/A as halted early
Dasatinib	Tyrosine kinase inhibitor	21.5 *vs.* 21.2 months
OGX-011	Antisense clusterin inhibitor	23.8 *vs.* 16.9 months (Phase II) Phase III results pending

All trials except the estramustine trial compared results to docetaxel + prednisone. DLT, dose-limiting toxicity; GI, gastrointestinal; N/A, not available; OS, overall survival; VEGF, vascular endothelial growth factor.

Phase II and III clinical trials. Further, better selection of patients for these non-AR therapies is another need which may improve the success of new agents in clinical evaluation.

The molecular classification of CRPC will continue to evolve and is expected to play a large role in selecting patients for future trials of targeted therapy. Most of the molecular targets under investigation are not specific to prostate cancer, with overlap with other advanced cancers. While the AR plays a unique role in prostate cancer, the
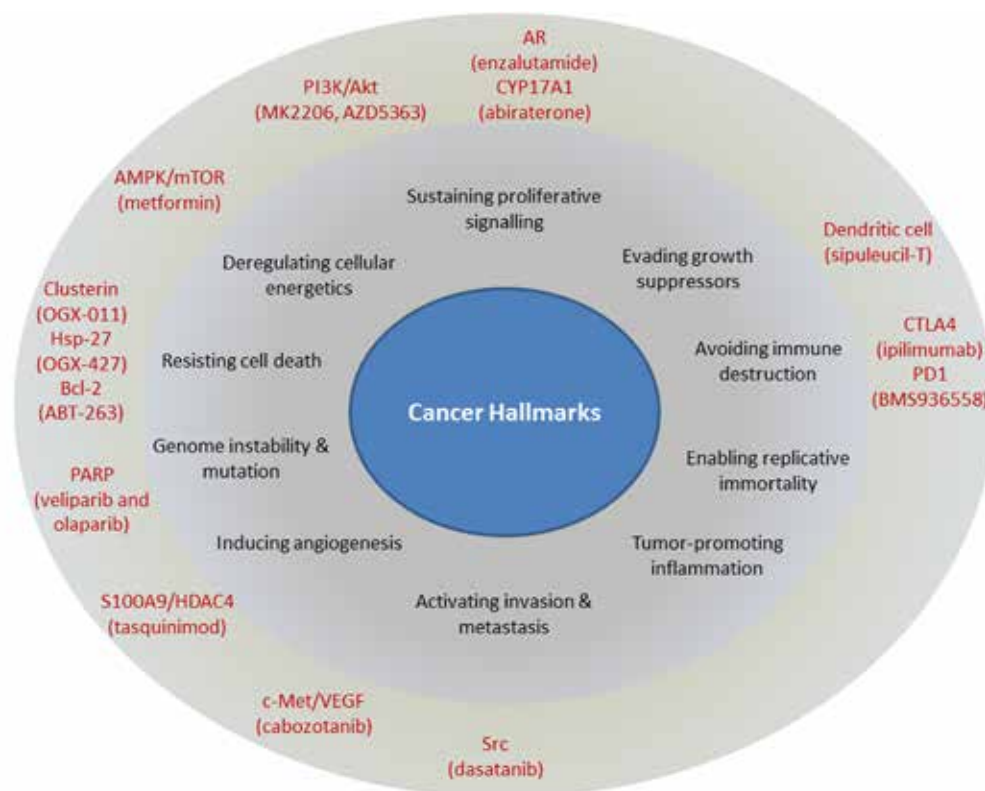
Figure 1 Current targets in castrate-resistant prostate cancer according to targeted cancer hallmarks. Adapted from Hanahan *et al*. Weinberg [2011]. PI3K, phosphoinositol triphosphate kinase; CTLA4, cytotoxic T-lymphocyte antigen 4; PD1, programmed cell death protein 1; AMPK, adenosine monophosphate-activated protein kinase; VEGF, vascular endothelial growth factor; mTOR, mammalian target of rapamycin; PARP, poly-ADP ribose polymerase.

division of molecular targets in CRPC into AR targets *vs.* non-AR targets can be misleading. Many non-AR cellular targets appear to support continued activation of the AR-axis through complex signalling and structural pathways. For example, microtubules are considered important in AR cellular transport (9). Similarly, the Akt pathway regulates and is regulated by the AR (10,11). Further characterization of the molecular changes which occur with CRPC progression may allow for identification of predictive and prognostic biomarkers for personalized targeted therapy.

This review will highlight some of the recently approved and currently researched molecular targets in CRPC beyond novel AR antagonists and steroid synthesis inhibitors. These targets, with their corresponding therapeutics, focus on many of the hallmarks of cancer (*Figure 1*). We will focus on discussing both the biologic mechanisms and clinical experience with these targets in CRPC. Particular emphasis is on agents which have reached Phase II-III clinical trials. With a particular concern that neuroendocrine cancers (also

known as small cell cancer of the prostate) will be more prevalence as a result of recent treatment improvements, we will also discuss briefly this relatively rare subtype of CRPC.

Targeting microtubules

Docetaxel and cabazitaxel are the two taxanes which are in clinical use in CRPC. Taxanes function by stabilizing the dynamic polymerization of microtubules. The ability of microtubules to assemble and disassemble is critical for mitosis and thus targeting microtubules preferentially targets rapidly dividing cancer cells. It also affects AR signalling through its alteration of microtubules-associated AR cellular transport and nuclear translocation (9,12). Docetaxel is the first approved agent outside of hormonal therapy which has a demonstrated survival benefit in CRPC (13,14).

Two studies demonstrated the survival benefit of docetaxel in CRPC patients. The TAX327 study showed that docetaxel every 3 weeks plus daily prednisone was superior to docetaxel

every week plus prednison or weekly mitoxantrone plus prednisone (14). Updated survival results indicate a median survival of 19.2 months in the q3weekly docetaxel plus prednisose arm versus 17.8 months in the weekly docetaxel arm plus prednisone versus 16.3 months in the mitoxantrone/ prednisone arm (13). The SWOG9916 study also found a 2-month survival benefit of docetaxel plus estramustine compared to mitoxantrone plus prednisone (15). However, no benefit in pain palliation or quality of life was noted; subsequently, estramustine is not in clinical use in CRPC.

Resistance to taxanes may be mediated through overexpression of the multi-drug resistant P-glycoprotein efflux pump (16), mutations in the microtubule binding sites, and mutations in microtubule-associated proteins giving greater stability to cellular microtubule assembly (17,18). Cabizataxel is a newer taxane which was selected through pre-clinical studies which found it had the greatest activity against docetaxel-resistant cell lines *in vitro* and *in vivo* (19). However, there is no clear definition of clinical docetaxel resistance. Similarly, the optimal duration of treatment with docetaxel is usually based on physician judgement. In the TAX-327, patients received up to 10 cycles; however it appears that more can be given if patients are receiving a tolerable response. Further, re-challenging patients with docetaxel after the recurrence of CRPC has also demonstrated some clinical success (20,21).

The TROPIC study established the role of cabazitaxel as second line therapy in CRPC after docetaxel. This study randomized men with progressive disease during or after docetaxel to receive cabazitaxel plus prednisone versus mitoxantrone plus prednisone (22). Cabazitaxel improved overall survival by a median of 2.4 months in this second-line setting. Cabazitaxel had a higher rate of adverse effects, particularly myelosupression, though even with mitoxantrone adverse events were higher than prior trials (14,15), highlighting the selection of sicker patients in this trial. Side effects of neutropenia and diarrhea were common (82% versus 58% and 6% versus <1% cabazitaxel versus mitoxantrone). Significantly, 28 patients (8%) in the cabazitaxel group had febrile neutropenia during the study versus 5 (1%) in the mitoxantrone arm. Two phase III trials are ongoing: FIRSTANA assesses cabazitaxel prior to docetaxel, while PROSELICA evaluates a lower dose (20 versus 25 mg/m^2) in men treated with prior docetaxel.

Newer therapeutics targeting the microtubules are in development. In contrast to the parenterally administered docetaxel and cabizataxel, tesetaxel is a novel, orally available taxane (Tesetaxel in Chemotherapy-naive Patients with Progressive, Castration-resistant Prostate Cancer http://clinicaltrial.gov/ct2/show/NCT01296243).It is currently in Phase II trials of men with CRPC. Epothilones also target microtubules through a different mechanism of action. Patupilone in a Phase II study recently demonstrated antitumor activity and safety as second line therapy (23). The oral synthetic epothilone, ixabepilone, demonstrated better activity in chemo naïve patients (24-26) compared to use as second line therapy, but has not been advanced to phase III trials.

Targeting the immune response

The goal of immunotherapy is to boost the tumour suppressive response of the patient's own immune system. This approach has been validated in CPRC with the approval of sipuleucel-T. Phase III randomized trials demonstrate an overall survival benefit (27) in men with minimally or asymptomatic metastatic prostate cancer, though an effect on progression-free survival or PSA-response was not seen. The treatment consists of re-infusing patient's autologous peripheral blood monocytes and antigen-presenting cells which have been exposed *ex vivo* to the fusion protein of prostatic acid phosphatase and granulocyte-macrophage colony-stimulating factor (GM-CSF).

The approach of priming of the immune system to tumour antigens has several advantages. It may result in a durable decreased rate of tumour growth. While this has yet to be identified, this theory corroborates with data suggesting that immunotherapy is of greatest benefit in patients with a lower burden of disease (28). The benefit of immunotherapy in lower volume disease is not unique to prostate cancer (29). The comparison of concurrent or sequential treatment of sipuleucel-T with abiraterone in patient with CRPC is underway and may help further understand the sequencing of immunotherapy with other available treatments. Further, in the case of sipuleucel-T, the side effects of this therapy are very tolerable, limited mostly to flu-like symptoms. However, the cost of this treatment remains a challenge to its implementation in many jurisdictions.

The PROSTVAC-VF vaccine similarly aims to boost natural immunity against tumor cells in CRPC. The vaccine consists of transgenes for PSA, as well as three co-stimulatory molecules (B7.1, leukocyte function-associated antigen-3 (LFA-3), and intercellular adhesion molecule-1 (ICAM-1) to enhance immune memory against the weakly immunogenic PSA antigen (30). A priming injection is

followed by monthly booster injections. An ongoing Phase III placebo-controlled study evaluates the efficacy of this vaccine +/- GM-CSF on the overall survival of men with minimally symptomatic metastatic prostate cancer. A whole cell vaccine, GVAX, has had two phase III trials terminated early due to an absence of benefit and an increased incidence of deaths in the treatment arm.

Checkpoint modulators of the immune system aim to remove the negative feedback signals in the patient's own immune system, thereby decreasing the immune system's tolerance of tumour antigens. Both ipilimumab and programmed death-(PD-1) inhibitors employ this strategy. Ipilimumab has previously demonstrated success in treating metastatic melanoma (31) It is a fully human monoclonal antibody which targets CTLA-4. CTLA-4 is an important negative regulatory receptor on T-cells. By blocking CTLA-4, ipilimumab releases the homeostatic negative feedback on T-regulatory cells which the immune system normally establishes in order to avoid autoimmunity. As a result, the immune system's tolerance of tumour antigens should decrease, resulting in greater immune-mediated destruction of tumour cells. Two randomized trials comparing ipilimumab versus placebo prior to and after chemotherapy are underway in CRPC. Further, there has also been reported some clinical success using an anti-PD-1 monoclonal antibody in refractory solid tumours (32). PD-1 is an immune inhibitory receptor expressed on T cells which also modulates the immune response. Blocking this receptor reduces some of the negative self-regulation of the immune system, conceptually similar to CTLA-4 inhibitors. In the first large clinical trial of solid tumours, treatment responses were observed in patients with lung, melanoma and renal cell carcinoma, many of whom had failed multiple therapies (32). In the small subset of 17 patients with CRPC, no objective responses were seen, though clinical evaluation in CRPC is ongoing (32). It is possible this target will have fewer side effects than targeting CTLA-4 as a result of a greater specificity for the tumour microenvironment.

Targeting angiogenesis and c-Met

Several trials evaluating anti-angiogenic agents in CRPC have reported universally disappointing results, including aflibercept, bevacizumab, lenalidomide, sunitinib and sorafenib in combination with docetaxel or as second-line monotherapy. Anti-angiogenic inhibitors currently in phase III evaluation include tasquinimod and cabozantinib. Other novel angiogenesis inhibitors currently in Phase II trials include TRC-105, which is a monoclonal antibody against endoglin, a receptor overexpressed on proliferating endothelium and the vascular epithelial growth factor(VEGF)/fibroblast growth factor receptor(FGFR) inhibitor dovitinib (30).

Tasquinimod is a novel orally administered quinoline-3-carboxamide with anti-angiogenic and anti-tumorigenic effects which is now in Phase III trials. In contrast to other angiogenesis inhibitors targeting VEGF and/or tyrosine kinases receptors, tasquinimod disrupts cross talk within the tumour microenvironment by modulating HDAC4 and also targets S100A9 (33). Phase II results demonstrated in men with minimally symptomatic metastatic disease a median PFS of 7.6 compared to 3.3 months in the placebo arm (P=0.0042) (34).

Cabozantinib (XL184) is a dual c-MET and VEGF-receptor inhibitor. c-MET is a receptor tyrosine kinase which binds hepatocyte growth factor. When activated through phosphorylation, c-MET activates downstream signalling pathways involved in survival, growth and invasion. These downstream pathways include the phosphatidylinositol triphosphate (PI3K)/Akt pathway and the mitogen activated protein kinase (MAPK) pathway (*Figure 2*). In reported Phase II clinical trial results with cabozantinib, 68% of patients at 12 weeks experienced an improvement on bone scan, with 12% having complete remission (36). Further, the median profession-free survival of those on treatment was 23.9 versus 5.9 weeks for placebo. However, randomization was halted early in the trial due to the benefit seen, so the numbers were small (36). Tivantinib is another c-MET inhibitor which in is in Phase II clinical evaluation in CRPC (NCT01519414).

Targeting cellular energetics

Metformin has been demonstrated in several retrospective and large cohort studies to confer an overall survival benefit in men with prostate cancer (37,38), though the literature is not entirely consistent (39,40). The survival benefit appears particular to metformin, as no benefit was seen with other anti-diabetic medications (37). The relative safety, availability and cost of metformin make it an appealing agent for further investigation in CRPC.

Metformin functions as an adenosine monophosphate-activated protein kinase (AMPK) activator. Activated AMPK regulates cellular energetics through inactivation of enzymes involved in adenosine triphosphate consumption such as fatty acid and protein synthesis. It also functions
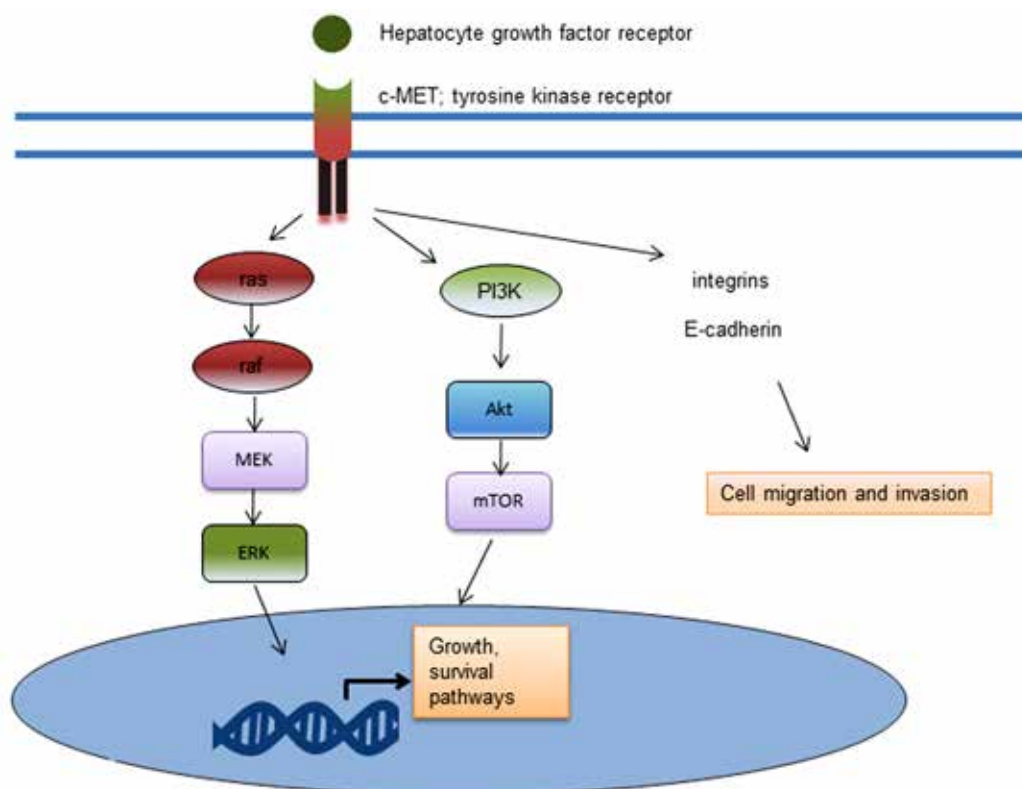
Figure 2 Schematic of the c-MET pathway. Adapted from Loriot *et al*. (35). ERK, extracellular related kinase; mTOR, mammalian target of rapamycin; PI3K, phosphoinositol triphosphate kinase.

through negative regulation on the mammalian target of rapamycin (mTOR) pathway (37). Clinical studies evaluating metformin alone and in combination with docetaxel for CRPC are now underway (NCT01796028, NCT01215032).

Targeting the stress-response

Molecular chaperones have an important role in the cellular stress response through maintaining protein homeostasis and regulating pro-survival networks. Chaperone proteins stabilize intracellular proteins against against misfolding and aggregation during stress, as well as facilitating intracellular and compartmental transport (41). In CRPC, two stress-activated cytoprotective chaperones, clusterin and Hsp27, are targets in ongoing clinical trials.

Clusterin exists in two forms, nuclear clusterin and secretory clusterin. Secretory clusterin (sCLU) functions as a cytoprotective chaperone which is up-regulated in CRPC. sCLU has been demonstrated to play a role in inhibiting endoplasmic reticulum stress, cytosolic protein aggregation

and also inhibits mitochondrial apoptosis (42-44). Custirsen (OGX-011) is a second-generation antisense oligonucleotide against the clusterin mRNA. Preclinical studies demonstrate that OGX-011 potently suppresses sCLU levels *in vitro* and *in vivo* (45,46). Further, cotargeting of sCLU and AR delays CRPC progression in pre-clinical models models through inhibiting the adaptive stress response and regulating AR stability (47). In a Phase II clinical trial, a survival advantage of 6.9 months for custirsen plus docetaxel and prednisone over docetaxel and prednisone was seen (48). Phase III results of this combination are expected in 2014.

Heat shock protein-27 (Hsp27) is another abundant stress-induced cellular chaperone protein implicated with the AR signalling and treatment resistance (49,50). OGX-427 is a second-generation antisense oligonucleotide against Hsp27 now in phase II trials as second line treatment in metastatic CRPC in combination with abiraterone.

Targeting survival pathways

Deregulation of normal cellular functions of apoptosis is one

of the common characteristics of cancer. Bcl-2 is a regulator of apoptosis; several other anti-apoptotic family members include Bcl-2, Bcl-XL, Mcl-1, BCL-W and BFL-1 (51). The balance of cell survival or cell death is further regulated by multidomain pro-apoptotic proteins such as BAX, BAK and BOK. As in several advanced cancers, Bcl-2 gene expression it is up-regulated in CRPC (52), presenting a targetable oncogene.

A small phase II trial of 13-cis retinoic acid and interferon-alpha2b in combination with paclitaxel demonstrated the ability to modulate Bcl-2 levels in peripheral monocytes. However, the low treatment response rates and decreased quality of life halted further development. Similarly, the use of a Bcl-2 anti-sense oligonucleotide showed promising data *in vitro* (53,54) and demonstrated proof-of principle target inhibition in phase I trials (55). However, challenges with a short half-life upon infusion contributed to sub-optimal target inhibition and disappointing phase II results (54,56).

BH3 mimetics target the function of Bcl-2 family proteins through hydrophobic binding which displaces the BH3-only proteins, allowing them to activate Bax or Bak proteins and subsequently signal cell death (51). Examples of BH3 mimetics include ABT-737 and its oral-derived enantiomer ABT-263. A clinical trial aims to compared ABT263 plus abiraterone to treatment with ABT263 plus abiraterone and an autophagy inhibitor hydroxychloroquine (NCT01828476).

Targeting DNA-damage repair

PARP inhibitors represent a different class of therapeutics and include veliparib and olaparib. The enzyme poly-ADP ribose polymerase (PARP) is responsible for repairing single strand breaks in DNA. Inhibition of this enzyme leads to alterations in the ability of DNA replication to occur, causing cell death (57). It may have a specific benefit in tumours with BRCA1 or BRCA2 mutations, both of which are implicated in more aggressive prostate cancer (58). BRCA1 and BRCA2 proteins are responsible for repairing double-strand DNA breaks in DNA. With the inhibition of PARP, single-strand breaks may become non-repairable (and thus lethal) double-strand breaks in BRCA1/2 mutant cancers. Similarly, a synthetic lethality using DNA-damage repair inhibitors has also been proposed to apply to the common PTEN-deletion CRPC tumours, which are reported to have defects in homologous recombination (59). Therefore, this represents a possible tailored therapy for

patients with these mutations. A clinical trial is also underway for patients with ETS fusions, based on pre-clinical data suggesting the involvement of TMPRSS2 gene fusions with DNA repair cellular machinery, including PARP1 and topoisomerase II (60).

Targeting the tumour microenvironment

With increased understanding the importance of the tumour microenvironment on the progression of CRPC, therapeutic strategies are emerging to target the adjacent stroma (61,62). Further, it appears that treatments which target both the stroma and epithelium compartments may be expected to be more successful. Both androgen androgen deprivation and cabozantinib are examples of this approach: the AR and c-MET are both active in both the stroma and epithelial compartments during CRPC (63,64). IGF-IR inhibitors also target both stroma and tumour components (65). However, with the failure to date of several angiogenesis inhibitors in CRPC, agents targeting the microenvironment are likely best evaluated in rationale combination strategies with other treatments. For example, pre-clinical research suggests that IGF-IR blockade may enhance Src inhibition (66).

Hedgehog signalling is an important paracrine factor during organogenesis and appears to be de-regulated during prostate cancer progression. Sonic hedgehog secreted by the tumour appears to alter the tumour microenvironment to ultimately increase oncogenic Gli-1/2 transcription factors through paracrine signalling. Sonic hedgehog ligands signal via Patched-1 and result in the loss of the Smoothened repression on Gli-1 and Gli-2. Hedgehog signalling appears to be up-regulated following androgen deprivation conditions (67,68). Preclinical data on TAK-441 and GDC-0449 (vismodegib) in CRPC models (68,69) and an ongoing neo-adjuvant study of GDC-0449 should lead to upcoming clinical trials in CRPC patients.

Another novel drug which targets the tumour microenvironment is the monoclonal antibody sibrotuzumab. It targets fibroblast-activated protein (FAP). This protein expressed in cancer-associated stroma, but not normal stroma-associated with epithelial cancers. It is considered to play a role in tumor growth and proliferation (70).

Targeting the bone micro-environment

Therapeutic targeting of the bone microenvironment addresses side effects associated with androgen deprivation

therapy as well as the commonest metastatic location of CRPC. Several clinical trials have now established new treatment options for patients with CRPC and should be used appropriately alongside lifestyle changes and calcium supplementation. Bisphosphonates were the first agents approved for men with metastatic prostate cancer. Zoledronic acid was approved based on studies which demonstrated an improvement in skeletal related events, though no survival benefit was observed (71,72). More recently, denosumab has been approved has been approved for me with CRPC. It functions as a monoclonal antibody against RANK-L, which prevents bone loss through the inactivation of osteoclasts. Further, denosumab also appears to have an effect on the metastatic niche, with a delay in the appearance of bone metastasis (73). However, this did not result in differences in overall survival. Compared to zoledronic acid, denosumab appears to have superior potency, with a greater reduction in skeletal-related events (74).

Radiopharmaceuticals also target the bone metastatic environment. Historical agents Rhenium-186 and Samarium-135 have demonstrated improved bone pain in patients with metastatic CRPC in small randomized trials (75,76). Strontium-89 is another radiopharmaceutical which as a calcium mimetic has a strong propensity for the bone microenvironment (77,78). An ongoing phase III trial evaluates Sr^{89} plus docetaxel and prednisolone versus docetaxel and prednisolone. Radium-223 chloride is a newer calcium mimetic radiopharmaceutical. In contrast to the aforementioned agents which emit beta-radiation, it emits alpha radiation. Alpha-radiation has a shorter penetration depth with higher energy and is therefore less toxic to the bone marrow. Bone marrow toxicity is a challenging toxicity in men with CRPC and bone metastasis who often have anemia to begin with. Clinical experience has demonstrated now significant differences in hematologic side effects using radium-223. Notably, radium-223 has also demonstrated an improvement in overall survival in recent clinical trials of men with painful bone metastasis (79,80). In a placebo-controlled trials, the radium-223 treated arm had a hazard ratio of 0.70 for overall survival at the interim analysis (81). As well as validating the benefit of this drug, these studies suggest that a survival benefit may be achieved through targeting of metastatic disease.

Targeting intra-cellular signalling transduction pathways

Further understanding of the molecular biology of cancer

has led to several intracellular transduction pathway inhibitors now in clinical evaluation. Often, these agents are evaluated in combination with current treatment strategies to synergize anti-cancer activity and minimize toxicity. For example, studies suggest a synergist effect of targeting of both AR and signal transduction pathways such as PI3K/Akt and MAPK pathways (11,82).

The phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR pathway represents the most commonly activated pathway in CRPC. Alterations in this pathway have been identified in 42% of primary tumours and 100% of metastases (83). This pathway is also active in many other advanced cancers (84). Loss of function of the PTEN repressor results in increased levels of activated Akt and downstream effects, as does an activating mutation of the PIK3A gene. The activated downstream effectors, including GSK3β and S6 kinase, result in cell survival, proliferation, migration and invasion (*Figure 3*) (84-87). Activation of the PI3K/Akt pathway is associated with higher Gleason score, decreased metastasis free survival (87). Due to the reciprocal interactions of this pathway, combination targeting strategies with AR antagonists are under investigation using novel therapeutics targeting nodes of this pathway (11,88).

The Src family nonreceptor tyrosine kinases are another intra-cellular target of interest in CRPC. There are nine members of this kinase group (Blk, Fgr, Fyn, Hck, Lck, Lyn, Src, Yes, and Yrk) (89). Src is the most studied as it relates to prostate cancer progression. Src and related kinases are associated with prostate cancer progression through various mechanisms, including proliferation, invasion and interactions with the AR (90-92). Src signalling is also involved in regulating bone turnover in prostate cancer (93) and may therefore be important in the progression of bone metastasis, a common event in CRPC.

Dasatinib and saracatinib (AZD0530) are two Src inhibitors which have completed phase II trials in CRPC. Dasatinib is a small-molecule multi-tyrosine kinase inhibitor of with broad activity against receptor tyrosine kinases, Src family kinases, c-Kit, PDGFR, Bcr-Abl and ephrins (94). In a Phase I/II trial of dasatinib plus docetaxel, 30% of patients had disappearance of lesions on bone scan and 57% of patients experienced a durable PSA response (95). In Phase II dasatinib monotherapy trials in men prior to chemotherapy, no responses were seen but there was a lack of progression in 43% of patients at 12 weeks (96). Results of the phase III READY trial of dasatinib in addition to standard docetaxel in metastatic CRPC presented at GU-ASCO 2013 showed no benefit to OS (97). Dasatinib is currently in Phase III
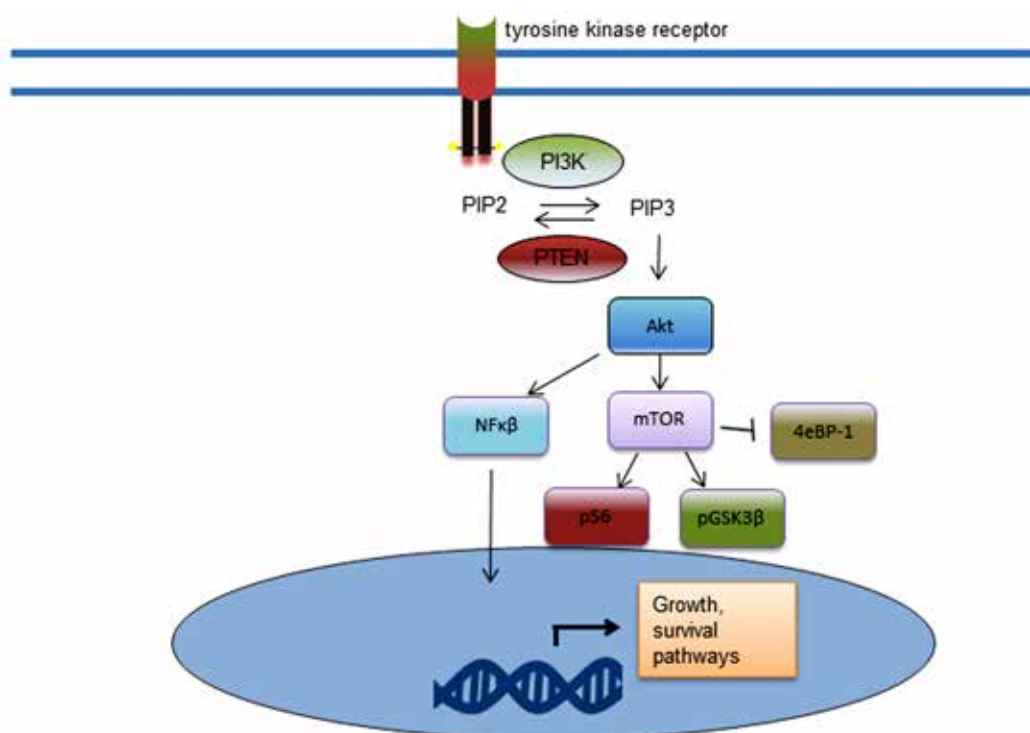
Figure 3 Schematic of the PI3K/Akt pathway. mTOR, mammalian target of rapamycin; PTEN, Phosphatase and tensin homolog; NFκβ, nuclear factor kappa beta; PI3K, phosphoinositol triphosphate kinase.

trials in combination with abiraterone.

Targeting neuroendocrine prostate cancer

Neuroendocrine prostate cancer is a separate entity from most cases of CPRC. Clinically, it remains a relatively rare and thoroughly aggressive phenotype. Disease progression appears entirely unrelated to the AR axis with patients usually identified through a disproportionately low PSA. Visceral and brain metastases are more common. Serum and tissue markers of chromogranin A, NSE and synaptophysin are commonly elevated. Recently, protocadherin-PC has also been suggested to be a marker of neuroendocrine transdifferentiation (98).

There is a renewed interest in this subtype of CRPC for a couple reasons. Firstly, the increasing use of potent AR antagonism is postulated to increase the incidence of NEPC, though this requires further research to validate this hypothesis. Secondly, new treatments for this aggressive entity are now in development. Aurora kinase inhibitors such as MLN8237 target neuroendocrine/small cell prostate cancer. Sequencing studies have identified overexpression and gene

amplification of aurora kinase A and N-myc in 40% of NEPC *vs.* 5% of prostate cancers (99). Phase II trials of MLN8237 are ongoing in men with elevated NEPC markers.

Conclusions

An improved understanding of the molecular biology of CRPC has led the way to a relative explosion in the number of targets and novel treatments for this lethal disease. Many new targets beyond the AR have the potential to further improve the outcomes for patients. Past failures of non-AR agents in clinical trials highlight the need for rigorous evaluation of agents which are selected to proceed to Phase III clinical evaluation. Combination strategies will likely optimize the efficacy of targeting alternate pathways. Further, the molecular classification of tumour subtypes will further aid in patient selection for these targeted therapies.

Acknowledgements

Footnote

Conflicts of Interest: The University of British Columbia has submitted patent applications, listing Dr. Gleave as inventor, on the antisense sequence described in this paper. This IP has been licensed to OncoGenex Pharmaceuticals, a Vancouver-based biotechnology company that Dr. Gleave has founding shares in. The other author has no conflicts of interest to declare.

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Steroid hormone synthetic pathways in prostate cancer

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Abstract: While androgen deprivation therapy (ADT) remains the primary treatment for metastatic prostate cancer (PCa) since the seminal recognition of the disease as androgen-dependent by Huggins and Hodges in 1941, therapy is uniformly marked by progression to castration-resistant prostate cancer (CRPC) over a period of about 18 months, with an ensuing median survival of 1 to 2 years. Importantly, castration does not eliminate androgens from the prostate tumor microenvironment. Castration resistant tumors are characterized by elevated tumor androgens that are well within the range capable of activating the AR and AR-mediated gene expression, and by steroid enzyme alterations which may potentiate *de novo* androgen synthesis or utilization of circulating adrenal androgens. The dependence of CRPC on intratumoral androgen metabolism has been modeled *in vitro* and *in vivo*, and residual intratumoral androgens are implicated in nearly every mechanism by which AR-mediated signaling promotes castration-resistant disease. These observations suggest that tissue based alterations in steroid metabolism contribute to the development of CRPC and underscore these metabolic pathways as critical targets of therapy. Herein, we review the accumulated body of evidence which strongly supports intracrine (tumoral) androgen synthesis as an important mechanism underlying PCa progression. We first discuss the presence and significance of residual prostate tumor androgens in the progression of CRPC. We review the classical and non-classical pathways of androgen metabolism, and how dysregulated expression of these enzymes is likely to potentiate tumor androgen production in the progression to CRPC. Next we review the *in vitro* and *in vivo* data in human tumors, xenografts, and cell line models which demonstrate the capacity of prostate tumors to utilize cholesterol and adrenal androgens in the production of testosterone (T) and dihydrotestosterone (DHT), and briefly review the potential role of exogenous influences on this process. Finally, we discuss the emerging data regarding mechanisms of response and resistance to potent ligand synthesis inhibitors entering clinical practice, and conclude by discussing the implications of these findings for future therapy.

Keywords: Steroidogenesis; intracrine; CYP17A; castration resistant prostate cancer (CRPC); androgen receptor; splice variant; steroid transport; abiraterone; enzalutamide

Presence of intratumoral androgens despite castration

The efficacy of androgen deprivation therapy (ADT) is routinely based on achieving castrate levels of serum T, defined as <20 ng/dL. However, tissue androgen levels in the setting of benign prostatic hyperplasia (BPH), primary prostate tumors, locally recurrent prostate cancer (PCa), or metastatic castration-resistant prostate cancer (CRPC) have consistently demonstrated that castration does not eliminate androgens from the prostate tumor microenvironment.

Geller *et al*. examined prostatic DHT levels by radioimmunoassay (RIA) and demonstrated that castration by orchiectomy (or megace plus DES) reduced prostatic DHT levels by 75-80% to 1 ng/g in some but not all patients. Epithelial and stromal cell protein synthesis was strongly

correlated with tissue DHT levels, and prostatic DHT levels were further reduced when castration was combined with adrenal androgen blockade by ketoconazole (1-6), suggesting the goal of therapy should be to decrease prostatic DHT to as low as possible, a concept similarly framed in early studies by Labrie (7).

Incomplete suppression of prostate tissue androgens by castration has been subsequently confirmed in numerous studies of short and long term castration therapy (8). Treatment of BPH patients for 3 months with an LHRH agonist decreased intraprostatic T levels by 75%, to about 0.1 ng/g, and DHT levels by 90%, to 0.48 ng/g (9). In men with PCa 6 months of neoadjuvant ADT with castration and flutamide reduced prostatic DHT levels by 75% to about 1.35 ng/g (10). Notably, tumor differentiation based on Gleason grading has been correlated with change in tissue DHT, with an 85% decrease measured in Gleason 6 cancers, but only a 60% decrement in Gleason 7-10 tumors (11). This finding indicates that tumor type-specific changes in androgen metabolism may impact responses to systemic T suppression.

Residual androgens have also been demonstrated in both locally recurrent and metastatic castration resistant tumors. Testosterone levels in locally recurrent tumors from castrate patients were *equivalent* to those of BPH patients, and DHT levels were only reduced 80%, to about 0.4 ng/g (12). Compared to primary prostate tumors from untreated patients (T 0.25 ng/g, DHT 2.75 ng/g) androgen levels in metastatic CRPC tumors obtained via rapid autopsy showed 3-fold higher T levels and an inverted ratio of T to DHT (T 0.74 ng/g; DHT 0.25 ng/g) (13). Adrenal androgens have also been detected at significant levels in prostate tissue of castrate men. Prostatic levels of dihydroepiandrosterone (DHEA), DHEA-sulfate (DHEA-S), and androstenedione (AED) were decreased by about 50% in castrate patients and far exceeded values of T and DHT in recurrent tumors (12). No decrease in prostatic levels of 5-androstenediol were found after castration (14), which is of particular significance as this androgen has been shown to bind wild type AR without being inhibited by flutamide or bicalutamide (15).

Two recently reported studies demonstrate that the addition of androgen synthesis inhibitors to castration therapy can lower prostate androgens below that achieved with standard androgen blockade. The addition of dutasteride and ketoconazole to combined androgen blockade (CAB) for 3 months prior to prostatectomy lowered prostate DHT from 0.92 ng/g (in the CAB arm) to 0.03 ng/g (16). In a second study, the potent CYP17A

inhibitor abiraterone was added to LHRH agonist therapy for 3 or 6 months prior to prostatectomy. Abiraterone decreased prostate tissue DHT from 1.3 ng/g (in men treated with LHRH agonist therapy alone) to 0.18 ng/g and also decreased prostate levels of AED and DHEA (17).

Significance of intratumoral androgens in progression of CRPC

These findings clearly demonstrate that achieving castrate levels of circulating T does not eliminate androgens from the prostate tumor microenvironment. The ability of DHT in the range observed in castrate tumors (~1 nm, 0.5 to 1.0 ng/g) to activate the AR, stimulate expression of AR-regulated genes, and promote androgen mediated tumor growth has been convincingly demonstrated in both *in vitro* and *in vivo* studies (12,18-21), and is evidenced by the nearly universal rise in serum PSA that accompanies CRPC progression.

Residual tissue androgens are implicated in driving the majority of mechanisms whereby persistent AR-mediated signaling drives castration resistant disease. These mechanisms include AR overexpression, AR mutations that broaden ligand specificity and/or confer sensitivity to adrenal androgens, alterations in AR coactivators and/or corepressors that modulate AR stability and ligand sensitivity, and activation of the AR or downstream regulatory molecules by "cross talk" with other signaling pathways. Restoration of AR expression and signaling in a xenograft model was both necessary and sufficient to drive progression from androgen-dependent to castration resistant growth, allowing tumor cell proliferation in 80% lower androgen concentrations (22). Importantly, ligand binding was required for hormone refractory growth, and modest increases in AR expression were sufficient to support signaling in a low androgen environment.

The clinical relevance of intratumoral androgens in promoting CRPC tumor growth is confirmed by the clinical responses to agents targeting residual androgen pathway activity. These include historical responses described in response to adrenalectomy and/or hypophysectomy (23,24); the limited but consistent ~5% overall survival benefit seen in meta-analyses of CAB (25-27); the observation that nearly 30% of recurrent prostate tumors demonstrate at least transient clinical responses to secondary or tertiary hormonal manipulation (28); and most recently, the striking clinical response observed with novel ligand synthesis inhibitors such as abiraterone, and potent AR inhibitors
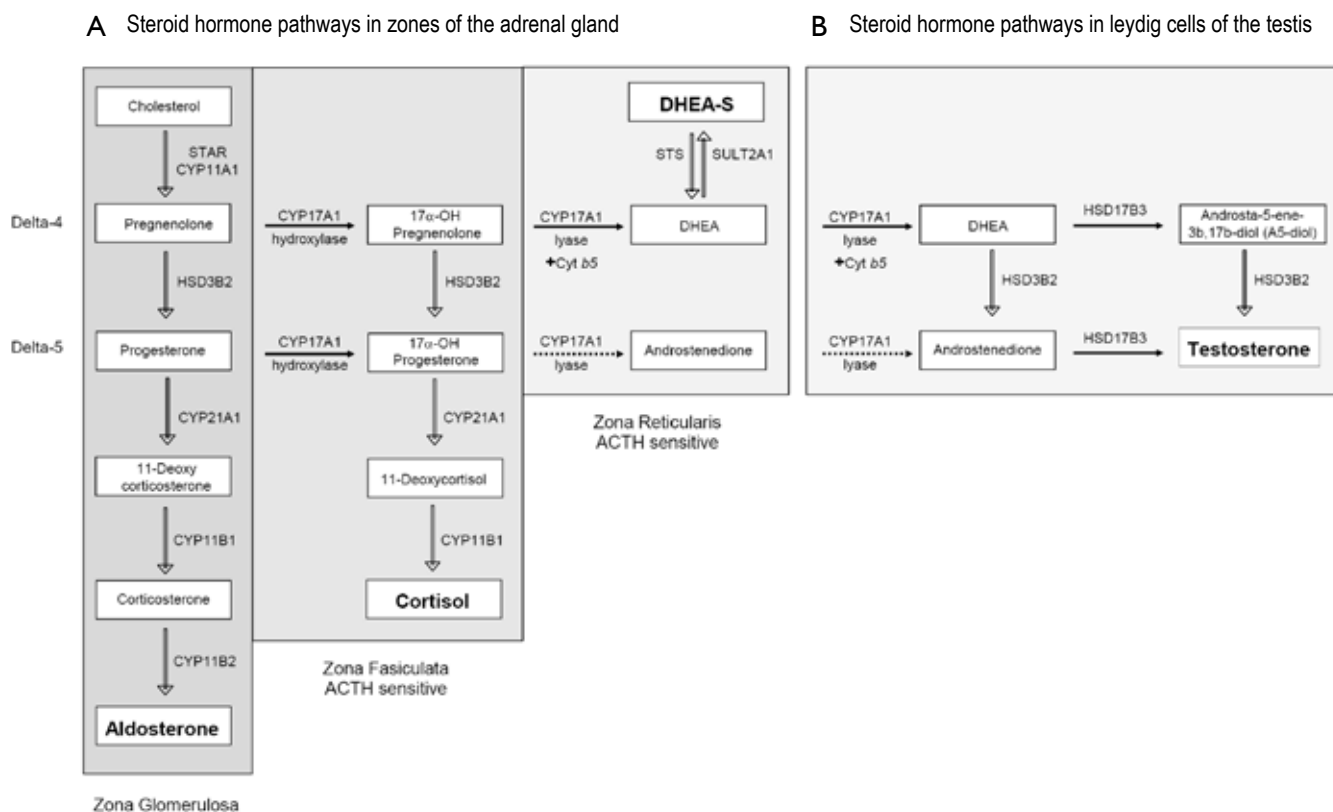
Figure 1 Steroid hormone synthesis pathways in the adrenal gland and testis. A. Steroid synthesis in the adrenal gland occurs in three zones, each with a specific complement of enzymes. The zona glomerulosa contains the enzymes necessary to produce aldosterone. The zona fasciculata and reticularis additionally express CYP17A. The hydroxylase activity of CYP17A is active in the zona fasiculata resulting in the production of cortiso. Due to tissue-specific expression of the cytochrome b5 coregulator, the lyase activity of CYP17A is only present in the zona reticularis and drives efficient production of DHEA which is then sulfated to DHEA-S. 17α-OH progesterone is a poor substrate for CYP17A lyase (dotted arrow) and thus androstenedione is formed at lower levels. The zona fasciculate and zona reticularis are sensitive to the ACTH feedback stimulation that occurs when cortisol production is suppressed by inhibition of CYP17A. Agents specifically targeting the lyase but not hydroxylase activity of CYP17A would not inhibit cortisol synthesis and are anticipated to induce less ACTH feedback stimulation; B. Testicular androgen synthesis follows a similar pathway to DHEA formation as that in the zona reticularis. Due to the absence of SULT2A1, and the presence of HSD3B2 and HSD17B3, DHEA is efficiently converted to testosterone.

such as enzalutamide (29,30). Perhaps most importantly, emerging studies suggest that response and resistance to abiraterone is associated with tumoral evidence of upregulated androgen synthesis, clearly demonstrating the importance of intratumoral androgen metabolism in CRPC tumor survival (31-33).

Pathways of androgen metabolism

The source of residual androgens within prostate tumors of castrate men has not been fully elucidated, but is generally attributed to the uptake and conversion of circulating adrenal androgens (14,34), and somewhat more

controversially, to *de novo* biosynthesis of androgens from progesterone or cholesterol precursors (35). Here we review the classical pathways of *de novo* androgen synthesis in adrenal and peripheral tissues [*Figure 1*, reviewed in (36)], the enzymatic pathways mediating prostate androgen metabolism, and the so called 'back-door' pathway of androgen synthesis. A general outline of the classical and non-classical steroidogenic pathways is provided in *Figure 2*.

Androgen synthesis in the adrenal gland and peripheral tissues

Steroid hormone synthesis begins with transfer of a
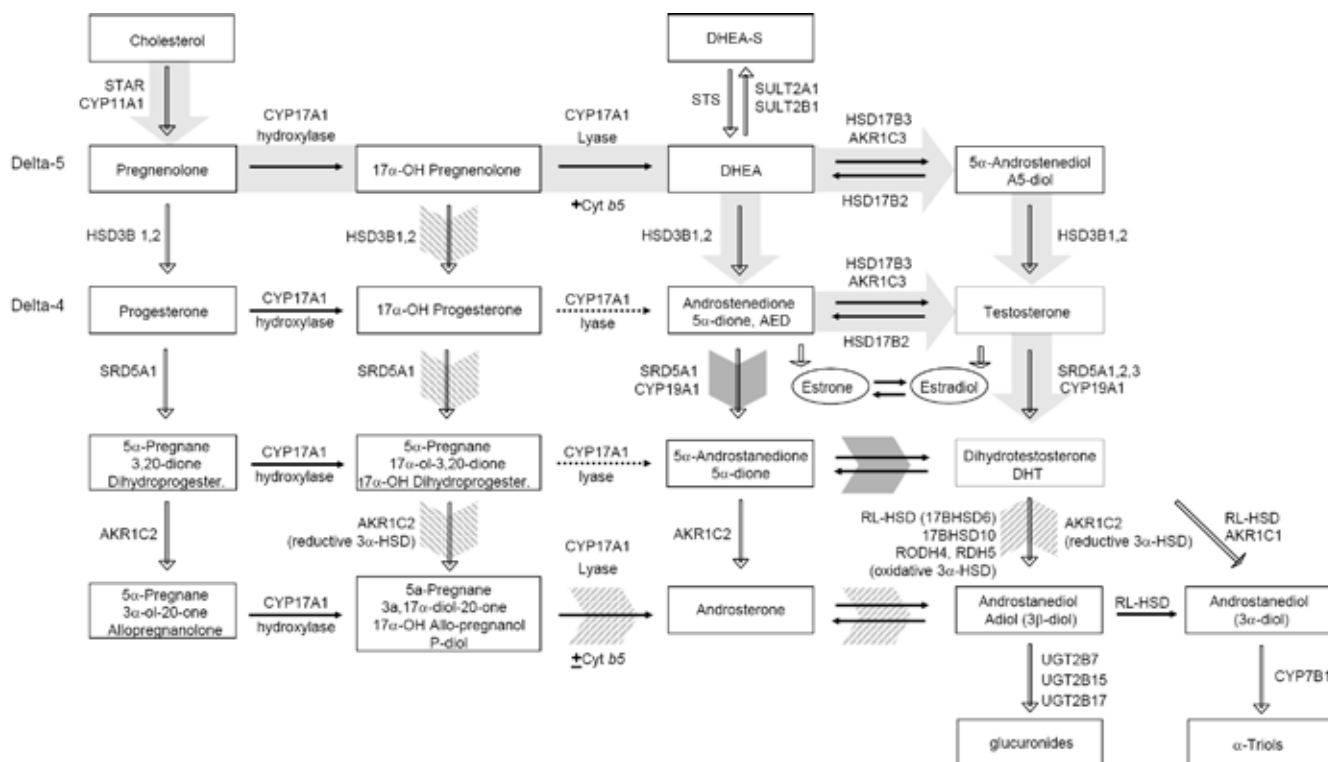
Figure 2 Classical and non-classical pathways of androgen biosynthesis. Cholesterol is converted to C21 precursors (pregnenolone and progesterone) by the action of STAR and CYP11A. In the classical pathway (light gray arrows) C21 steroids are converted to the C19 adrenal androgens DHEA and androstenedione (AED) by the sequential hydroxylase and lyase activity of CYP17A. Due to the substrate preference the lyase activity of CYP17 (which requires the cytochrome b5 cofactor) favors production of DHEA. DHEA (from intrinsic or circulating sources depending on the tissue) is subsequently acted on by HSD3B and HSD17B3 (or AKR1C3) to form testosterone (T) which is converted to DHT via SRD5A. In the backdoor pathway (hatched arrows) the progestin intermediates are acted on first by the activity of SRD5A and the reductive activity of AKR1C2 prior to the lyase activity of CYP17A. Androsterone is then acted on by HSD17B3 (or AKR1C3) and must undergo an oxidative step mediated by RL-HSD (or others) to generate DHT. In a third pathway, termed the 5α-Androstanedione pathway (dark gray arrows) DHEA and AED are produced as in the classical pathway. However, instead of conversion of AED to T followed by the activity of SRD5A to produce DHT, the enzymatic sequence is reversed such that AED is converted first by SRD5A to 5α-Androstanedione and then by HSD17B3 (or AKR1C3) to DHT.

27-carbon (C-27) cholesterol molecule from the outer mitochondrial membrane to the inner membrane by steroidogenic acute regulatory protein (STAR), followed by its conversion to the C-19 steroid, pregnenolone via CYP11A (side change cleavage enzyme). Subsequent metabolism to progesterone, mineralocorticoids, glucocorticoids (all C-21steroids), androgens (C-19) or estrogens (C-18) is dictated in a tissue-specific manner, driven by the expression of specific enzymes and catalytic cofactors.

CYP17A, expressed in the adrenal gland, testis and ovary, is a single enzyme with one active site which catalyzes sequential but independent hydroxylase and lyase

reactions, both of which are required for converting C21 progestins to androgens and estrogens, either along the delta-5 pathway from pregnenolone or the delta-4 pathway from progesterone. The hydroxylase activity of CYP17A for pregnenolone and progesterone is similar, but its lyase activity for delta-5 and -4 substrates requires the activity of the cytochrome *b5* cofactor, and is approximately 50 times more efficient for converting the delta-5 substrate 17-OH pregnenolone to DHEA than the delta-4 substrate 17-OH progesterone to AED (36). HSD3B enzymes catalyze the conversion of delta-5 to -4 steroids. Whereas HSD3B2 is the primary isoform expressed in adrenal, testis and ovary, HSD3B1 (10 fold more efficient) is the isoform expressed in

peripheral tissues such as skin, breast, prostate, placenta and brain (36).

In adrenal steroidogenesis [*Figure 1A*, reviewed in (37)], the zona glomerulosa lacks CYP17A activity and produces aldosterone via the sequential activity of HSD3B2, CYP21A1, and CYP11B on pregnenolone. Both the zona fasiculata and zona reticularis express CYP17A, but the zona fasiculata does not express the cytochrome *b5* cofactor required to catalyze the lyase activity of CYP17A, channeling precursors to production of glucocorticoids. The differential expression of cytochrome *b5* in the zona reticularis catalyzes the lyase activity of CYP17 10-fold, leading to robust production of DHEA, followed by conversion to DHEA-S via the sulfotransferase activity of SULT2A1. The zona reticularis is characterized by low expression of HSD3B2, favoring conversion of pregnenolone to DHEA and DHEA-S, although small amounts are converted to AED (38).

Less recognized is that the human zona reticularis also expresses AKR1C3, which mediates the final step in T synthesis from AED. Notably, in a small study of 8 women, adrenal vein T levels increased 6-fold (18.5 to 116 ng/dL) before and after ACTH stimulation (39). In a much earlier study, selective adrenal vein catheterization in men also demonstrated adrenal to peripheral venous T gradients, although a compensatory increase in adrenal production of T was not observed in castrate *vs.* intact men (40).

Leydig cells of the testis (*Figure 1B*) express similar metabolic machineries, including STAR, CYP11A, and the preference of CYP17A for delta-4 substrates, allowing them to produce DHEA from cholesterol, but with several key differences, including absence of SULT2A1, preventing conversion to DHEA-S, and abundant expression of HSD3B2, which mediates the delta-4 to -5 conversion required to generate T. The final steps in T biosynthesis are catalyzed by HSD17B3 and/or HSD17B5 (called AKR1C3). HSD17B3 is primarily expressed in testicular Leydig cells, while AKR1C3 mediates production of T and DHT in peripheral tissues. The activity of HSD3B2 sand HSD17B3 thus drives the stepwise conversion of DHEA to T, via either AED or androst-5-ene-3, 17-diol (5-androstenediol or A5-diol).

Androgen synthesis in the prostate and pre-receptor control of DHT metabolism

The uptake of circulating androgens and the local synthesis of active steroids in peripheral target tissues such as breast, prostate and skin has been termed intracrinology and involves the paracrine diffusion and conversion of steroid substrates among neighboring cell types with different enzyme capacities (41). In the prostate, circulating T from the Leydig cells is converted to DHT by SRD5A2 present in both basal and luminal epithelial cells. Circulating DHEA-S can be converted to AED, T and DHT via the activity of HSD3B1, AKR1C3 and SRD5A2 present in basal epithelial cells (42,43). Circulating T or that produced in the basal cells diffuses to the AR positive luminal cells where it is then converted to DHT by SRD5A2 (41).

Prostate tissue also demonstrates epithelial cell expression of phase I (reducing) and phase II (conjugating) DHT catabolizing enzymes that act in concert to regulate access of DHT to the AR. AKR1C1 is the primary enzyme responsible for the irreversible reduction of DHT to the weak metabolite, 5α-androstane-3,17-diol (3α-androstanediol or 3α-diol, a low affinity AR ligand), whereas AKR1C2 catalyzes the reversible conversion of DHT to 5α-androstane-3,17-diol (3β-diol, a pro-apoptotic ligand of estrogen receptor beta, ER) (44). The reductase activity of AKR1C2, coupled with the reverse oxidative activity of specific 3α-HSD enzymes is a critical molecular switch regulating access of DHT to the AR (44-47).

Candidate enzymes mediating the reversible conversion of 3β-diol to DHT include RL-HSD (17BHSD6), 17BHSD10, RODH4, RDH5, and DHRS9. Transcripts of RL-HSD and 17BHSD10 are highly expressed in the prostate, however several studies suggest RL-HSD is more active in converting 3β-diol to DHT in prostate cells (48,49). Basal epithelial cell expression of RL-HSD is present at the protein level, while transcript profiling of cultured epithelial and stromal cells detects stromal expression as well (48,50). RL-HSD also acts as an epimerase to convert 3β-diol to 3α-diol, although at much higher substrate concentrations (51). Recently, RL-HSD was also shown to directly catalyze conversion of physiologic levels of DHT to 3α-diol, suggesting RL-HSD is involved in maintaining the intraprostatic balance of DHT, 3α-diol and 3β-diol (50).

The glucuronidating enzymes UGT2B15 and UTG2B17 located in prostate luminal and basal epithelial cells, respectively, irreversibly terminate the androgen signal by glucuronidation of 3β-diol (as well as T, DHT and other metabolites), and are major determinants of the androgen signal in PCa cell lines (52-54). UGDH is required to generate the substrate for glucuronide conjugation (UDP-glucuronate), and over-expression of UGDH increases the generation of glucuronidated androgens (55).

Thus, the relative activity of AKR1C2 in converting DHT to 3β-diol, and of RL-HSD and UGT2B17 in competing for conversion of 3β-diol back to DHT or to 3β-diol-G, respectively, will collectively determine the amount of active steroid available for AR ligand occupancy.

Classical and backdoor pathways of androgen metabolism

In the classical pathway of androgen synthesis discussed above (*Figure 2*, light gray arrows), C21 steroids generated from cholesterol such as pregnenolone and progesterone are first converted to the C19 steroids DHEA and AED via sequential hydroxylase and lyase activity of CYP17A and are then acted on by HSD17B3 to generate T, with peripheral conversion of T to DHT carried out by SRD5A2 in target tissues. However, in steroidogenic tissues in which both CYP17A and SRD5A are co-expressed, an alternate route to DHT, called the 'back-door' pathway (*Figure 2*, hatched arrows) is possible wherein C21 steroids undergo 5α-reduction by SRD5A *prior* to being acted upon by the lyase activity of CYP17A (56). In fact, 17α-OH progesterone is a better substrate for SRD5A (especially SRD5A1) than either AED or T (57). Since 17α-OH dihydroprogesterone (the 5α-reduced product of 17α-OH progesterone) is a poor substrate for the lyase activity of CYP17A, synthesis proceeds via the 3α-reduction of 17α-OH dihydroprogesterone by ARK1C2, which yields 17α-OH allo-pregnanolone, an excellent substrate for CYP17A lyase activity that is minimally dependent on cytochrome *b5* (58). Androsterone generated by the lyase activity of CYP17A is then acted upon by HSD17B3 or AKR1C3 to generate 3β-diol. In this case, a reverse oxidative step catalyzed by RL-HSD (not required in the classical pathway) is required to generate DHT (36). This pathway, e.g., 5α-reduction of C21 steroids prior to the action of CYP17A lyase, occurs in the testis of the immature mouse and the tammar wallaby, is also hypothesized to occur in ovarian hyperandrogenism and polycystic ovarian syndromes, as the human ovary expresses both CYP17A and SRD5A (56).

Interestingly, production of DHT in mouse testis via this mechanism is specifically mediated by type 1 and not the type 2 isoform of SRD5A (59). This observation is of relevance to prostatic androgen metabolism in that a clear shift from SRD5A2 to SRD5A1 expression occurs in the transition from benign to neoplastic prostate tissue (discussed below). Moreover, human CYP17A displays markedly more robust lyase activity for the 5α-reduced progesterone intermediate 17α-OH allo-pregnanolone than for the classical substrates 17α-OH pregnenolone or 17α-OH progesterone, such that the combination of increased SRD5A1 activity in conjunction with expression of CYP17A in PCa tissue may favor *de novo* synthesis via the backdoor pathway over the classical pathway (60). Importantly, while these 'backdoor' pathways to DHT bypass conventional intermediates of AED and T, it is worth emphasizing that *the backdoor pathway requires the same enzymatic conversions which produce DHT via the conventional pathway*; all that differs is the order in which the enzymes mediate the reactions.

Altered expression of steroidogenic enzymes in progression to CRPC

Primary PCa and castration resistant tumors are characterized by a number of changes in steroidogenic gene expression that are consistent with either promoting conversion of adrenal androgens to DHT, inhibiting conversion of DHT to inactive metabolites, or in case of CRPC tumors, mediating *de novo* synthesis of androgens from cholesterol and/or progestin precursors. Here we review the alterations observed in prostate tumors during the progression to CRPC and discuss implications of these changes for determining intra-tumor androgen levels.

Altered expression of steroidogenic genes in primary PCa

Perhaps the most consistently observed alteration in prostate tumors is a subtotal loss of tumoral SRD5A2, the principle isoform of this enzyme expressed in benign prostate tissue (61), and a relative shift in primary and recurrent prostate tumors to expression of SRD5A1 (34,62,63) [although some studies have shown Gleason grade-related increases in both SRD5A1 and SRD5A2 (64)]. The significance of this shift was recently elucidated by the group of Sharifi who demonstrated that (I) the 5α-reduction of AED to 5α-androstanedione is a required step for DHT synthesis in PCa cells (rather than direct 5α-reduction of T to DHT); (II) this conversion is specifically mediated by SRD5A1; and (III) that in PCa cells T and AED are actually negligible substrates for SRD5A2 (60) (possibly related to the altered redox environment of tumor cells as SRD5A1 and 2 have different pH optima). These data support previous findings that SRD5A activity in PCa cells has a preference for AED rather than T (65), as well as initial studies of SRD5A1 which found AED to be a better substrate for 5α-reduction than T (66,67).

Sharifi *et al.* call this the 5α-androstandione pathway

(*Figure 2*, dark arrow) and suggest that the upregulation of SRD5A1 observed in the transition to CRPC reflects selection of tumors cells capable of efficiently synthesizing DHT via this pathway. Interestingly, a recent report demonstrated that progression to CRPC was correlated with a higher pre-treatment ratio of T to DHT in prostate biopsies taken before the start of ADT [T:DHT ratio 0.19 pg/mg (0.98 to 4.92 pg/mg) *vs.* 0.05 pg/mg (0.45 to 16.89 pg/mg) in patients who did not develop CRPC] (68). It is tempting to speculate that this elevated ratio of T to DHT may reflect tumor cells with pre-treatment loss of SRD5A2 activity, followed by induction of SRD5A1-mediated DHT production via 5α-androstanedione under the selection pressure of ADT. Altered expression of a third SRD5A isozyme, SRD5A3, has also been reported, with increased expression observed in primary and castration recurrent prostate tumors (69). The importance and/or activity of this enzyme in PCa progression awaits further evaluation (70).

Differential changes in the expression of reductive and oxidative enzyme pairs favoring the conversion of inactive diones to active androgens (e.g., AED to T, or androstanedione to DHT) have been observed in primary prostate tumors, including increased tumor expression of the reductive enzymes HSD17B3 (71) and AKR1C3 (34,43,72), and decreased expression of the oxidative enzyme catalyzing the reverse reaction, HSD17B2 (71,73), suggesting a shift in tumoral androgen metabolism to the formation of T and DHT. While increased prostate tumor expression of HSD17B4, which has unidirectional oxidative activity, has been observed, this isoform (also known as D-bifunctional protein or DBP) has a unique peroxisomal targeting sequence and acts primarily in peroxisomal -chain oxidation of fatty acids (74).

Similarly, primary PCa demonstrates a selective loss of both AKR1C2 and AKR1C1 versus paired benign tissues, accompanied by a reduced capacity to metabolize DHT to 3β-diol, resulting in increased tumoral DHT levels (47). Increased expression of HSD17B10, one of the oxidative enzyme capable of mediating the back conversion of 3β-diol to DHT, has also been observed in malignant epithelial cells compared to normal, similarly consistent with an increased capacity to generate DHT in tumor tissue (75). In contrast, epithelial expression of RL-HSD (which can mediate either conversion of 3β-diol to DHT or of DHT to 3α-diol) is lost in primary PCa, which is hypothesized to reflect loss of the 3β-diol/ER mediated growth inhibition pathway during malignant transformation (50).

Another enzyme which may modulate prostate tissue androgen levels is SULT2B1, which shows selective loss of expression in tumor *vs.* benign prostate epithelial cells (76). While SULT2A1 is the primary phase II enzyme responsible for sulfonation in the adrenal gland, SULT2B1b is highly expressed in the prostate and may limit the pool of unconjugated DHEA available for conversion to AED. This is consistent with a report demonstrating increased DHEA-stimulated LNCaP proliferation in cells with knockdown of SULT2B1b (77).

Altered expression of steroidogenic genes in castration resistant prostate tumors

CRPC tumors demonstrate altered expression of numerous genes in the steroid synthesis pathway, including genes involved in cholesterol metabolism, *de novo* steroidogenesis, as well as utilization of adrenal androgens, suggesting that castration resistant tumors may have the ability to utilize cholesterol, progesterone and/or adrenal precursors for conversion to T and DHT (13,34). Changes related to cholesterol metabolism include increased expression of squalene epoxidase (SQLE), the rate-limiting enzyme in cholesterol synthesis, as well other genes in this pathway such as HMG-CoA synthase, squalene synthetase and lanosterol synthase (35). In a study comparing CRPC with primary tumors, the relative expression of numerous transcripts involved in *de novo* androgen biosynthesis and adrenal androgen utilization were altered, including increased expression of HSD3B2 (1.8), AKR1C3 (5.3), SRD5A1 (2.1), SRD5A2 (0.54), AKR1C2 (3.4), AKR1C1 (3.1) and UGT2B15 (3.5). Another study of CRPC metastases in which elevated levels of tumor T and DHT were also measured (T 0.74 ng/g, DHT 0.25 ng/g), showed elevated expression of STAR, CYP17A, HSD3B1/2, HSD17B3, AKR1C3, SRD5A, UGT2B15/17, CYP19A and decreased SRD5A2 (13,78,79). Interestingly, CYP17A has also been demonstrated to have squalene epoxidase activity in assays using recombinant CYP17A and in a mouse Leydig tumor cell line (80), suggesting it may have a dual role in CRPC steroid metabolism. Other studies have not specifically found increased expression of CYP17A in CRPC tumors, but have demonstrated similar findings suggestive of intracrine utilization of adrenal androgens, including increased expression of HSD17B3 and AKR1C3 (34,81,82). Also of note, AKR1C3 has recently been identified as an AR coactivators and thus may play dual roles in promoting ligand synthesis and AR activation (83).

A gain of function SNP in HSD3B1 (1245C; N367T, population frequency 22%) has recently been identified as a somatic mutation in CRPC tumors (84). Three of 25 CRPC tumors with wild type germline DNA at this site had acquired the gain of function mutation in the tumor, and 3 of 11 CRPC tumors with heterozygous germline DNA, showed loss of heterozygosity of the wild type allele. Expression of this variant resulted in increased protein levels of HSD3B1, rendered the protein resistant to ubiquitination and degradation, and lead to increased levels of intratumoral DHT production. Compared to the poor conversion of DHEA to AED in LAPC4 cells which do not have this mutation, the mutant allele was shown to account for the efficient flux of DHEA to AED in LNCaP cells, and was also detected in the VCaP cell line.

Notably, the expression of enzymes involved in *de novo* steroidogenesis, including MLN64 (homolog of STAR), CYP11A, CYP17A and HSD3B has also been demonstrated in primary prostate tissues (78,85). While a role for *de novo* steroidogenesis *per se* in primary prostate tumors is less likely, these observations suggest that the selection pressure of ADT may lead to upregulated expression of these enzymes and reconstitution of tumor androgen levels in CRPC.

Intracrine steroidogenesis in the continuum from normal prostate to CRPC

The ability of prostate tissue and prostate tumors to mediate the intracrine conversion of adrenally derived androgens or cholesterol to downstream androgens of T and DHT has been evaluated in normal rat and human prostate, in primary prostate tumors, in CRPC tumors, and *in vitro* and *in vivo* models of CRPC. Here we review the evidence in each of these setting that demonstrate the activity of steroidogenic pathways in the continuum from normal prostate to CRPC.

Evaluation of steroidogenesis in normal prostate and PCa tissue

A number of early studies attempted to directly examine the steroidogenic ability of rat and human prostate tissue by evaluating the conversion of exogenously administered radiolabeled-adrenal androgens to T or DHT. Bruchovsky administered radioactively labeled androgens including T, DHT, and the adrenal androgens DHEA and AED to castrated male rats and evaluated prostatic metabolites at 60 minutes after injection (86). Following administration

of DHEA approximately 1% and 8% of the recovered radioactivity was found in T and DHT respectively, with AED it was 2% and 12%, respectively, compared to 37% conversion of exogenously administered T into DHT. Labrie *et al.* demonstrated that administering DHEA or AED to castrate adult rats at levels found in the serum of adult men led to increased prostatic DHT levels and increases in ventral prostate weight (87). In the Dunning R3327 prostate carcinoma model, administration of adrenal androgens to castrate male rats increased tumor DHT levels and stimulated tumor growth to the level of intact controls (88).

In studies of human prostate tissue, Harper *et al.* evaluated prostate androgen metabolism by infusing eugonadal men with ^{3}H-T, ^{3}H-AED or ^{3}H-DHEA-sulfate (DHEA-S) thirty minutes prior to performing radical prostatectomy for BPH (89). The major metabolite present in prostate tissue after ^{3}H-T infusion was DHT (about 65% conversion). Infusion of ^{3}H-AED resulted in approximately 7-10% radioactivity associated with either T or DHT. ^{3}H-DHEA-S was primarily converted to DHEA (70-90%), with 1-3% conversion to T, DHT and AED. Consistent with these observations, a more recent study using mass spectrometry to identify metabolites formed from *ex-vivo* incubation of human prostate homogenate with DHEA demonstrated production of 5-androstenediol, T, DHT and androsterone (90). Together, these studies in rat and human prostate tissues suggest that while the most efficient substrate for DHT production in non-tumor prostate tissue is T, a limited amount of DHT is also formed from exogenous DHEA or AED, consistent with intracrine steroidogenesis.

In PCa tissues Acevedo *et al.* investigated the metabolism of C14 progesterone in primary cancer, but did not observe significant metabolic conversion beyond the formation of immediate progesterone derivatives (91). This finding is not necessarily unexpected, as studies have now clearly demonstrated that it is CRPC tumors in which steroidogenic genes capable of *de novo* synthesis are upregulated. Klein *et al.* evaluated the presence of adrenal androgens and steroid metabolizing activity (including SRD5A, HSD3B, and HSD17B) *ex vivo* in hormone naive tumors and lymph node metastases. Although malignant tissue had a sub-total loss of SRD5A activity, they found that primary tumors and metastases possessed the capacity to metabolize adrenal androgen precursors along the pathway to DHT (61). Di Silverio *et al.* demonstrated the conversion of DHEA-S to DHEA within PCa tissue extracts from both eugonadal and castrate men (92), and Klein *et al.* subsequently confirmed

the presence of the steroid sulfatase required for conversion of DHEA-S to DHEA within prostate epithelial tissue (93). Consistent with their observation that the primary route to DHT in PCa cells is from AED to androstenedione rather than from AED to T, Sharifi's group demonstrated robust conversion of AED to androstenedione and limited to no metabolism of AED to T in biopsy tissue from two patients with CRPC (60).

Experimental models of de novo steroidogenesis in CRPC

Studies using *in vitro* and *in vivo* models of CRPC support the concept of intratumoral androgen synthesis, including both adrenal androgen utilization and *de novo* androgen synthesis (94). Numerous studies using CRPC xenograft models in castrate mice have demonstrated measurement of substantial intratumor androgen levels (31,32,95-99). As rodent adrenal glands do not synthesize significant amounts of adrenal androgens, these findings are suggestive of *de novo* steroidogenesis from cholesterol or progesterone precursors. Notably, circulating levels of exogenously administered cholesterol were associated with tumor size (R=0.3957, P=0.0049) and intratumoral T levels (R=0.41, P=0.0023) in subcutaneous LNCaP tumors grown in hormonally intact mice, and were directly correlated with tumoral expression of CYP17A (R=0.4073, P=0.025). Since the hypercholesterolemia did not raise circulating androgen levels, these data suggest the administered cholesterol led to increased intratumoral androgens via *de novo* steroidogenesis.

More directly addressing this question, a number of groups have carried out *in vitro* studies with radiolabeled cholesterol precursors to demonstrate intratumoral conversion to downstream metabolites. The androgen-independent LNCaP derivative (C81) showed higher expression of STAR, CYP11A and CYP17A compared to its androgen-dependent counterpart (C33) and was shown to directly convert radioactive cholesterol into T (100). Increases in expression of genes responsible for accumulation of free cholesterol and cholesterol synthesis including LDLR, SRB1, ABCA1, STAR, ACAT, HMG-CoA and CYP11A were demonstrated in a xenograft LNCaP model (97,101,102) (as well as increases in transcripts encoding CYP17A, AKR1C1, AKR1C2, AKR1C3, HSD17B2, and SRD5A1) (101). Conversion of C14-acetic acid to DHT was observed in these xenografts, and tumors were shown to metabolize H3-progesterone to six different intermediates upstream of 5α-DHT, suggesting

occurrence of steroidogenesis via both classic and "backdoor" pathways (101). In a study of six prostate cell lines (LnCaP, 22Rv1, DU145, RWPE1, PC3 and ALVA4), expression of CYP11A, CYP17A, HSD3B2, 17BSHD3 was detected in all, with conversion of C14-labled cholesterol to T and DHT demonstrated in each cell line, albeit with different efficiencies (78). It should be noted that other studies have not detected expression of CYP17A or evidence for *de novo* steroidogenesis in PCa cell lines (103,104).

Exogenous influences on intratumoral androgen biosynthesis

A number of exogenous factors including cytokines, growth factors and paracrine cellular interactions have been found to promote steroid production in PCa cell lines. IL-6 is implicated in cross-talk and regulation of AR activity and PCa growth, but may also play a role in modulating androgen synthesis. Treatment of LNCaP cells with IL-6 induced the expression of steroidogenic enzymes including CYP11A, HSD3B2, AKR1C3 and HSD17B3, and increased levels of T in lysates of cells grown in serum free media by 2 fold (105).

In a study designed to evaluate the effects of insulin on steroidogenesis, exposure of LNCaP cells to insulin caused an increase in transcript levels of cholesterol and steroid synthesizing genes, including SREBP1, STAR, CYP11A, CYP17A, HSD3B2, HSD17B3, and SRD5A1, which were confirmed at the protein level for a number of genes including CYP11A1 and CYP17A1. In parallel, insulin increased intracellular levels of pregnenolone, 17α-OH progesterone, DHEA and T, and incubation of insulin-treated LNCaP and VCaP cells with C14-acetate resulted in detection of radiolabeled pregnan-3,20-dione, AED, T and androsterone (99). In similar studies evaluating the effect of IGF2 on steroidogenesis, these authors demonstrated increased conversion of C14-acetate to pregnan-3,20-dione, pregnan-3,17-diol-20-one, androsterone, AED, and T (106).

Receptors for luteinizing hormone (LH), the target of LH releasing hormone (LHRH) agonist therapy in the brain, have also been demonstrated in PCa specimens and may play a role in steroidogenesis (107). Exposure of both androgen-sensitive (LNCaP) and androgen-independent (22RV1 and C4-2B) PCa cell lines to LH increased the protein expression of steroidogenic enzymes including STAR, CYB5B, CYP11A, and 3BHSD, and a 2.5 fold increase in progesterone synthesis was observed in LH

treated C4-2B cells compared to controls (108). These data suggest that LH may have a role in the regulation of steroid biosynthesis in PCa cells and identify the LH receptor as a potential therapeutic target.

Several studies have indicated that bone-marrow and PCa-derived stromal cells may play an important role in facilitating androgen biosynthesis in PCa cells. Whereas DHEA induced little or no PSA expression in monocultures of LAPC-4 PCa cells, co-culture with PCa-associated stromal cells resulted in marked stimulation of PSA expression, likely mediated by stromal cell generation of T from DHEA (as T was detected in a time and dose-dependent manner in PCa-stromal cell monocultures treated with DHEA) (109). Similarly, the impact of DHEA on PSA promoter activity in LNCaP cells was markedly enhanced in the presence of PCa-derived stromal cells (110). Knockdown of AR in the LNCaP cells abrogated this effect, while coculture with PCa-stromal cells transfected with AR shRNA did not, suggesting paracrine factors secreted by the stromal cells act on the LNCaP AR. Furthermore, following DHEA treatment, T and DHT concentrations were ~5-fold higher in the PCa-stromal/LNCaP coculture *vs.* the LNCaP monoculture. Interestingly, normal-prostate stroma, bone-marrow stroma, lung stroma and bone-derived stromal cells also induced an increase in PSA expression, although the strongest effects were noted with PCa-stromal cells. In a separate study of bone-marrow stromal cells, resting mesenchymal cells were found to express HSD3B and SRD5A protein, while incubation with DHEA resulted in the additional expression of HSD17B5 (111). These findings indicate that metabolism of androgen precursors in PCa-associated stromal cells may facilitate and/or potentiate the maintenance of intratumoral androgen levels in CRPC tumors.

Together these studies provide evidence supporting the role of steroidogenesis in reactivating AR signaling in CRPC, and highlight the interplay between cytokines, growth factors, and paracrine stromal and epithelial cell interactions in this mechanism.

Response and resistance to potent steroidogenesis inhibition in CRPC

Collectively, these studies demonstrate the capacity of primary and castration resistant prostate tumors to carry out the intracrine conversion of adrenal androgens to DHT, while the *in vitro* and *in vivo* experimental models clearly show that PCa cells are capable of *de novo* steroidogenesis starting from cholesterol and/or progesterone precursors. These findings cannot address the efficiency with which these pathways are active in human CRPC tumors *in situ*, but they strongly support the premise that the residual androgens measured in CRPC tumors reflect the increased expression and activity of enzymes mediating *de novo* steroidogenesis and adrenal androgen utilization. These data provide mechanistic support for the role of intracrine androgen production in maintaining the tumor androgen microenvironment in CRPC and underscore these metabolic pathways as critical therapeutic targets.

Given its central role in the production of either adrenal or tumor-derived androgens, CYP17A has emerged as a primary target of novel therapeutics. Abiraterone, a pregnenolone derivative that acts as a selective irreversible inhibitor of both the hydroxylase and lyase activity CYP17A, is the first of these agents to enter clinical practice. While clinical responses have been impressive, not all patients respond, the duration of response is variable, and a majority of men eventually progress with a rising PSA. Although the mechanisms determining response and mediating resistance to CYP17A inhibition have not been fully elucidated, emerging clinical and pre-clinical data suggest several possibilities.

Perhaps most importantly, pre-clinical studies provided the first *in vivo* confirmation that the clinical effect of abiraterone was associated with suppression of tumor androgen levels. Clinical studies have clearly demonstrated abiraterone-mediated suppression of serum androgens, including suppression of DHEA by approximately 75% and of DHEA-S, AED, and T to essentially undetectable (112,113). As well, higher levels of AR and CYP17A staining in pre-treatment tumor-infiltrated bone marrow biopsies from men with CRPC were associated with longer responses to abiraterone treatment, supporting CYP17A mediated androgen production as the target of abiraterone activity (41). However, the efficacy of abiraterone in suppressing tumor androgens in men with CRPC remains to be demonstrated.

In this regard, treatment of castration resistant LuCaP35 and LuCaP23 xenografts significantly inhibited tumor growth, serum PSA, and intratumoral androgen levels, supporting the hypothesis that abiraterone's primary mechanism of action is through effects on tissue androgens (31). Seven days after starting treatment levels of T and DHT decreased from 0.49 to 0.03 pg/mg and 2.65 to 0.23 pg/mg, respectively in LuCaP23, and from 0.69 to 0.02 pg/mg and 3.5 to 0.24 pg/mg

in LuCaP35. A similar impact of abiraterone on T and DHT levels was observed in separate study of castration resistant VCaP tumors (32). Notably, while androgen levels remained suppressed in LuCaP23 tumors recurring after therapy, increasing levels of T and DHT were observed in LuCaP35 tumors recurring on abiraterone.

Further evaluation demonstrated that these CRPC models responded to CYP17A inhibition with multiple mechanisms directed at maintaining AR signaling. This included upregulated expression of full length AR and ligand independent AR variants, as well as induction of steroidogenic genes (including the target gene, CYP17A), several of which showed strong correlations with DHT levels in recurrent tumors. Moreover, tumor biopsies from patients treated with the CYP17A inhibitor ketoconazole also demonstrated increased expression of transcripts encoding CYP17A compared to biopsies from CRPC patients not treated with ketoconazole (114).

These findings are consistent with clinical observations that patients progressing on abiraterone have a rise in PSA, suggesting reactivation of AR signaling. Development of resistance to abiraterone has not been associated with a rise in serum androgen levels or in bone marrow aspirate T levels (although 5α-androstanedione may be more appropriate to assess if the route to DHT bypasses T). However, numerous studies (reviewed above) show that circulating androgen levels do not reflect tumor cell androgen concentrations. Thus, in the setting of tumor progression on abiraterone, the rationale for focusing further therapeutic efforts on more potent AR antagonists and agents suppressing AR ligands remains strong.

Conclusions

Data regarding the molecular response of PCa to hormone therapy continues to emerge, providing critical insight into cellular growth and signaling pathways that may be exploited as therapeutic targets. The presence of residual androgens and persistent activation of the AR signaling axis in CRPC suggest that a multi-targeted treatment approach to ablate all contributions to AR signaling within the prostate tumor will be required for optimal anti-tumor efficacy.

The molecular alterations occurring in CRPC tumors following abiraterone treatment suggest tumor-specific methods of addressing resistance, either through optimizing steroidogenic blockade or by inhibiting AR signaling. Importantly, a 2 to 3 fold increase in AR expression can render low androgen levels (in the range detected in the abiraterone-treated tumors) physiologically relevant in promoting AR driven growth (22). Combining CYP17A blockade with inhibitors of other critical components of the pathway such as HSD3B1 or SRD5A2 or with AR inhibitors could offset adaptive upregulation of CYP17A (115). Abiraterone at higher (but clinically achievable) concentrations can strongly inhibit HSD3B1 and 2 (116), and can antagonize the promiscuous T877A mutant AR (117), providing a rationale for dose-escalation of abiraterone at time of progression. However, data demonstrating the induction of full length and ligand-independent AR splice variants in abiraterone-treated tumors suggests combined strategies directed at targeting ligand synthesis with AR inhibitors may have the greatest efficacy.

The introduction of potent steroidogenic inhibitors such as abiraterone and novel AR inhibitors such as MDV3100 holds significant promise for improving the treatment of men with CRPC. However, the optimal timing, sequence, and potential combinatorial strategies using new AR pathway inhibitors are critical unanswered questions. Delineating mechanisms and biomarkers of resistance will be critical for rational trial design and for the stratification of men to treatment strategies with the highest likelihood of durable efficacy.

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Footnote

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Translating insights of AR signaling from mouse models

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Abstract: Androgen receptor (AR) signaling plays a critical role in the physiology of the prostate and thus the biology of prostate cancer. Agents targeting the AR pathway have been the mainstay of treatment for patients with locally advanced and metastatic prostate cancer. In this review we will cover the role of androgen signaling in prostate cancer mouse models with an emphasis on how tumorigenic molecular alterations impact response to AR pathway inhibition and downstream AR target gene expression. Both of these concepts have meaningful implications for the management of patients with prostate cancer.

Keywords: Mouse models; prostate cancer; androgen receptor (AR)

Androgen receptor (AR) signaling in prostate cancer

The AR gene located on the X chromosome, encodes for a 110 kDa nuclear hormone receptor protein that mediates the transcription of target genes. Androgens bind to AR (ligand binding domain) and orchestrate a transcriptional program mediating cell growth, proliferation, differentiation, and homeostasis of androgen dependent cells. AR signaling is crucial for the development and maintenance of male reproductive organs including the prostate gland.

Huggins and Hodges first demonstrate that prostate cancer was dependent on androgen signaling by observing disease regression in men with prostate cancer following bilateral orchiectomy (1). Since that time, androgen deprivation therapy has been the treatment of choice for patients with locally advanced and metastatic prostate cancer. Despite the initial response to androgen deprivation therapy in patients with metastatic prostate cancer, the majority of patients develop progressive disease (castrate resistant prostate cancer), which recently has been shown to still be dependent on persistent androgen signaling (2-4). Through the identification of the molecular mechanisms promoting AR signaling in the setting of castration (AR amplification, AR over expression, AR mutation, peripheral

androgen production) a number of novel therapies (enzalutamide, zytiga) have successfully been developed and have been shown to improve survival in patients with castrate resistant disease (5-8). However, even with the advances of the second-generation AR pathway inhibitors, the majority of patients still suffer disease progression with active AR signaling, highlighting the importance of the need to identify the mechanisms of resistant disease and further explore alternative pathways that may promote cell death alone or in combination with AR targeted therapies.

In the current manuscript we will review the knowledge of AR signaling gained through pre-clinical mouse models with an emphasis on how this has translated to our clinical understanding and management of prostate cancer.

Murine prostate response to castration

Unlike humans, mice do not express the enzyme required for the production of adrenal androgens and hence surgical castration results in complete hypogonadism. In the wild-type mice, surgical castration induces a wave of luminal epithelial cell apoptosis over an initial 3 days period followed by prostate gland atrophy (9). Despite this tremendous decrease in prostate gland size and luminal cell death, non-androgen dependent basal epithelial cells and

a small percentage of luminal cells persist in the prostate. Studies have demonstrated that following castration the add back of testosterone is capable of reconstituting prostate gland size and cellular differentiation (9). Through studies of castration and regeneration, several groups have isolated progenitor cells from the prostate with the capability of giving rise to basal and luminal epithelial cells (9,10). These castrate resistant progenitor cells have been identified through lineage tracing to be present in the basal cell and luminal cell compartments. While linage different progenitor cells have been observed (basal and luminal) they share common features with regards to being castrate resistant, the ability to reconstitute basal and differentiated luminal cells, and following oncogenic insult promote a prostate cancer phenotype (9,10). This work has led to an improved but not complete understanding of the cell of origin of prostate cancer and castrate resistant phenotypes.

AR knock-out mice

To further explore the role of AR signaling in the prostate, a series of genetically engineered mouse (GEM) models using the Cre-Lox system have been developed. The models generate knock-out the AR gene leading to diminished transcript and absent protein production in a Cre inducible fashion, where the expression of Cre can be driven by cell/tissue specific promoters (11,12). This technology allows the AR gene to be knocked-out in a cell specific fashion, with the caveat that Cre expression can be leaky and heterogeneous.

Ubiquitous knock-out of AR using an actin regulated Cre model revealed that the male progeny had ambiguous external genitalia with a hypospadiac microphallus, testicular atrophy, and agenesis of the vas deferens, epididymis, seminal vesicles and prostate gland (12). This work confirmed the critical role of AR in male reproductive development. Furthermore, through this work, androgen signaling was demonstrated to play a critical role in the immune and skeletal organ systems. Using tissue specific Cre driven models, several investigators have evaluated the role of AR in various prostatic compartments (luminal, stomal) to evaluate physiologic and oncogenic prostate biology. Using the probasin promoter to drive Cre expression and knock-out AR in the luminal epithelial cells of the prostate post-pubertal resulted in a phenotype of basal epithelial cell hyperplasia without differentiation to a luminal cell phenotype (13). This data confirmed the role of androgen signaling in promoting luminal cell

differentiation. Knock-out of AR in the prostate stroma smooth muscle component, using the transgelin promoter to drive Cre expression, resulted in a relatively normal prostate epithelial phenotype with a slight reduction in luminal cell infolding (14). The most striking phenotype observed in this mouse model was a reduced stromal cell proliferation which was associated with a decrease in IGF-1 levels and signaling. Collectively these GEM models have allowed us to evaluate the inhibition of AR in a cell and tissue specific manner to elucidate the role of AR signaling in a cell specific context that could not be obtained from androgen deprivation therapies alone.

AR inhibition in GEM models of prostate cancer

Inhibition of the AR axis is the mainstay of treatment for locally advanced and metastatic prostate cancer. Genomic profiling studies in prostate cancer have revealed that loss of the tumor suppressors *PTEN* and *TP53*, amplification of *MYC*, and genomic rearrangements involving *ERG* are amongst the most common alterations present in prostate cancer. *PTEN* loss is reported to occur in approximately 50% of metastatic prostate cancer specimens and is significantly associated with concomitant loss of *TP53* and *ERG* genomic rearrangements. Based on these findings, several GEM models of prostate cancer have evaluated the biologic role of these oncogenic events in prostate tumorigenesis leading to the development of mouse models that spontaneously develop prostate cancer (15-18). Using these models, several groups have evaluated the impact of specific genetic alterations on response to AR pathway inhibition in these GEM models to determine molecular predictors of response and resistance.

The Pb-MYC model developed by Sawyers and colleagues has been shown to display sensitivity to androgen deprivation by surgical castration at early time points while aged mice reveal castrate resistant disease that is still sensitive to combined androgen blockade (surgical castration + enzalutamide, an AR antagonist) (15,19). The Pten loss series of mouse models developed by the Pandolfi lab demonstrate castrate and AR inhibitor resistant phenotypes, despite significant down regulation of AR target gene expression (19). This data is further reinforced through work by Mullholland *et al.*, where epithelial knock-out of AR did not promote tumor regression in a GEM model of Pten loss (20). These studies highlight that loss of PTEN in prostate cancer is associated with tumor cell survival independent on AR pathway activation. Ongoing

AR target gene expression in primary prostate cancer
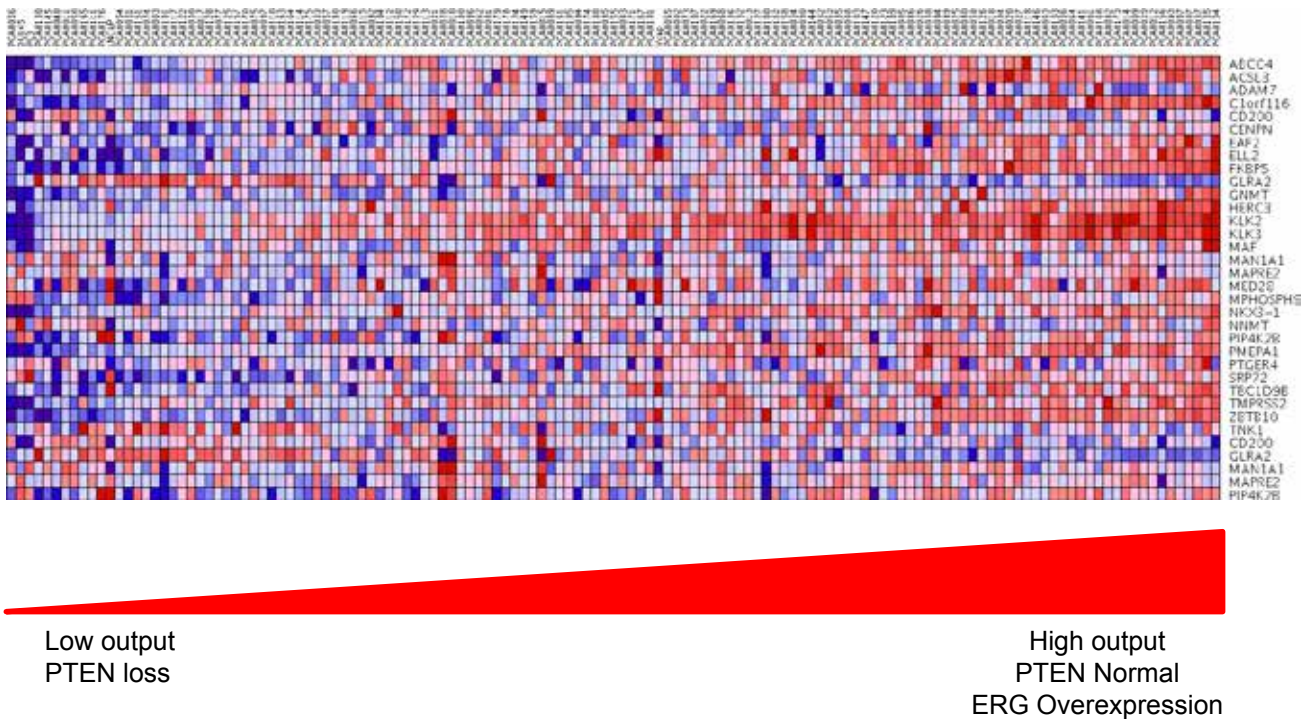
Figure 1 The spectrum of AR activity as measured by target gene expression in primary prostate cancer [adapted from data originally published by Carver *et al*. (19), Chen *et al*. (20)].

studies are evaluating the ability of loss of PTEN to serve as a predictive biomarker for response to AR inhibition in patients with advanced metastatic prostate cancer.

Genetic determinants of AR signaling

Molecular profiling studies in GEM models of prostate cancer have improved our understanding of the pathways regulating AR activity and downstream target gene expression. By analyzing the prostate transcriptome profiles of wild-type mice pre- and post-castration, Carver and colleagues developed a murine AR responsive gene signature (19). This gene signature allows investigators to determine the degree of AR activity across differing genetic context or AR targeted therapy in the mouse prostate. Based on this it has been demonstrated by several investigators that loss of the tumor suppressor Pten, resulting in activation of PI3K signaling, is associated with reduced AR target gene activity and repressed AR output (19,20). This may explain in part the resistance of AR targeted therapies in the setting of Pten loss as these tumors are inherently less dependent

on AR signaling. Importantly, castration in the setting of AR pathway inhibition further suppresses the murine AR responsive gene signature indicating that this pathway is still functional. Additionally, inhibition of the PI3K pathway in the setting of Pten loss resulted in increased AR target gene expression. Through a series of experiments if has been established that the PI3K and AR pathways Based on these findings, studies have demonstrated that AR target gene activity in primary and metastatic prostate cancer specimens is quite variable and also dependent on genetic context with tumors displaying loss of *PTEN* having reduced AR target gene expression compared to tumors with a normal *PTEN* status (*Figure 1*) (19).

Furthermore, Chen and colleagues have recently demonstrate that genomic rearrangements of ERG, which are presented in approximately 60% of *PTEN* loss tumors, can partially restore AR target gene expression in the setting of Pten loss (21). Chromatin IP experiments in GEM models demonstrated that over-expression of ERG dramatically increased the number of AR binding sites, thus priming the chromatin for AR binding. These findings

were also observed in patient derived metastatic prostate cancer specimens. Collectively this data has improved our understanding of the role that molecular alterations outside of AR may play in regulating AR target gene activity. It is becoming increasingly appreciated that AR activity is not just present or absent, but present across a wide spectrum of activity in both murine and human prostate cancer. This understanding will have a significant impact for predicting sensitivity to and quantifying the degree of AR pathway inhibition in prostate cancer.

Conclusions

The AR pathway plays a critical role in prostate cancer biology and thus targeting AR for inhibition is the mainstay of treatment for locally advanced and metastatic prostate cancer. Through mouse modeling work we have gained an improved understanding of genetic determinants of resistance to AR targeted therapies and an improved understanding of how oncogenic pathways regulate AR target gene expression. Collectively this work may allow for better prediction of which patients will respond long-term to androgen pathway inhibition and in which patients combination therapies may be required to optimize outcome.

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Footnote

Conflicts of Interest: The author has no conflicts of interest to declare.

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Epithelial mesenchymal transition (EMT) in prostate growth and tumor progression

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Abstract: Epithelial-mesenchymal transition (EMT) and its reversal, mesenchymal-epithelial transition (MET), are essential morphological processes during development and in the regulation of stem cell pluripotency, yet these processes are also activated in pathological contexts, such as in fibrosis and cancer progression. Multi-component signaling pathways cooperate in initiation of EMT and MET programs, via transcriptional, post-transcriptional, translational, and post-translational regulation. EMT is required for tissue regeneration and normal embryonic development as it enables epithelial cells to acquire the mesenchymal phenotype, conferring them migratory and dynamic properties towards forming three-dimensional structures during gastrulation and organ formation. Uncontrolled activation of such phenomenon and the pathways signaling EMT events in adult life, leads to cancer growth and orchestrated by signaling interactions from the microenvironment, epithelial tumor cells with enhanced polarity, become invasive and rapidly metastasize to distant sites. Loss of epithelial markers (E-cadherin) and gain of mesenchymal markers (N-cadherin), at the leading edge of solid tumors is associated with progression to metastasis. This review will explore the contribution of EMT to embryonic development of GU organs and the functional consequences of EMT-MET cycles in prostate tumorigenesis. Recent insights identifying key players driving EMT and its reversal to MET during prostate cancer progression to metastatic castration-resistant disease will be discussed, with specific focus on androgen receptor (AR) and transforming growth factor-β (TGF-β) signaling in the context of their predictive and targeting value in prostate cancer progression.

Keywords: Androgens; cell invasion; adherens junctions; therapeutic targeting; moving front; embryonic growth; predictive markers

Introduction

In 2013 prostate cancer continues to be one of the most commonly diagnosed cancers in men, with an estimated 241,740 men newly diagnosed with prostate cancer in 2012 (1). The majority of deaths associated with prostate cancer are attributed to the failure of current therapies to cure metastatic disease (2). The process of epithelial-mesenchymal transition (EMT) plays a pivotal role in the development of metastatic castration resistant prostate cancer (mCRPC) (3) The androgen receptor (AR) functions not only to control prostate normal development and tumorigenic growth, but also contributes to metastasis by facilitating EMT and promoting signaling interactions (*Figure 1*).

The prostate is an androgen-dependent organ that is regulated by the androgen/AR-signaling axis in both organogenesis and tumorigenesis (4). During the early stages of development, differentiation and development of prostate cells are dependent on androgens. Stimulation of the AR
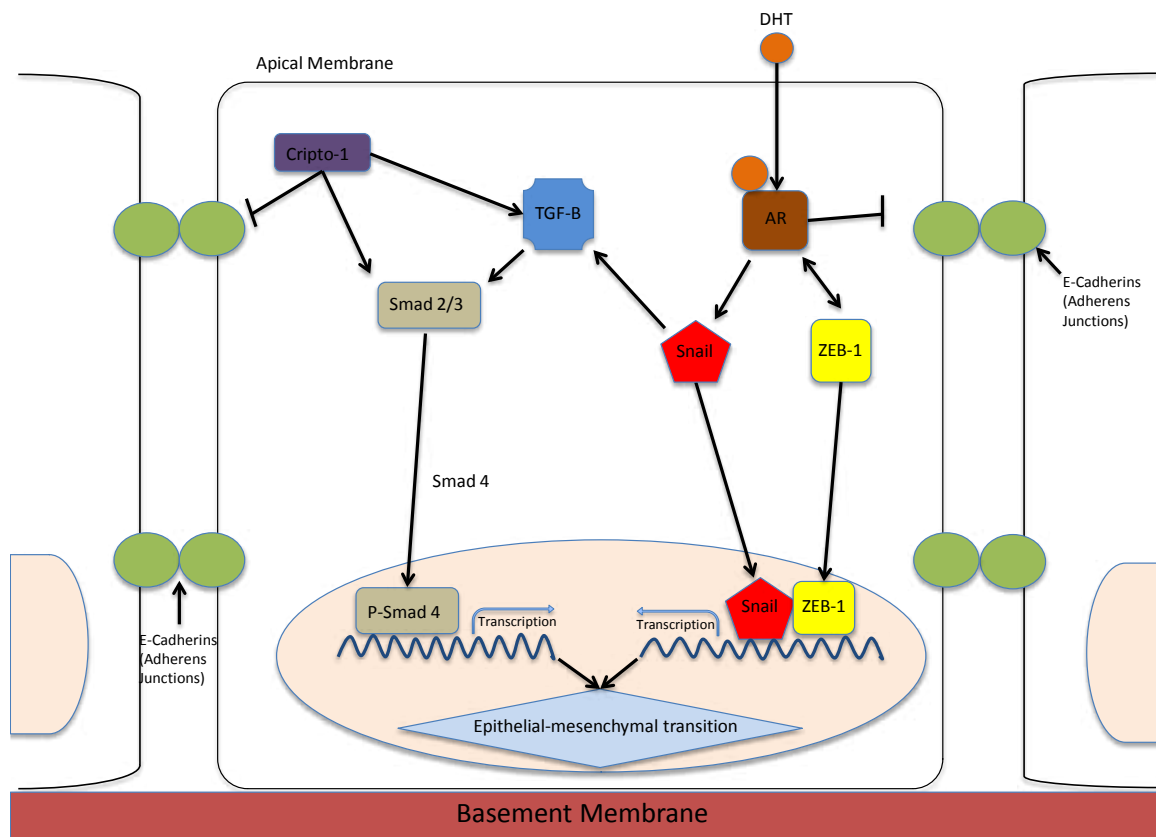
Figure 1 EMT control by the AR signaling. EMT in prostate epithelial cells is mediated by TGF-β and AR signaling interactions. AR induces EMT through activation of Snail transcription factor or via repression of E-cadherin. Activation of Snail, ZEB-1 or Smad transcription factors initiates allows for mesenchymal gene expression. Snail increases the expression of mesenchymal markers and proteins associated with invasion. E-cadherin is transciptionally suppressed by Snail; this loss of E-cadherin, which mediates intracellular adhesions at adherens junctions, leads to collapse of cell-cell communications and onset of EMT. Cripto-1 is a critical effector of the TGF-β signaling that upregulates mesenchymal expression during embryonic development. ZEB-1 is involved in a feedback loop with AR where downregulation of AR leads to uncontrolled ZEB-1 expression.

signaling in the urogenital sinus mesenchyme by testicular androgens induces epithelial budding, proliferation and differentiation towards formation of ductal structures. A non-functional AR results in testicular feminization-like syndrome with the absence of the prostate (5). Circulating testosterone is converted into dihydrotestosterone (DHT) via 5-alpha reductase activity, the active metabolite that interacts with the AR to ensure maintenance and function of adult prostate gland. Once bound to DHT, the AR undergoes a conformational change and releases heat shock proteins (HSP). The AR translocates to the nucleus where dimerization, DNA binding, and recruitment of coactivators occurs (6). In men with metastatic prostate cancer, androgen depravation therapy (ADT) improves bone pain, lessens lower UTI symptoms, and increases quality of life (7). Yet, metastatic disease develops in patients failing ADT and emerging with castration-resistant prostate cancer (CRPC), through an aberrant AR signaling mechanism activated despite androgen deprivation. Increased AR expression, elevated intraprostatic androgens and perpetually activated AR signaling in the face of castrate levels of androgens characterize this progression to CRPC (8,9). Prostate tumors employ several mechanisms to bypass or perpetuate AR signaling on the path towards CRPC, including alterations to the AR itself, the synthesis of androgens by prostate cancer cells, or the activation of AR by aberrant signaling pathways (10-12). There are two recognized mechanisms for castration resistance: ligand-dependent and ligand-independent. In the ligand-dependent model, adrenal androgens and intra-tumoral

androgens are involved in the development of CRPC. In the ligand-dependent model the increased expression of steroid-5α-reductase isoenzyme-1 allows adrenal androgens to be converted to DHT, bypassing testosterone (13). Adrenal androgens have long been recognized as contributors to androgen production under condition of castration-induced androgen depletion. Still, early attempts to maximize adrenal androgen blockade via adrenalectomy have limited efficacy (14). Recently however, abiraterone acetate, a CYP17A1 inhibitor, blocks the synthesis of adrenal androgens and has been FDA-approved to treat CRPC (15). The presence of residual androgens in the prostate cancer microenvironment points to an autocrine pathway of androgen production that may drive castration resistance (16). Many novel endocrine therapies disruptive to the AR-signaling axis and residual androgen are currently undergoing clinical testing in Phase II clinical trials. The ligand-independent route includes mutations of the AR that may result in the AR responding to AR antagonists, potentially a mechanism underlying the disease remission with the discontinuation of anti-androgen therapy (17,18). Preclinical studies highlight the significance of epigenetic changes such as histone modification and DNA methylation leading to castration resistance (19), supporting optimism towards their therapeutic targeting in clinical trials for the treatment of CRPC (17). Additionally, AR splice variants, unlike wild-type AR, are constitutively active and promote tumor cell growth independent of the ligand (20). The AR signaling inhibitor MDV3100 inhibits the growth of prostate cancer in some cell lines containing the AR splice variants (21). Moreover, the entire AR signaling axis can be bypassed by overexpression of Bcl2, which is an apoptosis blocking protein (22). Innovative therapeutic targeting approaches directly impairing AR activity and localization are currently being interrogated (7).

EMT in control of cell polarity and movement

The onset of mobility requires a relaxation of static actin structures on order to form pliable membrane protrusions. Rigid actin fibers are disassembled upon dorsal circular ruffle formation leaving a fine actin network from which cell membrane protrusions (lamellipodia) are formed (23). Formation of a stable, polarized epithelium requires tight cell-cell and cell-matrix connections. E-cadherin is the major component of epithelial adherens junctions (AJ), which mediate intercellular adhesions. EMT is a cellular process that allows a polarized epithelial cell to assume a mesenchymal

phenotype and the first step in this process is the loss of E-cadherin and the collapse of cell-cell communications (24). The downregulation of E-cadherin not only leads to a mechanical disruption of AJ, but it also liberates proteins from the cytoplasmic cell adhesion complex, which exert ambivalent functions depending on the cellular localization. EMT has been identified as an important mechanism in organization of cells within the developing embryo, forming mesenchymal cells following tissue injury, and initiating the invasive and metastatic nature of epithelial cancers (24). EMT and mesenchymal to epithelial transition changes (MET) play critical roles in the development of the prostate gland, the seminal vesicles and kidney. Several studies have established that EMT facilitates malignant transformation of cells and plays an important role in cells' ability to metastasize (5,25,26). Mesenchymal cells provide support and structure to epithelial cells through the production of an extracellular matrix (ECM) and are highly mobile and invasive, unlike their epithelial counterparts. But, it is believed that insult to cells re-activates these developmental mechanisms out of context in adult cells to trigger oncogenesis. However, EMT is not the only example of epithelial cell plasticity. Another process entails the movement of epithelial cells in a physically and functionally collected group, termed collective migration (27). It is possible that collective migration falls on a spectrum somewhere between EMT and MET. This would make sense in the context of cancer, which lacks the orderly and coordinated induction of EMT (28). In EMT during development, E-cadherin is replaced by N-cadherin and Vimentin and fibronectin replace cytokeratins. These changes also occur in mammary gland tumors undergoing EMT (29).

EMT permits a polarized epithelial cell that normally interacts with a basement membrane via its basal surface to undergo multiple changes that allows it to assume a mesenchymal phenotype. This mesenchymal cell has an elevated resistance to apoptosis, an amplified production of ECM components and has the capacity to migrate and invade (24). One of the classic examples of EMT in organ formation, is the occurrence of EMT in kidney formation, which is driven by genes such as paired box 2 (*Pax2*), bone morphogenetic protein 7 (*Bmp7*), and Wilms tumor 1 (*Wt1*). In fact, several rounds of EMT and MET are necessary to construct the three-dimensional structure of internal organs and to complete the differentiation of specialized cell types (30). Some metastatic cancer cells have shown the ability to re-express E-cadherin after migration and colonization (31) Morphological profiling of EMT consists of several

cellular markers. For instance, mesenchymal markers that are increased in EMT include: N-cadherin, Vimentin, Fibronectin, Snail, Slug, Twist, FoxC2, and MMP's-2, 3, 9. Subsequently, epithelial markers that are decreased in EMT include: E-cadherin, B-catenin, Cytokeratin, and Desmoplakin (32). Snail and Twist are transcription factors which act as repressors of E-cadherin. TGF-β superfamily members induce *Snail1 and Snail2* (33). Moreover microRNAs recently emerged as potent regulators of EMT-MET inter-conversions, with their abilities to target multiple components involved in epithelial integrity or mesenchymal traits, thus impacting tumor progression, metastasis and colonization (34).

EMT-MET cycles in urogenital growth and organ development

The cycling of EMT-MET processes, is instrumental in inprinting urogenital growth during embryomic development. The signaling activities of mesenchymal cells facilitate migration and survival of epithelial cells in an anchorage-independent environment. Expression of the transcription factors *Snail1* and *Snail2*, is necessary for gastrulation to proceed during embryogenesis, by signaling TGF-β mediated EMT. Elegant studies have established that *Snail*-deficient embryos fail to gastrulate and mesodermal-like cells that are unable to downregulate E-cadherin accumulate at the gonadal streak. These mesodermal cells eventually undergo MET to become the notochord, the somites and the precursors of the urogenital system (30). In male reproductive tracts the Mullerian-inhibiting substance induces EMT in the Mullerian duct, causing its regression. Testicular cords form following the mesonephric endothelial cells that have undergone EMT (30). *Snail* is thus considered a "master regulator" that upregulates expression of mesenchymal proteins associated with invasion such as: vimentin, fibronectin, metalloproteinase-2, -9, ZEB1 And LEF-1 (35).

EGF-CFC proteins have been implicated as essential signaling cofactors for Nodal, a transforming growth factor β family member whose expression has been defined as embryo specific. *Cripto-1* (CR-1), an embryonic gene that encodes for an epidermal growth factor-CFC (EGF-CFC) family member, performs key functions during embryonic development, while it dramatically disappears in normal adult tissues, with the possible exception the stem cells (36). *Cripto-1* is highly expressed in a subpopulation of human embryonal carcinoma cells with prostate cancer stem-

like characteristics (37). *Cripto-1* re-expression in human tumors promotes cell proliferation, migration, invasion, EMT and angiogenesis. This diversity of biological effects is functionally dictated by the interaction of Cripto-1 with an extensive array of signaling molecules. Specifically, Cripto-1 modulates signaling of TGF-β family members, including Nodal, GDF-1/-3, Activin, and TGF-β1, activates c-src/MAPK/Protein Kinase B (AKT) pathway in a Glypican-1 and GRP78-dependent manner (36). It also are cross-talks with erbB4, Wnt/β-catenin, Notch, Caveolin-1, and ALK4 on the cell membrane of epithelial conferring a mesenchymal phenotype. Nodal is coexpressed with Cripto-1 in the mammary gland, and Cripto-1 can phosphorylate the Smad-2 TGF-β signaling effector in epithelial cells (in presence of ALK4) promoting induction of EMT. *Cripto-1* contributes to an upregulation of mesenchymal markers including vimentin, Snail and N-cadherin, while it reduces expression of epithelial markers such as E-cadherin during mammary cancer development (38). *Cripto-1* expression initially detected in the blastocyst during early embryonic mouse development, is indeed high in stem-like cells in embryonal, melanoma, prostate, and pancreatic cancer cells. Its essential role and contribution to embryogenesis is revealed by genetic studies identifying the embryonic lethality of *Cripto-1* knockout mice (38).

The prostate gland is formed during embryonic development from the urogenital sinus, a midline structure with an endodermally derived epithelium surrounded by a mesodermally derived mesenchyme (25). *SRY*-related high-mobility-group box (Sox) transcription factors are transcription factors that regulate development during male differentiation. One of those transcription factors, *SOX9*, is found in basal epithelial cells in a normal prostate and is essential for prostate development. SOX9 is highly expressed in fetal prostate cells as the epithelium is expanding into the mesenchyme (39). Moreover, SOX9 stimulates expression of anti-Mullerian hormone in the developing gonad (40), and is also elevated in recurrent prostate cancer (41). SOX9 is a critical signaling partner of both the Wnt/B-catenin and fibroblast growth factor signaling pathways and can induce AR expression, ultimately impacting growth and progression of metastatic tumors (41). During prostatic development, androgenic action within smooth muscle cells is suppressed by TGF-β via translocation of nuclear AR into the cytoplasm (26). TGF-β is an essential cytokine necessary for embryogenesis, as TGF-β null mutation mice embryos die within several

weeks due to an excessive inflammatory response (42). TGF-β signaling is required for testis development also by virtue of its effect in navigating EMT (43). During kidney development, the gene family encoding *Snail* are downregulated and Snail expression correlates with Cadherin-16 expression in a renal tissue specific pattern towards EMT outcomes (44). Testis cords form via an endothelial to mesenchyme transition following endothelial cell migration. Endothelial cells express cell surface factors that influence surrounding cells as well as remodeling of the surrounding ECM during development (45).

Benign prostatic hyperplasia (BPH) is an abnormal prostate growth condition that resembles embryonic awakening of the gland. Until recently EMT has only casually been linked to the onset of BPH, potentially dictated by macrophages associated with the benign growth of the prostate (24). Morphological-based evidence suggested that accumulation of mesenchymal cells derived from the prostatic epithelium contributes to BPH development, implicating TGF-β mediated EMT in the etiology the disease (46). EMT markers such as N-cadherin, TGF-β2, and Snail are overexpressed in prostate specimens derived from BPH patients and functional communication between macrophages and prostate epithelial cells may lead to induction of TGF-β2 (47). Mechanistically, modulation of AR activity in BPH induces characteristic EMT changes suggesting that AR in prostate epithelial cells may promote macrophage-mediated EMT in BPH (47).

EMT in prostate tumorigenesis

The cytoskeletal rearrangements that tumor cells endure during EMT and blood vessel invasion determine the cell-in-motion plasticity and sensitivity to ECM-adhesion-detachment. Snail and Slug are zinc-finger transcription factors that are instrumental in embryonic development via their regulatory roles in EMT. The functional requirement of the significant players is reflected by the knowledge that Snail knockout mice are embryonically lethal (48). Snail, expressed in response to FGF, binds to the promoter region of the E-cadherin gene to silence E-cadherin expression and induce EMT during gastrulation (49). The Wnt signaling pathway is involved in embryonic development and tumorigenic growth and progression. Components of the Wnt signaling pathway, including B-catenin, glycogen synthase kinase, lymphoid-enhancer binding factor 1 and cyclin D1 by functionally engaging the AR signaling axis, are capable of modulating the AR-driven transcriptional activity (50).

β-catenin is a leading cell-cell adhesion molecule that navigates the dynamics of the actin cytoskeleton remodeling to cell behavior via its functional interaction with E-cadherin. β-catenin acts to link the cytoplasmic domain of the cadherin family of transmembrane proteins to α-catenin and ultimately connecting the adhesion complex to the actin cytoskeleton. Non-invasive cells tend to exhibit β-catenin on the membrane, as opposed to invasive cells that have undergone EMT changes where β-catenin is confined more to the cytosol and the nucleus (51). Expression of β-catenin is distributed in two different cellular localizations: in the cellular membrane associated with E-cadherin, and in the cytoplasm or nucleus in functionally association with an activated Wnt signaling pathway. It is regulated by several signaling pathways through binding to other protein partners including Tcf/LEF family members, axin, APC, and cadherins. In an unorthodox twist of interaction, β-catenin binds to the AR but not to other nuclear steroid receptors, such as the progesterone receptor, the estrogen receptor or the glucocorticoid receptor. When bound to AR, β-catenin is able to translocate into the nucleus, where it increases the ligand-dependent transcriptional activity of AR, increasing expression of AR-dependent promoters such as MMTV and the PSA gene, and consequently contributing to a highly malignant and invasive phenotype. The effect of β-catenin on AR is further enhanced in cells not harboring E-cadherin expression (52). Since elevated β-catenin in association with increased AR has been detected in CRPC, the evidence implicates a value of β-catenin protein expression as potential predictive marker of prostate cancer progression. Indeed, overexpression of β-catenin may explain a mechanism for the emergence of the androgen independent-castration resistant state of prostate cancer (53). Attractive as this concept might be, one must also consider the differential cellular "zip code" of β-catenin that impacts the high expression of nuclear protein in BPH and low-grade prostate cancers, while a decreased level of nuclear β-catenin is associated with increasing Gleason scores and tumor progression to metastasis (53), potentially via deregulation of the EMT-MET phenotypic switching navigated by AR signaling. TGF-β overexpression is detected in the serum of patients with advanced prostate cancer (54). The fluidity of the microenvironment dynamic is enhanced in prostate tumors, as the cancer epithelial cells counterbalance the signals from the cancer-associated fibroblasts and neighboring endothelial cells. In a pre-clinical model of prostate cancer metastasis, the tumor suppressor DAB2IP, can reverse EMT

and prevent circulating tumor cells from spreading (55). This is evidence not only identifies a tumor suppressor that controls scaffolding, but it also provides a new platform for targeting tumor-circulating cells undergoing EMT at initiation of metastasis.

TGF-β and AR navigate EMT during tumor progression to metastasis

TGF-β is a multifunctional cytokine that controls critical cellular processes including apoptosis, immune responses, and EMT (56). The TGF-β serine threonine membrane kinases that activate intracellular Smad2/3 proteins, forming a complexthat upon nuclear translocation is responsible for transcription of TGF-β responsive genes. TGF-β promotes EMT and consequently leads to generation of cells with stem cell like properties (57). Moreover, this EMT protagonist (TGF-β) induces proliferation in mesenchymal cells, while it inhibits growth in epithelial cells (58). Cripto-1 is a cell-signaling marker that may play a role in the reversal of TGF-β from a tumor-suppressing gene to a tumor promoter (38). *Twist* is a helix-loop-helix transcription factor that imparts migratory and invasive characteristics to cells and controls multiple aspects of EMT, primarily by repressing E-cadherin expression (59), while concomitantly induces mesenchymal gene expression (60). Twist is indicative of high-grade tumors, malignant disease progression and resistance to anti-cancer therapies (61), via its ability to suppress apoptosis and promote angiogenesis. Moreover its significant regulatory role in EMT, has rendered *Twist* an attractive molecular target for CRPC treatment (32).

EMT-based molecular signatures as biomarkers for prostate cancer

The loss of E-cadherin is a hallmark of EMT induction, in association with changes in several interconnected signaling pathways that frame the cellular landscape in advanced tumors (*Figure 1*). E-cadherin loss correlates with prostate tumor progression and Gleason grade, establishing this EMT player as a prognostic factor for clinical disease progression (62). Elevated N-Cadherin has been shown to be a significant predictor of clinical recurrence in prostate cancer patients following radical prostatectomy (63), as well as an effective therapeutic target in CRPC (64). Activated AR decreases E-cadherin in metastatic prostate and breast cancer cells, leading to a mesenchymal phenotype (65).

TGF-β serves as suppressor of tumorigenesis via apoptosis induction and inhibition of proliferation, but during tumor progression, cells become insensitive to the growth suppressor actions of TGF-β, that it functionally switches to a metastasis promoter leading to tumor invasion and metastasis (66,67). In the prostate TGF-β takes a lead role in tumor-stroma interactions and input of the tumor microenvironment to the metastatic progression of primary cancer epithelial cells to metastasis (68). The stroma induces expression of TGF-β via multilayered signaling events that impact apoptosis, angiogenesis and adhesion, ultimately promoting prostate cancer progression (69,70). In a dynamic cross-talk, androgens enhance the apoptotic effects elicited by TGF-β in prostate cancer cells. Changes within the androgen signaling axis in prostate tumors occur due to altered stromal-epithelial cell interactions and aberrant recruitment of AR co-regulators towards enhanced vascularity and invasion (71). In a dynamic frunctional cross-talk AR enables prostate cancer cells to overcome the apoptotic and EMT action of TGF-β (70,72). Elevated TGF-β ligand correlates with increasing tumor grade in prostate cancer (73) and a dysfunctional TGF-β receptor signaling accelerates prostate cancer progression in a mouse model via EMT and cytoskeleton changes in the tumor microenvironment (68). ZEB-1 is a zinc-finger transcription factor that plays a role in loss of adherens junctions (AJ) during EMT (*Figure 1*). ZEB1 represses E-cadherin expression, facilitates transendothelial migration, and mediates progression to metastasis (74). Consequential to loss of ZEB1 in prostate cancer cells already undergone EMT, there is a moderate re-expression of E-cadherin, enabling the acquisition of epithelial characteristics (74). Of translational significance is the correlation between elevated ZEB1 expression, induced by androgens, and high Gleason scores in prostate cancer, evidence implicating its biomarker value in predicting the onset of metastatic spread (75), potentially via the involvement of ZEB1 with AR in a feedback loop. Downregulation of AR during ADT leads to unchecked ZEB1 expression, ultimately promoting EMT and metastasis (76). Mechanistic evidence supports an AR function, similar to *Snail*, in repressing E-cadherin expression and independently triggering EMT (65). In androgen sensitive and TGF-β responsive human prostate cancer cells, *Snail* is upregulated at the transcriptional and translational level by androgens alone or in combination with TGF-β, while *Snail2* expression can be directly enhanced by AR (68,70,77). Work from this laboratory first demonstrated that human prostate cancer cells undergo changes consistent with EMT and cytoskeleton reorganization in response

to androgens (70). Interestingly these androgen-mediated EMT changes occur independently of TGF-β and promote aggressive tumor cell behavior (70). The inverse relationship between AR expression and EMT induction suggests that a threshold level of AR observed after the onset of ADT in patients (18), may enhance prostate tumor cell metastatic spread (70). Activation of the β-catenin pathway dictated by AR signaling provides another mechanistic platform via which androgens indirectly impact EMT in prostate tumors (70). The central role played by the AR in EMT enables new insights into therapeutic targeting of androgen axis and AR function in CRPC (78).

Circulating tumor cells (CTCs) and bone turnover markers have been identified as possible biomarkers in the blood that could be used as potential surrogates for clinical benefit in men with CRPC. Detectable levels of CTCs are however found in only 50% of patients with widespread metastases. The problem may be linked to CTC's undergoing EMT, which could cause underdetection (78). Improved methods of capturing CTCs could enhance the promise of its biomarker value. Bone turnover markers such as bone type 1 collagen breakdown product N-telopeptide have been linked to survival, however the metastasis predictive value is limited by its normal values in patients with bone metastasis (79).

Most of the predictive markers for high-risk disease maybe indirectly associated with EMT-MET interconversions during progression to CRPC (80,81). Prostate Cancer Antigen 3 (PCA3), a noncoding RNA with expression confined to the prostate, is overexpressed in 95% of prostate cancers compared with normal or BPH (82). Progensa PCA3 is a commercially available diagnostic test that detects *PCA3* RNA expression in urine and prostatic fluid, with high sensitivity and specificity for prostate cancer (83). The notoriety of the transmembrane protease serine 2 v-ets erythroblastosis virus E26 oncogene homolog (*TMPRSS2-ERG*) translocation cannot be bypassed, as these gene fusions are detected in more than 50% of human prostate tumors, pointing to a specificity for prostate cancer detection (80). The gene coding AKR1C3, the enzyme responsible for the conversion of adrenal androgens to DHT, is elevated at the mRNA and protein level in CRPC compared to BPH or primary prostate cancer (84). The "universal" presence of AR gene changes in prostate cancer, potentially navigating EMT in disease progression to mCRPC, enables new avenues for therapeutic targeting and predictive screening (85). Decreasing costs of whole-genome sequencing and technology-driven discovery of molecular profiles, lead new avenues of identification of gene signature-based classification markers of prostate cancer progression, as well as therapeutic guidance towards treatment optimization of metastatic disease (18).

Summary

EMT is a morphological phenomenon involving disruption of cell polarity, acquisition of mesenhymal phenotype, and cytoskeleton organization remodeling. The process of EMT is necessary for normal embryonic development and GU organ differentiation, but it is hijacked by mechanisms that promote tumor initiation and progression. EMT in tumor epithelial cells results from transcriptional reprogramming of abnormal survival signals via growth factor receptor signaling regulating apoptosis, survival and cytoskeletal organization. Metastatic CRPC is driven by EMT, a process facilitating epithelial-derived tumors to invade and rapidly metastasize. EMT-MET interconversions via aberrant TGF-β and androgen signaling pathways confer distinct survival and invasive abilities to prostate tumor epithelial cells. How can we, in view of such uninhibited and functionally promiscuous behavior at the cellular level, build a case for EMT as an effective therapeutic target and attractive diagnostic test in patients with advanced prostate cancer? A morphologic reflection of transcriptional events governed by the prostate microenvironment and dictate tumor cell behavior in a controlled pattern of EMT-MET cycling that produces metastasis, may provide valuable insights. Therapeutic targeting of EMT in mRCPC by a proteasome inhibitor suppressing Snail and reducing RKIP is promising (35). Interrogation of disruptive mechanisms via which AR induces EMT under conditions of androgen depletion in CRPC, may define an EMT-MET signature interconversion that would predict therapeutic resistance and facilitate treatment. The tumor suppressor DAB2IP provides scaffolding to modulate prostate tumor EMT towards MET, blocking the metastatic spread at initiation point (55). Thus in a personalized medicine approach, directed by EMT profiling of individual tumors impacting tumor survival pathways and cytoskeleton remodeling, as validated in pre-clinical models of tumor progression (68,80), EMT mechanistic exploitation in prostate cancer metastasis is unlikely to be clinically insignificant.

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Footnote

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The role of microRNAs in prostate cancer progression

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Abstract: Prostate cancer (PCa) is the most common male malignancy and the second highest cause of cancer-related mortality in United States. MicroRNAs (miRNAs) are small non-coding RNAs that represent a new mechanism to regulate mRNA post-transcriptionally. It is involved in diverse physiological and pathophysiological process. Dysregulation of miRNAs has been associated with the multistep progression of PCa from prostatic intraepithelial neoplasia (PIN), localized adenocarcinoma to metastatic castration-resistance PCa (CRPC). Identification of unique miRNA could provide new biomarkers for PCa and develop into therapeutic strategies. In this review, we will summarize a broad spectrum of both tumor suppressive and oncogenic miRNAs, and their mechanisms contribute to prostate carcinogenesis.

Keywords: Androgen receptor (AR); castration-resistant prostate cancer (CRPC); epithelial-to-mesenchymal transition (EMT); microRNA (miRNA)

Introduction

Prostate cancer (PCa) is a typical hormone-dependent disease (1); however, almost all PCa patients with androgen-ablation therapy ultimately become castration-resistant prostate cancer (CRPC), which contributes to the majority of mortality in PCa. Androgen receptor (AR) is known as a key mitogen for the growth and morphogen for the development of prostate. In PCa, AR activity is critical for the disease progression and becomes hyperactivated in CRPC. Mounting evidences indicate that the development of PCa is highly associated with aberrant AR activity resulted from AR gene amplification and/or mutation, alternative splicing (2-7), the cross talk with growth factor signaling pathways (8-13), and the presence of AR co-activators and AR co-repressors (14-17). In particular, Dicer-mediated maturation of microRNAs (miRNAs) suppresses the expression level of AR co-repressors, NCoR and SMRT, leading to enhance AR transcriptional activity (18). Based on the interaction between Dicer and AR, the correlation between AR and microRNA signaling has been broadly examined to investigate the fundamental role of miRNAs in PCa progression.

miRNAs are a large family of small 20-25 nt single stranded noncoding RNAs, which can interfere with the expression of ~60% protein coding genes by post-transcriptional suppression, target mRNA degradation, or translational inhibition (19). In the past two decades, significant advances have been achieved in miRNA research. miRNAs are found to be highly conserved among the animal phylogeny. Based on their conserved sequences, miRNAs shared an identical seed region of 2-7 nucleotides are grouped into different family. Up to date, 63 miRNA families have been categorized and more than 1,000 miRNAs have been fully characterized in their expression, epigenetic regulation, biogenesis and functions. In general, miRNAs are either derived from non-coding RNA transcripts or located within the introns of protein-coding genes (20,21). Multiple miRNAs can be clustered in close proximity and encoded together as a single polycistronic primary transcript, such as miR-106a-363 (22) and miR-17-92 clusters (23). The transcriptional mechanism of microRNA is similar to that of mRNA. miRNA gene promoters are regulated by transcriptional factors that also regulate protein-coding gene expression. For example, the promoter region of miR-21 can be regulated by AR, activation protein 1 (AP-1) and

signal transducer and activator of transcription 3 (STAT-3) (24,25). Hence elevation of miR-21 in cancer is partially due to aberrant activation of AR and AP-1. Meanwhile, c-Myc is a well-known oncogene that is suggested to regulate an oncogenic miR-17-92 cluster. Overexpression of both c-Myc and miR-17-92 cluster is indicated to enhance the tumor aggressiveness (26). Moreover, the genomic organization of miRNAs reveals that about 52% of miRNA genes are localized at the fragile chromosomal regions, which are susceptible to amplification, deletion and translocation associated with cancer. A recent study indicates that let-7 miRNAs family is located in the genomic regions that are frequently deleted in multiple cancer types including PCa (27). Moreover, the miR-15a/miR-16-1 cluster is located at chromosome 13q14. The frequency of allelic loss at 13q increases from early, advanced to metastatic PCa (28). In addition, aberrant DNA hypermethylation at the CpG island is often observed in the promoter region or transcriptional start site of tumor suppressive miRNAs such as miR-200/-141, miR-205, miR-34, miR-143 and miR-145 associated with PCa (29-32). Thus epigenetic regulation is also a key regulatory mechanism for miRNA gene expression.

In addition to the regulation of miRNA gene expression, the biosynthetic process of miRNA maturation becomes an emerging area. The biogenesis of miRNAs composes sequential steps of RNase III-mediated endonucleolytic cleavage mechanisms (33,34). In brief, the primary transcripts of miRNAs are transcribed by RNA polymerase II and processed in the nucleus by Drosha and Pasha (DGCR8) into a 70-100 nucleotides-long precursor miRNAs (pre-miRNAs) (35). Pre-miRNAs are exported to the cytoplasm through Exportin 5 and further processed by Dicer, generating a 20-25 nt RNA duplex comprise of a matured guide strand and a complementary passenger strand (miRNA*). The single stranded matured miRNA is then incorporated into the RNA-induced silencing complex (RISC) associated with Agonaut (AGO2), and bound to the complementary sequence on the 3' untranslated region (3' UTR) of target mRNA, leading to mRNA degradation (36-38). Based on their post-transcriptional regulation on a variety of target genes, miRNA is expected to be involved in virtually every biologic process in cell. In cancer, based on their post-transcriptional repression on a variety of oncogenes or tumor suppressor genes, miRNAs are also divided into onco-miRNAs (oncomirs) and tumor suppressors miRNAs (*Figure 1*). Overall, the importance of microRNAs has become a key to gain more understanding of molecular mechanisms associated with prostatic

carcinogenesis. In this review, we will focus on key unique miRNAs (*Tables 1,2*) involved in PCa.

Tumor suppressive miRNAs

Let-7 family

The *let*-7 gene encodes a highly conserved miRNA family of let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let-7i and miR-98, which are significantly down-regulated in localized PCa, compared to adjacent benign tissues (39). The functionality of let-7 has been shown to target oncogenes involved in cell-cycle regulation, cell migration, proliferation, differentiation, and epithelial-to-mesenchymal transition (EMT) progression. In particular, let-7g can inhibit tumor growth via post-transcriptional suppression on RAS oncogene (44). On the other hand, loss of let-7 miRNAs is corresponded with elevated level of Enhancer of Zeste homolog 2 (Ezh2) correlated with PCa progression (45). Ectopic expression of let-7 results in the reduction of Ezh2, accompanied with diminished clonogenic ability and sphere formation in PCa cells (45). Another let-7 target gene is High-mobility group AT-hook 2 (HMGA2) (89) that is highly expressed in PCa compared to adjacent benign tissues. Indeed, HMGA2 was found de-repressed upon let-7 inhibition (43). Meanwhile, co-regulation of HMGA2 and Smad were found to orchestrate an EMT transcriptional network via targeting the promoter of SNAI1 in human hepatocarcinoma cell line (90). These results suggest a possibility that let-7 could inhibit EMT via targeting HMGA2 during PCa progression. Moreover, another study also imply that let-7 can induce cell cycle arrest and xenograft PCa tumor development by suppressing E2F2 and CCND2, which are found to be the direct target of let-7 (43). Lin-28 is a well-identified post-transcriptional suppressor of precursor let-7 maturation (91,92); An inverse correlation between lin28 and let-7 is also found in many cancer cell lines including PC3 (93). Based on these observations, lin28-mediated let-7 biogenesis has become an important mechanism to impact tumorigenesis. Conversely, let-7 can target the lin28 mRNA, suggesting that a reciprocal feedback loop exists between let-7 and lin28 (94-97). In addition, c-Myc is found to be a key factor involved in this interaction. c-Myc acts as a transcriptional activator for lin-28 gene expression and c-Myc is also found to be a target gene of let-7 family in multiple cancer types (40,98,99). Overall, the orchestrated interaction between lin28, let-7 and c-Myc is a complicated network of gene
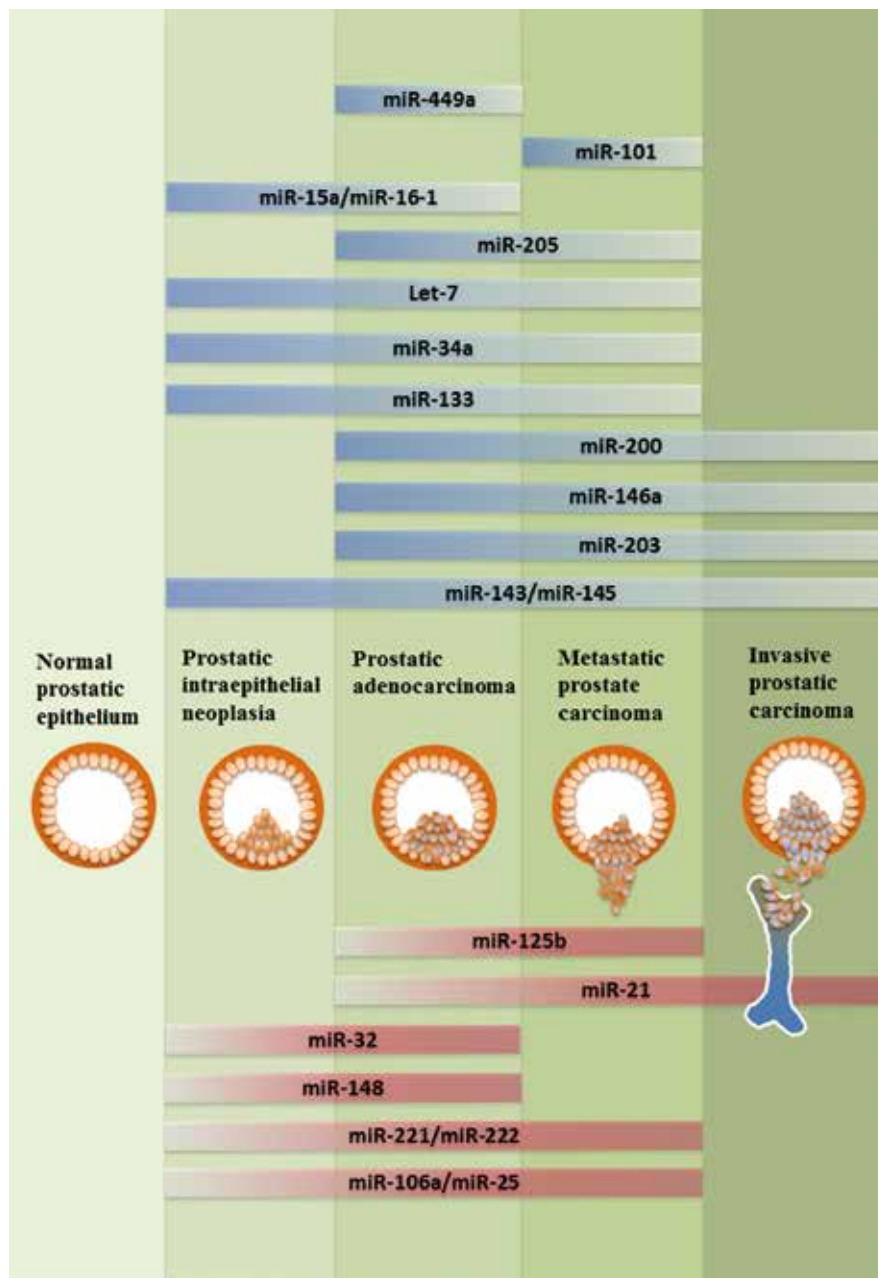
Figure 1 Schematic representation of tumor suppressive miRNAs (blue) and oncogenic miRNAs (red) in the prostatic tumorigenesis progression from PIN to metastatic CRPC.

regulation, which is often altered in cancer cells (100). Also, let-7c is shown to antagonize AR expression by targeting c-Myc (101). Overexpression of let-7 leads to AR suppression, accompanied with attenuated cell proliferation, clonogenicity and anchorage-independent growth in PCa cells (39,41). Overall, the let-7 miRNA family exerts tumor suppressor characteristics via targeting multiple oncogenes including RAS,

HMGA2, Ezh2, Lin28 and c-Myc. Therefore, let-7 could be a potential diagnostic biomarker and further developed into a new therapeutic strategy for PCa.

miR-143 and miR-145

Both miR-143 and miR-145 are derived from the same

Table 1 Tumor suppressive miRNAs in PCa

miRNA	Target genes	Function	Ref.
Let-7	AR, c-MYC	Suppress cell proliferation, clonogenicity and anchorage-independent growth	(39-41)
	HMGA2	Suppress advanced tumor progression	(42)
	E2F2, CCND2	Induce cell cycle arrest *in vitro* and suppress tumor development *in vivo*	(43)
	RAS	Inhibit tumor growth	(44)
	EZH2	Inhibit clonogenicity and sphere formation	(45)
miR-143	KRAS	Suppress cell proliferation, migration *in vitro*	(46,47)
		Attenuate bone metastatic invasion *in vivo*	
	ERK5	Arrest cell proliferation and abrogate tumor growth	(48)
	CD133, CD44, OCT4, KLF4, c-MYC	Suppress tumor sphere formation	(49)
miR-145	FSCN1	Inhibit cell proliferation, invasion, migration and arrest cell cycle	(50)
	OCT4, SOX2, KLF4	Suppress tumor stemness by inhibiting sphere formation	(51)
miR-200	ZEB1 ZEB2	Prevent PDGF-D induced EMT	(52)
	SLUG	Inhibit TGF-β induced EMT and suppress mesenchymal differentiation	(53,54)
miR-203	CKAP2, LASP1, WASF1, BIRC5, ASAP1	Suppress cell proliferation, promote cell apoptosis and inhibit metastasis dissemination	(55)
	RUNX2	Inhibit tumor invasion destined for bone metastasis	(55,56)
	PARK7, BRCA1	Impair cell growth by promoting apoptosis and cell cycle arrest	(57)
	ZEB2, Bmi, Survivin	Suppress bone metastasis via inhibition of cell motility, invasion and EMT	(56)
miR-205	ErbB3, E2F1, E2F5, PKCε,	Counteract EMT by attenuate cell migration and invasion	(58)
	BCL2	Promote cell apoptosis and cell cycle arrest in response to DNA damage	(59)
	PSAP, ARA24, HRAS, PARK7	Induce apoptosis and cell cycle arrest	(57)
	AR, NR4A2, EPCAM	Impair cell growth by promoting apoptosis and cell cycle arrest	(60)
miR-34a	CD44	Inhibit tumor progenitor cells and suppress metastasis	(61)
	AR	Suppress tumor metastasis	(62)
	CDK6	Induce cell-cycle arrest, cell senescence and apoptosis	(32)
	c-MYC	Inhibit cell proliferation and cell invasion	(63)
	BCL2, SIRT1	Induce cell senescence and apoptosis	(64,65)
miR-101	EZH2	Attenuate tumor cell invasiveness	(66,67)
miR-133	EGFR	Reduce cell proliferation, migration and invasiveness	(68)
miR-146a	ROCK1	Suppress cell metastasis to bone marrow endothelium	(69) N.Y.
	EGFR, MMP2	Inhibit cell growth, colony formation and migration *in vitro* Reduce tumorigenicity and angiogenesis *in vivo*	(70)
miR-15 miR-16	FGF-2, FGFR1	Impair the tumor-supportive capability of stromal cells	(71)
	WNT3A	Attenuate tumor expansion and invasiveness	(72)
	BCL2, CCND1	Induce growth arrest, apoptosis	(72)
miR-449	HDAC1, CCND1	Induce cell cycle arrest and loss of clonogenicity	(73,74)

miR-143/-145 cluster, which are found down-regulated in metastatic PCa samples (29). Both miR-143 and miR-145 share similar functions in tumor suppression.

First, miR-143 is found to exhibit a negative effect on PCa cell proliferation and migration by targeting ERK5 and KRAS, and inactivating subsequent epidermal growth

Table 2 Oncogenic miRNAs in PCa

miRNA	Target genes	Function	Ref.
miR-21	RECK	Promote tumor invasiveness, support xenograft tumor growth and induce castration-resistance phenotype	(75,76)
	MARCKS, PDCD4, TPM1	Promote cell apoptosis resistance, motility, and invasion	(77)
miR-125b	p53, Puma, BAK1	Promote xenograft tumor growth	(78,79)
	p14ARF	Enhance cell proliferation	(80)
miR-221	ARHI	Enhance cell proliferation, colony formation, invasion, and cell survival	(81,82)
miR-222	p27	Promote cell cycle progression and increase clonogenicity. Enhance tumorigenicity *in vivo*	(83,84)
miR-32	BTG2, PIK3IP1	Facilitate cell growth by inhibit apoptosis and enhance proliferation	(85,86)
miR-148	CAND1	Facilitate tumor growth by enhance cell proliferation	(87)
miR-106a miR-25	CASP7	Facilitate tumor progression	(88)

factor receptor (EGFR)-RAS-MAPK signaling pathway (46,48). On the other hand, miR-145 is shown to inhibit PCa cell proliferation by targeting Fascin homolog 1 (FSCN1) that is an actin bundling protein involved in cell motility, adhesion and cellular interactions during tumorigenesis and metastasis (50). Second, overexpression of both miRNAs in PC3 cells represses fibronectin and enhances E-cadherin expression and both can reverse EMT and further attenuate the tumor invasiveness in an *in vivo* bone metastasis model (47). Third, a recent study indicates that both miR-143 and miR-145 can suppress the stem cell characteristics in PC3 cell lines by inhibiting the stem cell markers or factors including CD133, CD44, Oct4, c-Myc and Klf4 (49). Similarly, some studies of embryonic stem cell (ESCs) indicate that miR-145 has been identified to repress pluripotency by targeting Oct4, Sox2, and Klf4 (51,102). Taken together, both miR-143 and miR-145 can suppress several cancer behaviors of PCa cells from tumor proliferation, invasion/metastasis and stemness.

miR-200 family

During embryogenesis, EMT is established to determine the transition between epithelial and mesenchymal phenotypes at different developmental stages (103,104). However, during prostatic carcinogenesis, EMT has been highly implicated in PCa progression by initiating the tumor invasiveness (105-107). The consequences of EMT result in the suppression of epithelial markers by transcriptional repressors including ZEB1, ZEB2, SNAI1 and SNAI2, which are found to be the target genes of

several tumor suppressive miRNAs including the miR-200 family. The miR-200 family consists of miR-200a, miR-200b, miR-200c, miR-141 and miR-449, which are significantly down-regulated during PCa progression and identified to suppress PCa tumor metastasis particularly via inhibiting EMT. A recent study using PC3 cell line indicates that miR-200 can inhibit the platelet-derived growth factor-D (PDGF-D)-induced acquisition of EMT via targeting of both ZEB1 and ZEB2 (52). Another group studying benign prostate hyperplasia (BPH) also shows that miR-200 can reverse the TGFβ-induced EMT phenotype in BPH cell line (53). Meanwhile, in kidney epithelial cell line, all miR-200 family members have been shown to suppress TGFβ-induced EMT via targeting ZEB1, ZEB2 and SNAI2 *in vitro* (108,109); Similar result is also found in unilateral urethral obstruction (UUO) model that miR-200 can protect renal tubular epithelial cells from mesenchymal transition via suppressing ZEB1 and ZEB2 *in vivo* (110). In addition, a regulatory feedback loop has been demonstrated between SNAI2 and the miR-200 family. While miR-200 can target SNAI2 mRNA, SNAI2 protein acts as a repressor to suppress miR-200 gene expression (54). Thus, down-regulation of miR-200 may disrupt the homeostasis between SNAI2 and miR-200. Overall, loss of the miR-200 family in PCa initiates EMT process, which is critical for PCa invasiveness.

miR-203

miR-203 is a well-characterized tumor suppressor and shared the similar anti-metastatic function to miR-200

family (111,112). MiR-203 has been demonstrated to induce MET in PC3 and DU145 cell lines via targeting CKAP2, LASP1, BIRC5, WASF1, ASAP1, and RUNX2, which are critical effectors involved in cell proliferation, migration, invasion and EMT (55). Meanwhile, other study also suggests that miR-203 exhibits its negative effect on multiple steps of the PCa metastatic cascade via targeting on pro-metastatic molecules including ZEB2, Bmi, survivin, and Runx2. As a result, restoration of miR-203 in PC3, VCaP, and MDA-PCa-2b cell lines attenuates the invasiveness of PCa bone metastasis *in vivo* (56). This evidence suggests miR-203 play an important role in the metastatic progression of PCa and that loss of miR-203 may further enhance the invasive characteristics of advanced PCa.

miR-205

Similar to miR-203, miR-205 regulates PCa progression by targeting EMT signaling mechanisms (113). Restoration of miR-205 in PCa cells can induce MET phenotype by up-regulation of E-cadherin, along with attenuated cell invasiveness. In a more detailed study, miR-205 is suggested to attenuate cell invasion and migration via targeting ErbB3, E2F1, E2F5 and protein kinase Cε (PKCε) (58). Meanwhile, another study using xenograft model with tail vein injection also demonstrates that miR-205 can inhibit PCa lung metastasis *in vivo* by targeting ZEB1 and vimentin (114). In addition to EMT regulation, miR-205 can also promote PCa cell apoptosis and cell-cycle arrest by targeting the anti-apoptotic gene BCL2 (59). Moreover, miR-205 is able to inhibit tumor cell growth by inducing apoptosis and cell cycle arrest via targeting AR co-regulators (DJ-1, PSAP, ARA24) and MAPK signaling components (57). These accumulating findings indicate that both miR-203 and miR-205 may suppress metastatic progression of PCa by impairing the EMT-induced invasiveness.

miR-34a

In PCa, miR-34a is identified as a tumor suppressor by inhibiting the stemness characteristics of prostate cancer stem cells (CSC) (61). The study demonstrates that miR-34a is down-regulated in the CD44 + PCa cells purified from xenograft tumors; overexpression of miR-34a can attenuate clonogenic expansion, tumor regeneration, and metastasis in CD44 + PCa cells. These results suggest that miR-34a is a negative regulator of prostate CSC and may exert its suppressive effect on PCa progression

before the onset of metastatic CRPC. Moreover, miR-34a appears to be a p53-regulated gene from a study (115) using doxorubicin and camptothecin-induced p53 activation that a significant up-regulation of both miR-34a and miR-34c is shown. However, this p53-mediated miR-34a up-regulation is abolished in both AR-knockdown LNCaP cells and AR-negative cell lines including PC3 and DU145, suggesting miR-34a expression is AR-dependent. On the other hand, AR has been identified as a direct target gene of miR-34a (62); AR activity can be repressed by de-methylation of epigenetically silenced miR-34a promoter in PCa cells. Overall, these findings imply a reciprocal transcriptional regulatory network among miR-34a, p53 and AR in PCa cells. However, whether this network is involved in PCa progression and its clinical significance require further investigation. Another target gene of miR-34a is c-Myc (63). By targeting the c-Myc expression, miR-34a is shown to suppress the signaling cascade of c-Myc-Skp2-Miz1, which leads to RhoA gene expression and subsequent attenuates cell migration and invasion. In addition to PCa stemness and metastasis, miR-34a also affect the PCa tumor growth by inducing cell-cycle arrest, cell senescence and apoptosis via targeting cell-cycle regulatory gene, such as CDK6 (32), and anti-apoptosis genes including Bcl-2 and SIRT1 (64,65). Overall, miR-34a may exert its tumor suppressor role via targeting various signaling molecules at different stages of PCa progression.

miR-101

Ezh2 is a histone methyltransferase that regulates epigenetic silencing and early studies have demonstrated that overexpression of Ezh2 in PCa contributes to the enhanced aggressiveness and metastatic potential of PCa cells (116-119). A study shows an inverse correlation between miR-101 and Ezh2 expression has been observed in human PCa. Meanwhile, miR-101 is found to suppress the expression and function of Ezh2 in PCa cell lines (66) and the overexpression of miR-101 in PC3, DU145 and LNCaP cells also results in the suppression of Ezh2, along with attenuated cell invasion and migration of these PCa cell lines *in vitro* (67,117). These findings clearly indicate the role of miR-101 in the epigenetic regulation critical for PCa progression via targeting Ezh2 expression.

miR-133 and miR-146a

Epidermal growth factor (EGF) and EGFR are known

to be key tumor promoter for PCa (120). A recent study indicates that, under hypoxia condition, EGFR can interrupt the biogenesis of mHESM (miRNAs regulated by hypoxia-dependent EGFR-suppressed maturation) resulted in reducing Dicer binding and abolishing miRNA maturation via targeting AGO2 phosphorylation (121). Implying that EGFR may certainly contribute to the modification of miRNA processing. On the other hand, both miR-133 and miR-146a have been shown to suppress PCa tumor progression via targeting EGFR. Down-regulation of miR-133 has been observed in PC3 and DU-145 cell lines. Ectopic expression of miR-133 can reduce cell proliferation, migration and invasiveness by targeting EGFR (68). Similar to miR-133, expression level of miR-146a is also significantly down-regulated in PCa (122,123). Overexpression of miR-146a has been demonstrated to suppress PCa cell growth, colony formation and migration *in vivo* via targeting EGFR. Additional studies also reveal that miR-146a can inhibit angiogenesis and bone metastasis *in vivo* by suppressing both matrix metalloproteinase-2 (MMP2) and Rho-associated, coiled-coil containing protein kinase 1 (ROCK1) expression (69,70). These findings indicate that loss of both miR-133 and miR-146a in PCa may attribute to enhancement of EGFR signaling, leading to aggressive PCa progression.

miR-15a and miR-16-1

miR-15a and miR-16-1 are in the same cluster; the expression of miR-15a/miR-16-1 is often down-regulated in PCa due to chromosomal deletion at 13q14, which is highly correlated with the progression of PCa (124). A study has demonstrated that miR-15a/miR-16-1 level is inversely correlated with B-cell lymphoma 2 (BCL2), cyclin-D1 (CCND1) and wingless-type 3A (WNT3A) in advanced PCa (72). The same group also found that both CCND1 and WNT3A are putative target genes of miR-15a/miR-16-1. As a result, restoration of miR-15a and miR-16 is shown to arrest cell growth and induce apoptosis and knockdown of mir-15a/miR-16-1 can promote survival, proliferation and invasiveness of PCa xenograft tumor *in vivo*. On the other hand, miR-15a and miR-16-1 also exert tumor suppressive effects by interfering the stromal support in the tumor microenvironment since interaction between tumor cells and the surrounding cellular components is critical for tumor development (125,126). It has been indicated that down-regulation of miR-15a and miR-16 in cancer-associated fibroblasts results in tumor expansion and invasion (72), which is supported by the

reconstitution of both miRNAs in fibroblast can interrupt the stromal support by targeting FGF-2 and FGFR1 (71). All these data indicate that the tumor suppressor role of miR-15a/ miR-16-1 is to suppress cancer cells or interrupt their communication with the microenvironment.

miR-449a

miR-449a is inversely correlated with the expression of histone deacetylase 1 (HDAC1)-an enzyme critical for epigenetic regulation. It has been indicated that increased expression of miR-449a in PCa cell lines leads to both cell cycle arrest and loss of clonogenicity by targeting HDAC1 (73). In addition, miR-449 can initiate cell cycle arrest and induce cell senescence by targeting cyclinD1 (74).

Oncogenic miRNAs

miR-21

The recurrence of CRPC is often associated with hyperactivation of AR. Recent studies have suggested that several oncogenic miRNAs is correlated with aberrant AR activation. In particular, miR-21 is an AR-regulated miRNA and its expression level is consistently elevated from androgen-dependent PCa to CRPC (127). Overexpression of miR-21 can support xenograft tumor growth and induce castration-resistant phenotype (75). In addition to androgen response element (ARE), other cis-elements such as AP-1 and STAT-3 are also found in the promoter region of miR-21 (24,25). AP-1 activity is closely associated with CRPC recurrence (128) and STAT-3 is also shown to be involved in PCa metastasis (129). Overall, the highly elevation of miR-21 may be attributed to the aberrant expression of transcriptional activators such as AR and AP-1. The subsequent effect of miR-21 overexpression in turn contributes to the development of prostate tumorigenesis. Several target genes of miR-21 have been shown to suppress tumor progression by inhibiting invasiveness, promoting apoptosis and cell cycle arrest. For example, myristoylated alanine-rich protein kinase c substrate (MARCKS) is a direct target of miR-21, which plays key role in cell motility, membrane trafficking and mitogenesis. Thus, miR-21 promotes the apoptosis resistance, cell motility and invasiveness of PC3 and DU-145 cells partly via targeting MARCKS (77). Meanwhile, a recent study demonstrates that reversion-inducing cysteine-rich protein with Kazal motifs (RECK) is another novel target of miR-21; An

inverse correlation between RECK and miR-21 has been shown from different stages of PCa (76).

miR-125b

Similar to miR-21, miR-125b is also an AR-induced miRNA. The induction of miR-125b in LNCaP cells inhibits apoptosis and enhances cell proliferation. Mechanistically, miR-125b promotes PCa xenograft tumor growth by targeting major pro-apoptotic genes including p53, Puma and BAK1 (78). Consistent with this observation, miR-125b is shown to modulate the p53 network by interrupting Mdm2 degradation via targeting p14[ARF], which mediates the Mdm2 sequestration (80). Overall, miR-125b can target the p53-p21 and Puma signaling network, leading to enhanced cell proliferation in both LNCaP and CWR22Rv1 PCa cell lines through p53-dependent and p53-independent manner, respectively.

miR-221 and miR-222

Both miR-221 and miR-222 belong to the same miRNA cluster. Overexpression of miR-221/miR-222 has been often found in PCa. The aberrant elevation of miR-221/miR-222 is highly correlated with metastatic CRPC phenotypes. Moreover, an inverse correlation between miR-221/miR-222 expression and p27[Kip1] level has been observed in primary PCa. Several studies demonstrated that miR-221/miR-222 can up-regulate S-phase kinase associated protein 2 (Skp2), cyclin A and cyclin D1via targeting p27[Kip1] suppression, leading to cell cycle progression at G1-to -S phase, increased clonogenicity *in vitro* and enhanced tumorigenicity *in vivo* (83,84,130). Meanwhile, Ras homolog member I (ARHI), a tumor suppressor identified in ovarian cancer (131), is also identified as the target gene of miR-221/miR-222. Overexpression of ARHI in PC3 cells results in the inhibition of cell proliferation, colony formation, cell invasion and survival (81,82,132), suggesting decreased ARHI mRNA could be an additional mechanism for miR-221/miR-222 contributing to the accelerated tumor growth in PCa. Thus, these data conclude the functional role of miR-221/miR-222 as PCa promoter by targeting tumor suppressor genes such as p27[Kip1] and ARHI.

miR-32

miR-32 is highly expressed in CRPC specimens compared to BPH specimens (85). A study demonstrated that miR-32 exerts oncogenic characteristics by targeting on both B-cell translocation gene 2 (BTG-2) and phosphoinositide-3-kinase interacting protein 1 (PIK3IP1), which regulates the inhibition of PI3K, a well-known regulator of cell proliferation, migration and survival (85). An inverse correlation between miR-32 and BTG-2 has been found in the CRPC specimens (85). In addition, numerous studies have identified BTG-2 as a critical target gene of AR-regulated miRNAs including miR-32, miR-148 and miR-21 (85,86). Loss of BTG-2 was implicated in the progression of PCa accompanied by the appearance of EMT markers (133). Meanwhile, a study using LNCaP cells demonstrated that miR-32 facilitates cell growth by inhibiting cell apoptosis and enhancing cell proliferation, respectively. Overall, miR-32 exerts its oncogenic characteristics by targeting on tumor suppressors critical for cell proliferation, survival and migration.

miR-148a

Similar to miR-32, miR-148 is elevated in advanced PCa compared to primary tumor (134). However, in contrast to the distinctive oncogenic role of miR-32, the role of miR-148a in PCa progression is more controversial. For example, miR-148a was identified as an androgen-responsive miRNA and facilitates LNCaP cell proliferation via targeting cullin-associated and neddylation-dissociated 1 (CAND1) (87). In contrast, miR-148a is shown to be down-regulated in both DU-145 and PC3 cell lines. Furthermore, overexpression of miR-148a in PC3 cells attenuates cell growth, migration, invasion, and enhances the drug sensitivity to Paclitaxel. This phenomenon is paralleled with the effect in MSK1-knockdown PC3 cells. In particular, MSK1 has been identified as the target gene of miR-148a, suggesting miR-148a attenuates the drug-resistance of CRPC cells via targeting MSK1 (135). Apparently, miR-148a represents a unique miRNA with dual function in PCa. Although the exact mechanism remains undetermined, AR may be an important factor involved in miR-148a function or the presence of different target genes of miRNA-184a in PCa cells derived from different origins.

miR-106b/miR-25

A study (136) using computational approach to identify PTEN-target miRNAs has identified miR-106b/miR-25 as a candidate that is concomitantly overexpressed with

its host gene, minichromosome maintenance protein 7 (MCM7), which result in enhanced cell transformation and initiated prostatic intraepithelial neoplasia (PIN) progression of PCa. This study has a significant finding to show a cooperative expression of an oncomir cluster with its oncogenic host gene, which could simultaneously generate "two-hits" effect on the malignant transformation of normal cells. In addition, a study using LNCaP cell line has demonstrated that miR-106b/miR-25 cluster is associated with PCa progression by targeting caspase 7, apoptosis-related cysteine peptidase (CASP7) mRNA, which is down-regulated in both primary PCa and metastatic lesions (134). Overall, miR-106b/miR-25 cluster is an oncomir cluster and it is often found altered in its expression level between PIN, primary, and metastatic PCa (86,137).

Conclusions

miRNA represents a new mechanism of regulating gene expression at either post-transcriptional or translational levels. Aberrant alteration of miRNAs has been clearly demonstrated in PCa. However, knowing complex regulatory mechanism and relationship of miRNAs and their multiple target genes have further complicated their functionality during carcinogenesis. Therefore, carefully dissecting the mechanisms and functional role of each miRNA in heterogeneous PCa cells will certainly generate new information that could be applied as biomarker(s) and developed into novel therapeutic strategies.

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Footnote

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Cancer stem cells in prostate cancer

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Abstract: Prostate cancer (P-Ca) remains a leading cause of cancer-related death in men. Lately, increasing evidence for a hierarchically organized cancer stem cell (CSC) model emerged for different tumors entities, including P-Ca. CSCs are defined by several characteristics including self-renewal, pluripotency and tumorigenicity and are thought to be responsible for tumor recurrence, metastasis and cancer related death. In this review we discuss the recent research in the field of CSCs, its limitations and therapeutical implications in general and specifically in P-Ca.

Keywords: Cancer stem cells (CSCs); castration resistance; hierarchy; prostate cancer (P-Ca)

Introduction

Despite enormous basic and clinical research efforts as well as progress in modern diagnosis and therapeutical options including surgery, radiation and chemotherapy the overall survival rates of human cancer barely increased in the last decades (1). Still patients die due to the continuous growth of metastases that most probably already occurred but were not detectable at the time of diagnosis. For a most effective cancer therapy it is critical to target the cancer cell sub-population with the ability for self-renewal, proliferation, invasive and metastatic growth. Many tumors contain phenotypically and functional heterogeneous cancer cells. In the traditional clonal evolution model tumors are believed to be homogenous and that all cells are able to repopulate and regenerate the tumor by themselves (2). Any heterogeneity is achieved by a subset of cells that acquire additional mutations after intrinsic (e.g., genetic, epigenetic) or extrinsic (e.g., microenvironment) stimuli that promote their aggressiveness and metastatic potential (3,4). As a consequence most of the currently used therapies aim to eliminate as many cancer cells as possible. Recently, a new model, arguing that tumors are malignant caricatures of normal development with an inherent hierarchical organization, presents an explanation for cancer heterogeneity (5). Only a small proportion of cells is capable of self-renewal and responsible for tumor initiation, growth and recurrence, while the majority of cells may be non-tumorigenic end cells. In parallel to normal tissues where cellular hierarchy is maintained by stem cells, this biologically distinct cancer cells have been termed cancer stem cells (CSCs, *Figure 1*). In this review we discuss the recent research in the field of CSCs, its limitations and therapeutical implications in general and specifically in P-Ca.

The history of CSC

The idea of a small subpopulation of cancer cells responsible for tumor initiation, hierarchical organization, growth and metastases has led to remarkable excitement in the field of cancer research, because it is thought to be responsible for the clinical observation that nearly all tumors are heterogeneous and that relapse often occurs in patients considered to be tumor free for many years. However, the idea has been around for some time. As early as 1855, Virchow postulated in his "embryonal rest hypothesis", based on the histological similarities between teratocarcinomas and embryonic tissue, that the former originated from the latter (6). In 1937 Furth and Kahn
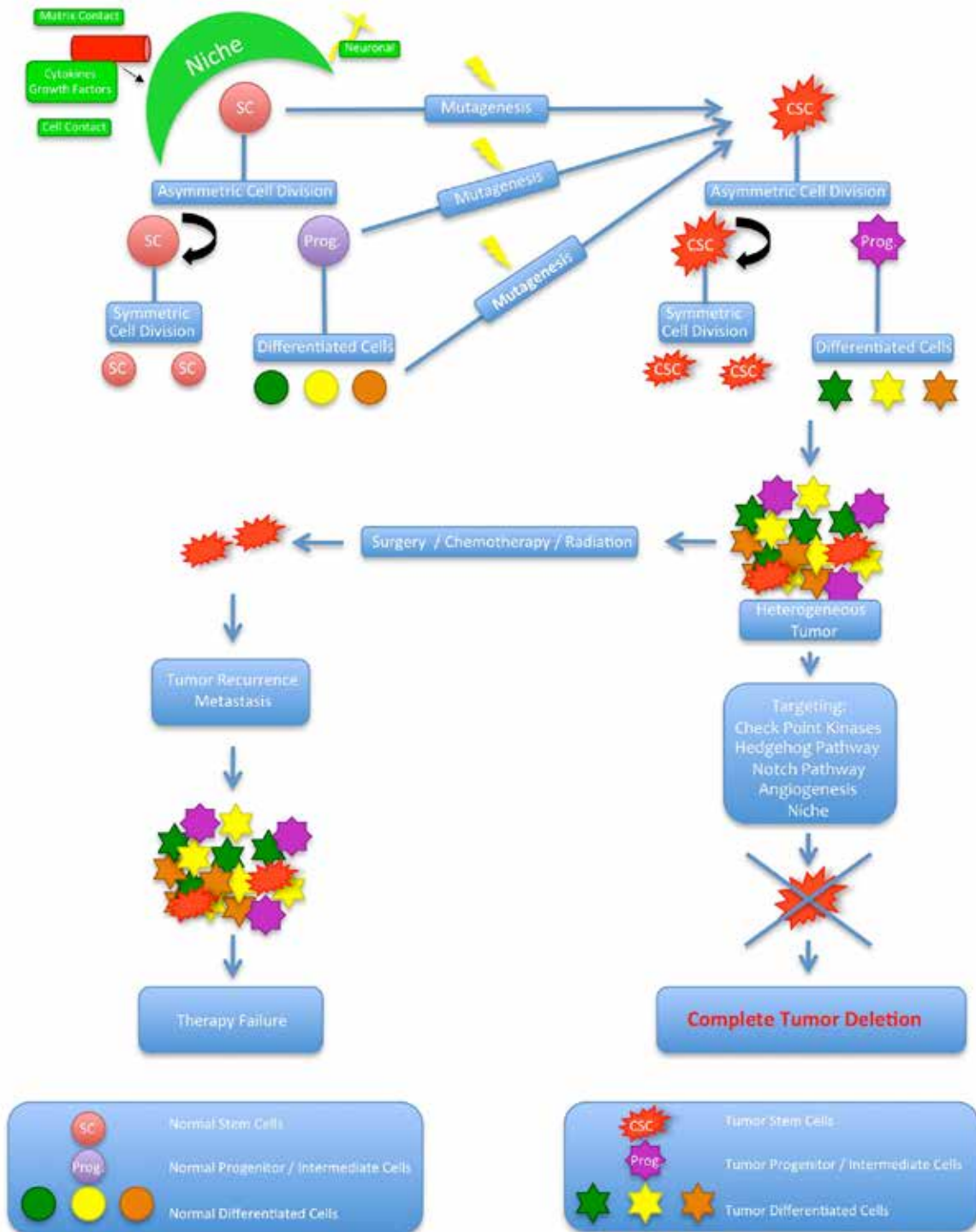
Figure 1 Cancer stem cell hypothesis.

described the transmission of leukemia in mice using only a single cell (7) and in the nineteen-sixties Pierce and colleagues demonstrated the clonal origin of mouse teratocarcinomas from single transplanted multipotent malignant cells (8). In 1997 Bonnet and Dick proved that human acute myeloid leukemias follow the CSC model (5). Single leukemia cell clones induced leukemia phenotypically identical to the parental tumor after transplanting them into immuno-deficient (NOD/SCID) mice. Those tumor-inducing cells revealed the surface markers $CD34^+/CD38^-$, characteristic of hematopoietic stem cells. Also solid tumor entities were described to follow the CSC hypothesis: Al-Hajj *et al.* transplanted only a few human breast cancer cells resulting in a tumor phenotypically identical to the original tumor. The tumor inducing cells ($CD44^+/CD24^{-/low}$) had in contrast to the non-tumor inducing cells ($CD44^-/CD24^+$) the capacity for self-renewal and massive proliferation (9). By today CSCs have been identified in many tumors including bladder (10), breast (9), brain (11), lung (12), prostate (13), ovary (14), colon (15), skin (16), liver (17), and other tumors (18).

CSC properties

Typically, CSCs are characterized to exhibit specific features similar to normal stem cells (SC): the ability for life-long and unlimited self-renewal allowing the maintenance of the CSC pool (19). This phenomenon is achieved by symmetric cell division into 2 new stem cells with the same fate. In contrast asymmetric cell division is thought to give rise to a new stem cell and a daughter cell that enters the differentiation process, loses multi-lineage potential and follows the hierarchical pattern (20). Experimentally, CSCs are defined by the ability to induce a phenotypic copy of the original tumor after serial transplantation into NOD/SCID mice (11). For some CSCs, self-preservation strategies including the activation of anti-apoptotic pathways, increased activity of membrane transporters, active drug efflux and enhanced DNA-repair activity has been described (21). Moreover the proposed ability of CSC to switch between an activated and quiescent state could serve as explanation for insufficient cancer therapies and long-term cancer recurrence (22), however the cell cycle distribution of most CSCs is unknown (23).

Identification/characterization of CSC

A common strategy for CSC identification is flow-cytometry using assumed specific CSC surface markers, e.g., CD44 or CD 133. However, many of the surface proteins used to identify CSCs are also expressed on physiological stem cells and/or progenitor cells (9,11,24). Moreover, since extensive research goes on discrepancies in marker expression of certain CSC entities as well as limited reproducibility has been reported, which could be due to differences in sample preparation and condition (fresh *vs.* passaged), dissociation techniques or even patient related (25,26). Marker based assays, especially single based, possibly enrich, but most probably do not isolate CSCs. Alternatively, different CSC clones with different marker expressions may coexist within primary tumors and/or functional different CSC clones might reside within defined cellular compartments (27,28). In order to reduce phenotypic variability the separation of live cancer cells based on functional measures e.g., signaling pathway activation has been demonstrated, however this is limited to mouse models with reduced variability using an inbred genetic background and targeted mutations (23). In label retaining assays all cells are labeled with a fluorescent marker which becomes more and more diluted with each cell division, therefore leaving the quiescent or low-cycling cell subpopulation positive (29). Utilizing the property of active efflux of the lipophilic dye (Hoechst 33342a) using ATP-binding cassette (ABC) transporters CSC containing "side populations" can be identified (30,31). In contrast to non-tumorigenic cells CSCs are able to form colonies from a single cell and have the ability to grow as spheres in serum free media (31,32). For genetic characterization of CSCs the expression of stemness genes as well as transcription factors can be used. Usually OCT4, Sox2 and Nanog are analyzed as they are essential for the maintenance of pluripotent embryonic stem cells (ESC). Other transcriptional factors are Bmi-1 (mediates gene silencing via regulation of chromatin structure) or Snail and Twist [promote epithelial-mesenchymal transition (EMT) (26,33,34)]. Xenograft models are considered to be the gold standard in the human CSC assay field. Mostly immuno-deficient mouse models are used due to the powerful xenogeneic immune response that kills most human cells before any proliferation. In these models CSCs are defined to have the ability to grow as serial transplantable tumors and to produce tumors showing the same biological heterogeneity as the parental tumor, hypothetically even after transplanting a single cell. However, these assays have limitations: the presence of species-specific signals, immune cells, tumor environment and niches as well as the site of injection are known to influence the efficiency of

tumor initiation. In some cancers the transplantation into highly immune-deficient mice (NOD/SCID/IL-2Rγ^{null}) can significantly increase the frequency of tumorigenic cells compared to transplantation into NOD/SCID mice, which retain an attenuated xenogeneic barrier (23,35). Therefore the different used mouse strains, method of tumor dissection and implantation influence experimental results and complicate the comparison of results.

CSC plasticity

The CSC concept should not be confused with the cell of origin. The cell of origin is the cell type first hit by an oncogenic mutation. Up to date the cell of origin for most cancers has not been yet precisely identified. CSCs can originate from stem cells through mutations that over-activate self-renewal mechanisms (36), however, it has also been demonstrated that they can arise from more differentiated progenitors. These acquire CSC properties by the accumulation of genetic and/or epigenetic abnormalities (37). Recent studies suggest that stemness may not be a fixed state but rather a flexible appearance (38). During the embryonic program of the epithelial-mesenchymal transition (EMT) cells acquire the ability to migrate, invade and to disseminate, which is mediated by transcriptional factors including Twist, Slug and Snail. The loss of epithelial markers and the gain of mesenchymal markers has been observed in epithelial cancer, whereas the overexpression of EMT regulators results in an enrichment of cells with CSC properties (39). In squamous cell carcinoma it has been demonstrated that CSC switch between a preferentially migratory or proliferative phenotype (40), leading to the theory of the existence of stationary and migratory CSCs and to the connection with circulating tumor cells (CTC). CTCs can be detected in blood from patients with primary and metastatic carcinomas. They are thought to be capable of self-seeding back to the original organs, which infers increased aggressiveness of the existing tumor or that they can settle in other organs such as bone marrow, a point at which they are termed disseminated tumor cells (DTC) and can serve as a reservoir of tumor cells responsible for future recurrence (41). Analogous to CSCs the EMT process is thought to be involved as the first step to allow the cells to enter circulation. The reverse process, [mesenchymal-epithelial transition (MET)] is thought to play a fundamental role after CTC have settled down in distant organs to form metastases in the new microenvironment (42). Both, CTCs and CSCs are able to become invasive, exhibit

an increased level of resistance and stem cell like properties, enabling them to initiate metastatic growth (41). Whether CTCs and CSCs are entirely different populations is still a matter of debate; however EMT seems to be important and to link both entities. Another interesting possibility for the CSC origin comes from the discovery that the process of differentiation is reversible through the four transcription factors Klf4, Sox2, Oct4 and c-Myc. This so called Yamanaka-factors are highly expressed in ESC and their over-expression can induce pluripotency in both mouse and human somatic cells (iPSCs), giving differentiated cells the possibility to acquire (C)SC properties (43). Inspired by iPSC, so called induced cancer stem cells (iCSCs) from somatic cells have been established, that similar to iPSCs have the potential to undergo self-renewal, to generate differentiated progenies, and to form tumors when transplanted into recipient mice (44). The exact relations and shared mechanisms of CSCs, CTCs, ESCs and iPSCs are still unknown. However this data suggest that CSC most probably display a dynamic phenotype giving multiple options for the cell of origin.

CSC regulation

The CSC niche is a highly important regulatory anatomical microenvironment. Different cell types as well as a vascular network actively regulate CSC fate and plasticity e.g., by direct cell contact, matrix contact, extracellular matrix (ECM) components, cytokines and growth factors (45,46). For human medullblastoma cells it has been demonstrated that the CSC are located next to capillaries and the transplantation in xenografts with endothelial cells (EC) resulted in increased numbers of CSCs, tumor growth and production of vascular endothelial growth factor (VEGF) compared to transplantation without ECs (47). VEGF in turn leads to the production of EC, underscoring the bidirectional relationship of the nice and CSC. The ECM is essential for anchoring CSC to the niches and probably also modulate CSC function. There is an ongoing controversy whether the CSC can modify the composition of the ECM within the niche. Additionally the niche has a regulative role for CSC drug resistance, making it to an attractive target for new anti-cancer strategies (48,49). A number of different regulatory pathways and proteins orchestrate the fine balance of SC and CSC. From the nine main signaling pathways involved in embryonic development and cancer, seven of them have been implicated in both cancer and stem cells. These are: the JAK/STAT pathway, NOTCH

signaling pathway, the MAP-Kinase/ERK pathway, the PI3K/AKT pathway, the NFkB pathway, the Wnt pathway and the TGFβ pathways (50). The dysregulation of signaling pathway networks plays an important role in enabling CSC to retain stem cell properties, however the detailed description is beyond the scope of this review.

Targeting CSC

Most of currently used anti-cancer therapies aim to kill cells in rapid expansion. Considering this from a CSC hypothesis point of view the results in a reduction of the non-tumorigenic tumor bulk, while the CSCs survive and later on may lead to metastasis and finally death. Treating leukemia in mice with valproic adic to induce growth arrest and apoptosis led to a fast tumor regression and prolonged animal survival, however after treatment withdrawal the disease recurred as a result of an increased self-renewal capacity of the CSCs (51). As mentioned above CSC self-preservation strategies include the activation of anti-apoptotic pathways, increased activity of membrane transporters, active drug efflux and enhanced DNA-repair activity. Since all these are potential targets multiple and different strategies are currently in the focus of extensive research (52). There is emerging evidence that CSCs enter quiescence in order to prevent self-renewal exhaustion. This would imply to either target the dormant CSC or to stimulate them to reenter the cell-cycle for a successful eradication. For chronic myeloid leukemia (CML) PML and FOXO were identified to be important regulators of quiescence. Targeting these genes in a mouse model resulted in an increase of CML proliferation and increased the sensitivity to the chemotherapeutic treatment with Imatinib (53). Therefore the combination of CSC stimulation and conventional drugs could be a reasonable treatment approach. Specifically aiming on CSC niches is an attractive concept as it simultaneously targets multiple signaling pathways, EMT and angiogenesis in the CSC microenvironment. In mice experiments the use of VEGF-Inhibitors led to a depletion of tumor blood vessels and reduction of medulloblastoma CSC (47). Clinically antiangiogenetic agents such as Bevacizumab improved outcomes in different cancer entities, especially in the combination with chemotherapy (54). Targeting surface markers has been demonstrated to successfully eradicate CSC, however it bears the risk of side effects as normal stem cells that exhibit the same markers are also targeted. A number of different regulatory pathways and proteins

orchestrate the fine balance of SC and CSC. Out of many more the Wnt/b-catenin-, Notch-, PI3K/AKT/mTOR-, PTEN, and Hedgehog pathways are responsible for the maintenance of stemness, self-renewal, differentiation and resistance to treatment. Most of them are currently explored as targets for CSC eradication (55), for example the inhibition of notch signaling substantially reduced the CD 133+ brain CSC population in glioblastoma (56). However these pathways are highly conserved cell signaling systems and blocking them might have significant negative side effects. As mentioned above EMT is crucial for tumor metastasis. Experimentally EMT can be targeted with small RNAs. Micro RNAs are small non-coding RNAs that regulate the stability of mRNAs through interaction with the 3' untranslated region of target genes (57). They are known to be important regulators for CSC self-renewal, differentiation, and tumorigenesis (58). In human breast CSC miR-200 inhibits TGF-b induced EMT, resulting in an inhibition of clonal expansion and tumor formation (59). Moreover one of the predicted target genes of miR-200c is Bmi-1, a regulator of self-renewal. In many cancers miR-34 expression is down regulated. Known targets of miR-34 are proteins important for apoptosis, cell cycle regulation and migration, mechanisms that are involved in CSC self-renewal and differentiation (60). Specifically one target gene is Bcl2, which improves CSC chemoresistance (61). Overexpressing miR-34 in pancreatic and gastric cancer sensitizes cells to chemotherapy, while tumor growth is inhibited (62,63). Moreover miR-34a leads to CSC differentiation through interaction with mRNA important for Notch1 and Notch2 in glioblastomas. Another currently unsolved problem is measuring CSC activity during and after treatment as specific markers/models still need to be established. However, they would be important for the correlation to outcome data in order to evaluate clinical efficacy.

The CSC hypothesis in P-Ca

Prostate stem cells (PSC)

The prostate consists of 3 cell types. Basal cells are relatively undifferentiated, androgen-independent, cells. They express CK 5 and 14, CD44 and p63, but no or only low androgen receptors (AR-), PSA and PAP. Secretory luminal, and glandular epithelial cells are differentiated cells of the mature prostate with androgen-receptor (AR+), PSA, PAP, CK 8 and 18 expression. The neuroendocrine cells appear to be

androgen-independent and fully differentiated cells containing chromogranins without expression of AR or PSA (64). The existence of physiological stem cells in prostate was deduced from the finding that androgen ablation leads to involution of the androgen-dependent compartments of the prostate, but that subsequent androgen replacement results in total reconstitution of the organ. Based on these observations, Isaacs and Coffey developed a PSC model (65). According to this model, androgen-independent stem cells give rise to progenitor cells that are androgen-sensitive, but not androgen-dependent, which then, under the influence of androgen, differentiate into androgen-de- pendent cells of the prostate epithelium. Following Isaacs and Coffey's theory the PSC were thought to reside in the basal cell layer, as it remains intact during androgen ablation-caused prostate involution.

Prostate CSC

CSCs in P-Ca are not well understood yet. There exists conflicting data for putative markers, the cell of origin as well as the location of P-Ca stem cells (PCSC) within the organ. However, ongoing research in mice and human provides convincing evidence that P-Ca follows the hierarchical model (66).

Markers used to study PSC and PCSC

Most studies investigating PCSC used established cell lines, primary tumors or xenografts in immuno-deficient mice (67). Multiple markers for the characterization of PSCs and PCSCs have been proposed, including cell surface markers, marker of self-renewal, pluripotency and markers of resistance to therapy (68). Collins isolated rare cells from human primary P-Ca using the combination of the surface markers CD44$^+$α2β1integrin^{high}CD133$^+$ that were able to self-renew *in vitro* (13). Using the same combination prostate CSCs were isolated from the cell line DU145 (69). Interestingly CD44, a glycoprotein involved in cell-cell interactions, cell adhesion and migration, has been identified as a marker of stemness of CSC for many different organs/cancer (70). Patrawala revealed that CD44$^+$ P-Ca cells from xenograft human tumors were enriched in tumorigenic and metastatic progenitor cells compared to CD44$^-$ cells (71). Hurt demonstrated the tumor forming ability of CD44$^+$CD24$^-$ prostate stem-like cells isolated from LNCaP cell line was after the injection of as few as 100 cells in NOD/SCID mice (72). Holoclones from the PC3 P-Ca

cell line were shown to contain cells expressing high levels of CD44, α2β1 and β-catenin, and could initiate serially transplantable tumors after subcutaneous injection (73). CD133 has been identified as CSC marker for a variety of malignant tumors (74). In prostate, CD133$^+$ cells were demonstrated to be able to possess a high *in vitro* proliferative potential and to reconstitute prostatic-like acini in immunocompromised male nude mice (24). However, recent studies suggest that CD133$^-$ cells in certain human tumors also possess tumorigenic activity after serial transplantation in NOD/SCID mice (74,75). Aldehyde dehydrogenase (ALDH1A1) acts in retinoic acid signaling, has important function in SC self-protection and high ALDH activity was correlated with the stem/progenitor cell state (76). For P-Ca it was found to be positively correlated with Gleason score and pathologic stage, and inversely associated with patient survival (77). In contrast to ALDH$^-$ cells, ALDH$^+$ P-Ca cells showed CSC-like characteristics such as increased self-renewing and colony forming capacity and tumorigenicity. In addition, ALDH$^+$ cells revealed an increased expression of putative P-Ca stem cell markers (CD44 and integrin α2β1) (78). Yu reported conflicting data, as they found that ALDHl^{ow}CD44$^-$ cells were also able to develop tumors with longer latency periods, although with lower capacity compared to their ALDH^{high}CD44$^+$ counterparts (79). Investigating PSA$^{-/lo}$ALDH$^+$CD44$^+$α2β1$^+$ phenotypes Qin described these cells to be quiescent and refractory to stresses including androgen deprivation. The cells expressed stem cell genes, and were able to undergo asymmetric cell division generating PSA$^+$ cells. Importantly they initiated robust tumor development, resisted androgen ablation and harbored highly tumorigenic castration-resistant PCa cells. In contrast, the PSA$^+$ PCa cells possessed more limited tumor- propagating capacity, underwent symmetric division and were sensitive to castration (80). Lin$^-$Sca1$^+$; CD49f^{hi} (LSC^{hi}) cells have been demonstrated to be useful for isolation of murine stem cells. In the Pten-null P-Ca model the LSC^{hi} subpopulation is sufficient for cancer initiation (81). Addition of CD166 further enriched sphere-forming activity of WT LSC^{hi} and Pten null LSC^{hi}. Moreover expression of CD166 is upregulated in human P-Cas, especially CRPC samples (82). Nevertheless, in the Pten null mouse model downregulation of CD166 did interfere neither with sphere formation nor with progression and metastasis. Identifying the ABCG2 side population, which is associated with multidrug resistance, in combination with the surface marker CD133$^+$/CD44$^+$/ CD24$^-$ have been also reported to increase CSC isolation (83).

Another method to possibly identify the clinical relevant cell population is the exposure to chemotherapeutic agents such as Doxetacel. Using this method in DU145 and 22Rv1 cells with elevated levels of Notch and Hedgehog signaling were identified. Moreover these cells were also detected in human primary and metastatic prostate tumors (84). Taking another approach, an EMT phenotype with the loss of epithelial and gain of mesenchymal markers was described in isolated PC3 cells with CSC characteristics. Interestingly the cells overexpressed multiple stem cell genes such as Nanog, Oct4, and Sox2 (85). Recently Rajasekahr demonstrated the ability of a subpopulation of cells (TRA-1-60+CD151+CD166+) from human prostate xenografts to recapitulate the cellular hierarchy of the original tumor (86). The variability of the different marker combinations suggests that CSC may be more than a distinct subpopulation and underscores the idea of a dynamic CSC phenotype and plasticity.

Castration resistance

Androgen deprivation leads to reduction of the AR+ cell bulk of P-Ca (65,87). Castration resistant P-Ca expresses stem cell genes within the basal cell layer. The putative CD44+α2β1integrin^{high}CD133+ primary human prostate CSC identified by Collins *et al.* are AR-; they displayed a high capacity for self-renewal and differentiation into AR+ cells (13). The same was described for CD44+AR- tumor initiating cells from prostate xenografts that express stemness genes such as Oct3/4 or BMI1, suggesting a multi-lineage differentiation capacity (88,89). Lee described that P-Ca patients who received ADT had increased PCa stem/progenitor cells population. The addition of the anti-androgen, Casodex, or AR-siRNA in various PCa cells led to increased stem/progenitor cells, while in contrast, addition of functional AR led to decreased stem/progenitor cells population, but increased non-stem/progenitor cell population, suggesting that AR functions differentially in PCa stem/progenitor *vs.* non-stem/progenitor ßcells (90). This data propose that CSC in could contribute to castration resistance PCa. In the BM-18 xenograft model pre-existing stem cell (SC)-like and neuroendocrine (NE) PC cells are selected by castration and survive as totally quiescent and express the SC markers aldehyde dehydrogenase 1A1 (ALDH1A1) or NANOG, coexpress the luminal markers NKX3-1, CK18, and a low level of AR (AR*low*), but not basal or NE markers (91).

The cell of origin

Stem cell biology and tumor biology are closely related, therefore lineage tracing studies in PSC can give insight in normal prostate regeneration, tumorigenesis and possibly the cell of origin in P-Ca, as this is highly relevant for understanding the applicability of a CSC model within the disease. According to the traditional model PSC reside in the basal layer, are AR- and give rise to AR+ luminal cells (92). Fitting into this model, intermediate cell types in transition between basal and luminal cells have been identified, expressing both basal and luminal markers (93). Stem cells in the basal layer are thought to be responsible for the regeneration of the prostate architecture after androgen ablation, however, this has also been demonstrated for a subset of luminal cells castration resistance (91,94). Recently, Zhou could show with the use of a mouse model for tracking cell fates and a mouse label-retaining assay that luminal cells are derived from a basal lineage and that slowly cycling cells, which may represent adult PSC, reside in the basal cell compartment (95). However for prostate CSC there is data supporting both, a basal and luminal cell of origin. In human PCa some believe that luminal cells are the cells of origin since the majority of cells in the tumor bulk are luminal and the disease is diagnosed based on the absence of basal markers. Moreover in human PIN the upregulation of c-MYC and shortening of telomere length was described exclusively in luminal but not in basal cells. Using a mouse Pten knockout P-Ca model all initial hyperplastic cells were luminal (67). Wang identified a rare luminal epithelial population with stem cell properties during prostate regeneration in mice, which they termed CARNs (Castration-resistant Nkx3.1-expressing cells). The deletion of Pten in CARNs resulted in high-grade PIN and carcinoma, indicating that CARNs are a cell of origin (96). The possibility of a human equivalent of CARNs was demonstrated by Germann *et al.* (91).

The CD44+α2β1integrin^{high}CD133+ CSC identified by Collins support the basal cell of origin theory as they comprised less than 1% of the tumor mass and were isolated from basal cells (13). Also the CD44+CD24- prostate stem-like cells described by Hurt revealed a basal phenotype (72). Cells within the basal fraction from human benign prostate tissues were able to regenerate benign prostate tissue in immuno-deficient mice. Interestingly the introduction of oncogenic alterations in the target cells induced a disease that mimics human P-Ca, while infected luminal cells failed to form tumors, supporting basal cells as one

cell-of-origin for P-Ca (97). The fusion between the androgen receptor-regulated gene promoter of *TMPRSS2* and ERG is present in about 50% of human P-Cas (98). In addition the *TMPRSS2*-ERG fusion was described to be present in the basal CD44+α2β1integrin^high^CD133+ prostate CSC (99). Given the complexity and heterogeneity of prostate cancer it is likely that the different models (especially mouse models) only recapitulate properties of specific subtypes of human P-Ca (67). It has been speculated that there may also be multiple cells of origin for P-Ca in analogy with breast cancer (100). As this might lead to individual behavior and treatment response the investigation of cells of origin for P-Ca might have important clinical implications.

Targeting P-Ca stem cells

Similar to CSC in other cancer entities targeting of prostate CSC is subject of intensive research. As described above P-Ca patients who received ADT had increased PCa stem/progenitor cells population. Targeting PCa non-stem/progenitor cells with AR degradation enhancer ASC-J9® (GO-Y025, Dimethylcurcumin) and targeting PCa stem/progenitor cells with 5-azathioprine (immunosupressor) and gamma-tocotrieno (Vitamin E Isomer) resulted in significant suppression of the PCa at the castration resistant stage in human PCa cell lines and mouse models (90). This suggests a combinational therapy that simultaneously targets both stem/progenitor and non-stem/progenitor cells will lead to better efficacy. Targeting the hedgehog pathway Nanta investigated the effects of Erismodegib on human prostate CSC's viability, sphere formation, apoptosis, EMT and tumor growth in NOD/SCID mice. The inhibition resulted in modulation of proliferation, tumor growth, EMT and apoptosis. Erismodegib inhibited CSC characteristics and regulation of Bcl-2 family members. Inhibition of Bmi-1 was meditated through upregulation of miR128 while the inhibition of EMT was regulated by induction of the miR-200 family. Targeting the hegdehog pathway could be a potential strategy for targeting prostate CSC (101).

Yang described a significantly higher expression of testicular nuclear receptor 4 (TR4) in PCa CD133+stem/progenitor cells compared with C133-non-stem/progenitor cells. The knockdown of TR4 led to increased drug sensitivity to two commonly used chemotherapeutic drugs, docetaxel and etoposide, mechanistic through the suppression of TR4 in these stem/progenitor cells led

to down-regulation of Oct4 expression, which, in turn, downregulated the IL-1 receptor antagonist (IL1Ra) expression, suggesting the possibility of targeting TR4 as approach to overcome chemoresistance (102). MicroRNA profiling revealed that miR-34a is relatively lower expressed in CD44+ prostate CSCs from xenografts and primary tumors. The enforced expression of miR-34a in CD44+ cells inhibited clonogenic expansion, tumor regeneration and metastasis. In contrast miR-34a antagomirs in CD44- cells promoted tumor development and metastasis. Interestingly CD44 was described as a direct target of miR-34a. Therefore miR-34a could serve as therapeutic agent against prostate CSC (103).

Limitation of the CSC hypothesis

The CSC hypothesis is an attractive concept of cancer development and has led to some enthusiasm in the field of cancer research. It serves as logical explanation for clinical phenomenons such as tumor recurrence even years after an initially successful therapy. Most brilliant discoveries are simple, but now it appears that the more insight researcher gain into CSCs the more complex it gets. There are many theoretical and experimental caveats to the CSC model that have remained unexplored. For a detailed description we suggest the excellent review of Hans Clever and emphasize below the most important points (104). The above-mentioned plasticity of CSCs has yet not been understood in detail; however, the stability of the CSC phenotype is a precondition for selective targeting and plasticity might interfere with therapy. The species barrier as well as the transplantation setting limits the validity of the commonly used xenograft assays. Importantly, Morrison pointed out that the transplantation of any stem cell can reveal the potential of the stem cell under the particular assay conditions, but it cannot reveal the actual fate of the transplanted cell in its original tissue or tumor (23). The heterogeneity and inconsistency of the putative stem cell surface markers have already been discussed above. Often these heterogeneously expressed FACS markers were selected for their ability to isolate certain cells and not on the basis of a deeper understanding of the underlying stem cell biology of the pertinent tissue from which the cancer originates. Moreover it has been demonstrated that the tumorigenic cell frequencies can sometimes increase dramatically as a result of changes in assay conditions. Therefore it will be necessary to systematically assess the degree to which changes in assay conditions affect the

spectrum of cancer cells that can form tumors.

Future perspectives

The exciting ongoing debate about the CSC theory will lead to further research elucidating the current controversies and open questions. Hopefully this will eventually result in the development of novel therapeutic strategies.

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Footnote

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Bacille-Calmette-Guerin non-responders: how to manage

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Abstract: Intravesical immunotherapy with bacille-Calmette-Guerin (BCG) is indicated in the treatment of high-risk and intermediate-risk non-muscle invasive bladder cancer (NMIBC). Our goal is to describe the various disease states following induction and maintenance BCG and to describe contemporary treatment options and the current and projected clinical trial landscape for patients who recur following BCG therapy.

Keywords: Bladder cancer; bacille-Calmette-Guerin (BCG); refractory; failure

Introduction

The definition of bacille-Calmette-Guerin (BCG) failure must be specified in the context of previous treatments and the time interval of recurrence. Valrubicin is the only FDA approved intravesical agent in this setting and the approval is restricted to patients with BCG refractory carcinoma in situ (CIS) and those who refuse or are not medically fit to undergo radical cystectomy. There are multiple other intravesical therapies available for these patients as well as others with Ta or T1 papillary urothelial cancer. Device assisted treatments designed to improved chemotherapy delivery are another option but await approval in the US. Ongoing clinical trials are testing strategies to augment the immune response including pre-treatment BCG vaccination, immune checkpoint inhibition, gene therapy and other novel delivery systems, targeted agents (e.g., FRFR3 inhibitors) and combination intravesical therapy (*Table 1*).

BCG is a live-attenuated mycobacterium originally developed as a vaccine against tuberculosis. Dr. Morales first demonstrated its therapeutic potential in bladder cancer in 1976 (1). While the initial application for NMIBC included the co-administration of transdermal and intravesical BCG, the current standard of care is full dose induction intravesical therapy weekly for 6 weeks followed by maintenance therapy (2).

BCG is approved by the US Food and Drug Administration for treatment of patients with CIS and high-grade papillary Ta and T1 urothelial bladder cancer. Meta analyses of EORTC trials suggest that BCG with maintenance is also an option for treatment of patients with multifocal and or recurrent or large solitary Ta and T1 low-grade disease (3,4).

In the current management of NMIBC at least eight different BCG strains are being used which all have been derived from the original BCG strain which was created from the attenuation experiments by Drs. Calmette and Guerin in 1921 at the Pasteur Institute in Lille, France (5). When the lyophilized form of BCG entered into mass production in 1961, the dispersion caused a drift in the genotype and with resulting substrains that were named after their site of origin and the manufacturer. The strains that are most commonly used are TICE (Chicago) and Connaught (Toronto). With a manufacturing shortage of the latter, the TICE strain is the most commonly available BCG strain at this time.

A Japanese study compared the efficacy of intravesical BCG with the Tokyo strain or the Connaught strain in a randomized study in 133 patients without prior intravesical treatment. The complete response (CR) rate was 90.3% and

Table 1 Ongoing clinical trials for optional treatments of BCG refractory urothelial carcinoma

Trial	Phase	Sponsor	Trial status
Efficacy study of recombinant adenovirus for non-muscle invasive bladder cancer (BOND)	II/III	Cold Genesys	Open—recruiting
Study of BCG combined with PANVAC versus BCG alone course of BCG in adults with high grade non-muscle invasive bladder cancer who failed at least one course of BCG	II	National Cancer Institute	Open—recruiting
AD001 and intravesical gemcitabine in BCG-refractory primary or secondary carcinoma *in situ* of the bladder	I/II	Memorial Sloan Kettering Cancer Center	Open—recruiting
Sunitinib malate in treating patients with recurrent transitional cell bladder cancer	II	Case Cancer Center	Ongoing—not recruiting
Phase 2b, trial of intravesical DTA-H19/PEI in patients with intermediate-risk superficial bladder cancer	IIb	Case Cancer Center	Ongoing—not recruiting
Radiation therapy and chemotherapy in treating patients with stage I bladder cancer	II	Radiation Therapy Oncology Group	Open—recruiting
Dovitinib in BCG refractory urothelial carcinoma with FGFR3 mutations or over-expression	II	Hosier Cancer Research Network	Ongoing—not recruiting
Intravesical administration of Instiladrin (rAd-IFN with Syn3) in patients with bladder cancer	II	FKD Therapies	Ongoing—not recruiting

BCG, bacille-Calmette-Guerin.

85.0% respectively, which was not statistically significantly different. Despite randomization significantly more patients with CIS were allocated to the Tokyo BCG arm (6).

A recent single center randomized clinical trial reported a significant superiority in the treatment of NMIBC with BCG Connaught versus BCG TICE significantly improving 5-year recurrence-free survival (7). This has been attributed to a more effective TH-1 immune response as shown in *in vitro* experiments in mice. A genomic analysis reported in the same study, demonstrated significant differences in the mutation patterns between the strains. While the trial has many limitations in design and power, it raises a provocative question regarding the potential that BCG strain differences may affect relative efficacy in the absence of maintenance BCG. The drift in the genome of BCG has been comprehensively studied as a potential modulator of vaccine efficacy (8). Investigators hypothesize that early BCG strains may be more effective than BCG strains widely used today likely due to independent tandem duplications (DU1 and DU2).

The AUA, EAU, and NCCN guidelines recommend treatment with BCG for high-grade non-muscle invasive cancer including Ta high grade, CIS and/or T1 high-grade disease, which do not meet the criteria for a primary cystectomy (9-11). BCG has been shown to have a durable response rate of about 50% over a median follow-up of 4 years but this number drops to only 30% of patients who are free of tumor recurrence or progression at 10 years. Level I evidence supports the use of full dose BCG plus 3 years of maintenance treatment in patients with high risk disease (12) and meta-analyses suggest that BCG is superior to Mitomycin or Epirubicin for intermediate risk disease but only when administered with maintenance treatment (3,4).

SWOG 8507 randomized patients with high-risk disease to BCG induction alone *vs.* induction plus maintenance BCG for 3 years. Maintenance BCG was associated with both reduced recurrence and disease worsening defined as biopsy proven invasive cancer or a change in treatment strategy implying progression or worsening of the disease state. Five-year RFS was 60% *vs.* 41% with and without maintenance treatment (2). EORTC 30962 compared low dose *vs.* high dose and 1- *vs.* 3-year maintenance therapy in a non-inferiority trial design. While the trial failed to meet the primary endpoint, subset analyses in patients with high-risk disease confirmed the requirement for full dose and 3 years of maintenance and suggested that patients with intermediate-risk disease could be treated with 1 year of maintenance therapy with a similar efficacy as 3 years of

maintenance (12).

The foremost challenge for the urologist who manages patients on BCG is to recognize and accurately define BCG failure and to determine the optimal treatment strategy. Failure to intervene with definitive radical cystectomy prior to progression to muscle invasive cancer is associated with a significant reduction in long-term survival probability (13). The definition of BCG non-responders has been modified over time. O'Donnell *et al.* have recommended the following categories (14): intolerance to BCG describes the inability of a patient to tolerate side effects from the treatment. Resistance to BCG refers to a recurrence or persistence of bladder cancer at 3 months after the first induction cycle but of lesser degree (stage or grade), which is absent at 6 months after either a re-induction cycle of 6 weeks or a first maintenance cycle of 3 weeks. Patients are refractory to BCG when there is persistent disease after a second course of BCG (either maintenance or second induction course). This also includes any progression or worsening of the tumor with regards to stage, grade and disease extent by 3 months after the first cycle of BCG. Formerly called BCG relapse refers to a recurrence of the disease after initial achievement of a disease free state within 6 months of initiation of treatment. A recurrence may be classified as early (within 12 months), intermediate (12-24 months) or late (>24 months). The 2013 FDA/AUA Workshop on clinical trial design in NMIBC expanded the patient population defined as BCG refractory to include those treated with two induction courses or induction plus 3 weeks of maintenance and fail to achieve a CR within 6 months of initiation of BCG (15). The FDA has also considered including patients who recur within 6 months after an initial CR. Single arm phase II trials may be considered for registration with this expanded patient population. Patients who recur with T1HG after an initial induction course may also be included in this group considered BCG unresponsive. BCG failure is a unique subset of patients with persistent TaHG or CIS after a single induction course of BCG or recurrent disease more than 1 year after an initial CR. Phase III trials comparing BCG to BCG plus an experimental drug should be considered in this patient population.

What to do when BCG fails the patient?

Patients who recur after an initial CR to BCG or have persistent disease not requiring a cystectomy after a single induction course can be re-induced with a second induction course or proceed to maintenance therapy with three weekly instillations. Patients who recur or have persistent disease after two induction courses or induction plus maintenance in general should not receive additional BCG. Cystectomy is the standard of care in these patients. For patients who refuse cystectomy or are at higher risk for morbidity or mortality due to co-morbidites can be re-induced with intravesical Valrubicin which is FDA approved for patients with CIS who meet these criteria. While patients with intermediate risk tumors i.e., multifocal and/or recurrent low-grade disease should be offered intravesical chemotherapy, any high-risk disease must be managed in a more aggressive fashion.

The initial CR rate for patient with CIS is 55% at 3 months following initiation of BCG therapy. Without any further BCG, an additional 10-15% of patients will have a CR by 6 months. With the addition of 3 weeks of maintenance, the 6-month CR rate for these patients improves to 84% (2). The message from these data from SWOG 8507 is that one should wait until the 6-month evaluation time point in order to determine whether a patient is refractory to BCG. The exception is patients with persistent T1 disease at the first evaluation at 3 months who should strongly consider cystectomy. An algorithm is presented in *Figure 1* describing the management of disease states following BCG therapy (16-18).

In a retrospective secondary analysis of the SWOG 8507 trial, we examined the association of a CR following induction BCG with overall survival (19). The 5-year survival probability was 77% for patients who had an initial CR *vs.* 62% who did not. Age and history of prior intravesical chemotherapy were also associated with worse survival.

In patients with a positive cytology but no visible disease in the bladder it is imperative to consider extravesical sites as the source of the malignant cells. This should include staging of the upper urinary tract (UUT) and the prostatic urethra as recommended by the EAU and NCCN guidelines. A recent study from Giannarini *et al.* reported that of 110 patients who were treated with a single or two courses of BCG, 52% had a second primary tumor either in the UUT or the prostatic urethra (20). Huguet *et al.* found that the involvement of the prostatic urethra was the strongest predictor for upstaging to muscle-invasive disease in patients treated with cystectomy for BCG failure (21). Moreover there is a clear association between the depth of invasion in prostatic urothelial carcinoma and survival as previously reported by our group and others (22). Ductal CIS or lamina propria invasion of the prostatic urethra and

CR: neg cysto/cytology
PR: neg cyto/+ cytology
NR: + biopsy
Cystx: Cystectomy

Algorithm

Induction BCG—weekly ×6

6 weeks

Cysto, cytology ± biopsy

CR   PR   NR

BCG weekly ×3   BCG weekly ×3   Cystectomy   BCG ± IFN weekly ×6
                 or weekly ×6

CR   + cytology   CR   + cytology   CR   PR   NR

BCG maint   Biopsy   BCG maint   Biopsy   BCG ± IFN maint   Biopsy   Cystx
SWOG 8507   SWOG 8507   SWOG 8507
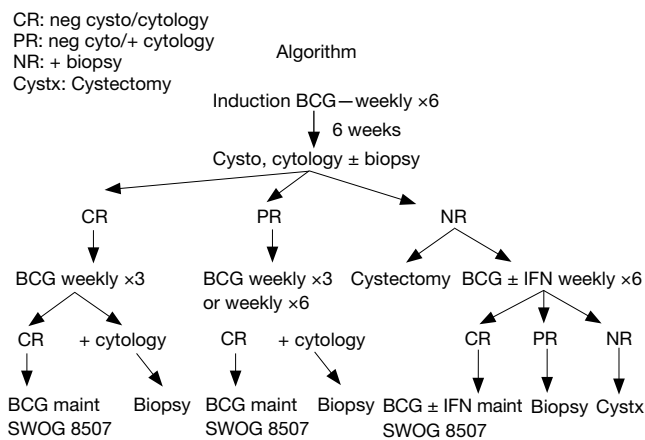
Figure 1 Algorithm for initial treatment with BCG and subsequent therapy depending on response (SWOG 8507 Phase III randomized trial comparing BCG induction and BCG induction plus maintenance to 36 months). BCG, bacille-Calmette-Guerin.

prostatic stromal invasion were associated with a 5-year survival of 44% and 32% respectively while the 5-year overall survival in the absence of any prostatic involvement was about 64%. Transurethral resection biopsy adjacent the verumontanum is imperative for the detection of CIS and invasive prostatic urothelial carcinoma arising from the prostatic urethra (23).

BCG failure—what next?

Radical cystectomy must be considered for patients who fail to achieve a CR or recur after treatment with BCG, but the best time point on when to abandon conservative treatment and consider radical cystectomy is unclear. There is a broad consensus among experts and this is also reflected by the guidelines that any patient not tolerating BCG or where BCG treatment is contraindicated should be offered a radical cystectomy. Contraindications include active tuberculosis or any form of congenital or acquired immunosuppression including (package insert for TICE http://www.fda.gov UCM163039):

(I)   Cancer therapy related immunosuppression (radiation or cytotoxic drug related);
(II) Long-term treatment of corticosteroids;
(III) Treatment with Remicade which is an inhibitor of tumor necrosis factor and blocks T cell immunity.

Moreover any patient with a BCG refractory tumor or any high-grade recurrence after BCG should be considered for radical cystectomy. This is especially important in

patients with a high grade T1 tumor who have a high risk of progression. An analysis from Denzinger *et al.* in 2008 revealed significant differences in the survival rates for patients with initial T1 high-grade tumors who opted for an early versus a deferred cystectomy with a 10-year CSS of 78% and 51%, respectively (24). In patients who opted for BCG treatment and underwent deferred cystectomy the median time in treatment delay from the initial TURBT to cystectomy was 11.2 months. Notable predictors of worse survival were delayed cystectomy and concomitant CIS.

For medically fit patients, radical cystectomy must be performed prior to the development of muscle-invasive disease. In a series of 3,200 patients who underwent RC for CIS, 36% of patients were upstaged to invasive cancer (pT1-T4) and 22.6% of patients were upstaged to muscle-invasive disease ($\geq$ pT2). Cancer specific survival at 3, 5 and 10 years was 91%, 85% and 73%, respectively (25).

There is no rationale for further BCG treatment after two courses unless the recurrence takes place $\geq$1 year after the last BCG treatment. However, there are several options for second line therapy that are available for patients who are medically unfit or unwilling to undergo cystectomy. Several of these must still be considered experimental.

Boosting the immune response

Transdermal vaccination prior to BCG

The mechanisms involved in the immune response generated by intravesical BCG immunotherapy for patients with non-muscle-invasive bladder cancer (NIMBC) are not completely understood, but evidence suggests a role played by both innate and adaptive arms (26). BCG induces a robust CD4 and CD8 T cell infiltrate and up-regulation of cytokine induction such as interferon-γ, IL-2 and IL-12, which are associated with a predominant T-helper cells (Th1) response. Previous trials have investigated the concomitant use of intradermal vaccination and intravesical therapy. The largest trial randomized 154 patients to intravesical therapy (BCG Pasteur strain) with or without combined intradermal inoculation (27). There was no benefit with regards to recurrence-free or progression-free survival similar to other trials (27-29).

Two clinical trials are in developments that revisit the concept of intradermal application of BCG. The European study BOOST and the US SWOG study PRIME will independently evaluate the role of transdermal vaccination with BCG and subsequent BCG intravesical treatment

versus BCG alone. In a pre-clinical experiment mice were treated with subcutaneous vaccination with BCG 3 weeks before the initiation of the intravesical induction BCG, which resulted in 100% survival compared to controls and a modest delay in tumor growth and mortality (30). The investigators found that an accelerated immune response was associated with rapid recruitment of T-cells. A retrospective analysis of a BCG treated patient cohort revealed that a previous exposure to BCG and positive Mantoux (PPD) test resulted in a better response to BCG. While simultaneous intradermal and intravesical therapy have been evaluated these upcoming trials might shine a light on whether intradermal BCG followed in 3 weeks by induction intravesical BCG treatment might provide better treatment responses (27-29).

BCG plus interferon-α (INF-α)

The treatment of high-grade bladder cancer with INF-α has been reported mostly as an intravesical therapy. Interferon monotherapy does not appear to have an effect on recurrence-free survival but has been shown to reduce progression to a higher grade or stage (31).

With the goal of reducing toxicity of BCG the immuno-modulator interferon-alpha has been studied in combination with dose-reduced BCG and as an enhancer of the immune response. A multicenter phase II trial led by the National BCG/Interferon Investigator group and published in 2006 studied a combination of BCG and INF-α, which was administered to patients who were BCG naïve or previously treated with one or two courses of BCG. All patients received 50 million units of IFN α-2b and BCG dosage was reduced in those previously treated with BCG or in those patients deemed BCG intolerant. Inclusion criteria allowed for patients with primary or recurrent bladder cancer, previous intravesical chemotherapy and patients who recurred after treatment with BCG. Of all 1,007 patients at 24 months, 59% and 45% were free of disease in the BCG naïve and the BCG failure group, respectively. Patients who received three or more cycles of BCG had an inferior outcome suggesting they had resistant disease. Based on these results a third cycle of BCG is not warranted as a general rule of practice. Importantly the study did not prove an advantage of the combination treatment to BCG monotherapy. A recent phase III trial however, showed no benefit to the addition of interferon alpha in a BCG naïve population (32). Rosevear et al. reported in a subset analysis data on patients with CIS from the same phase II trial.

While initial response rates were similar, 24-month disease-free rates were 60% in BCG naïve patients and 57% or 23% after one or two cycles of previous BCG (33). The treatment response to the drug combination was also associated with a significantly shortened disease-free survival in patients who recurred <12 months following BCG treatment compared to patients who recurred after >12 months. In practice many clinicians may consider adding INF-α in the setting of a relapse after 12 months or at the time of a second BCG induction course if there is persistent disease after the first 6-week induction course.

Checkpoint inhibitors

The class of immunotherapy drugs under the common term checkpoint inhibitors refers to monoclonal antibodies, which block the pathways of CTLA-4 (cytotoxic T lymphocyte antigen-4) or programmed cell death protein 1 (PD-1/PD-L1) (34). PD1 and CTLA-4 are negative regulators of the T cell activity and their expression may be up-regulated in the context of bladder cancer. Checkpoint blockade can reverse this suppression and enhance anti-tumor T cell activity. A single-arm phase II trial is planned to assess the efficacy of first-line gemcitabine—cisplatin with ipilimumab for metastatic urothelial cancer of the bladder (NCT01524991).

Nivolumab, an anti PD-1 antibody, is currently being evaluated in a phase II clinical trial to evaluate the efficacy of a combined treatment with nivolumab plus/minus ipilimumab in advanced or metastatic bladder cancer (NCT01928394).

While these drugs essentially enhance the T-cell response of cytotoxic CD8+ T-cells they may offer an opportunity to modulate T-cell response with BCG immunotherapy as well. Trials are planned to evaluate checkpoint inhibition in combination with BCG and as monotherapy for patients for whom BCG is no longer a treatment option (BCG unresponsive).

Mycobacterium cell wall-DNA complex

The treatment with an intravesical mycobacterium cell wall-DNA complex (MCNA) was first reported by Morales et al. in 2009 after treatment of 55 consecutive mostly BCG refractory patients as an alternative to BCG. The study was the first to report treatment efficacy as well as safety of the drug, which is formulated as an emulsion and diluted in saline for an intravesical administration. With a dose of

8 mg 46.4 % of patients had a CR at 12 and 26 weeks (35). Adverse events were mild to moderate in 90% of patients.

MCNA is believed to activate cytokine induction similar to BCG. More recently Morales *et al.* published data from an open label study with 129 patients from 25 study sites after treatment failure with BCG. The dose regimen was 8 mg of MCNA and treatments were given on a 2-year maintenance schedule similar to BCG. The overall disease-free survival rate was 25% after 1 year and 19% after 2 years but the best outcome was observed in patients with papillary disease only (36).

Valrubicin

Valrubicin is the only FDA approved therapy for patients with BCG refractory CIS or for patients who are intolerant. The drug was approved in 1998 in patients in whom "cystectomy would be associated with unacceptable morbidity and mortality".

In a phase II/III open-label study valrubicin was administered in 6 or 9 weekly instillation of 800 mg to patients with CIS (37). All patients had received at least one course of BCG and were either intolerant or BCG refractory. Thirty-five percent had no evidence of disease at the first control at 3 months, which included cystoscopy, biopsy and cytology. A positive cytology was allowed. A complete remission at 6 months was achieved in 18% of patients but the 2-year disease free probability was only 4% and 25% of patients underwent radical cystectomy as definitive treatment.

Gemcitabine

Several Phase I and Phase II studies indicate both the safety and potential efficacy of intravesical Gemcitabine. Dalbagni *et al.* found that the majority of high-risk patients who initially responded at 3 months had recurred by 12 months with a 1-year RFS of 21% (38). This suggests a potential role for maintenance gemcitabine therapy. The cost of this regimen is very high at about $1,000 per dose though gemcitabine is now off patent and the cost is likely to decrease. A prospective randomized phase II trial compared gemcitabine versus BCG in 80 patients with persistent disease after one BCG induction course. 2-year RFS were 19% and 3% respectively (39). The SWOG S0353 phase II trial investigated the role of gemcitabine in patients with NMIBC (intermediate and high-risk) after two prior courses of BCG (40). The treatment schedule included a 6 weeks induction treatment and monthly maintenance treatments up to 12 months. Forty-seven percent of patients responded at 3 months and 28% were free of recurrence at 1 year and 21% after 2 years. The response rates are similar to Valrubicin suggesting that this is an additional treatment option for patients unfit for major surgery.

Mitomycin

Intravesical mitomycin has been shown to have a higher efficacy when administered under optimized conditions as shown in a randomized phase III clinical trial. In the optimized arm, the dose and concentration were doubled (40 mg/20 cc), bicarbonate was given the evening and morning of treatment as MMC uptake in tissue is optimized under alkaline conditions. Patients were kept NPO in order to limit urine production and dilution of mitomycin. The bladder was scanned after catheter placement in order to minimize residual urine. Patients treated in the optimized were compared to patients treated with 20 mg/20 cc and no pharmacologic optimization demonstrated a longer median time to recurrence with 29 months versus 12 months for patients in the standard treatment arm (41).

Another promising approach has been device-assisted therapy for intravesical mitomycin using induced hyperthermia. Alfred Witjes *et al.* reported a response rate of 92% in patients with CIS (42). The series included BCG naive patients as well as patients with persistent or recurrent disease after previous BCG therapy but resulted in durable response rates which were around 50% after 2 years regardless of any previous BCG treatment. Side effects were transient and most commonly reported as pain and bladder spasms in about 13% of patients. Another report from Colombo *et al.* prospectively evaluated the response rate in patients with intermediate to high-risk bladder cancer in patients receiving thermo-chemotherapy versus chemotherapy alone (43). This study also included both BCG naïve as well as BCG pretreated patients. All patients received 8 weekly followed by 4 monthly treatments. The 10-year disease free survival rates were 53% and 15% respectively. Interestingly a history of multifocal tumor occurrence was associated with a reduction of disease-free survival only in patients treated with mitomycin alone while it had no bearing on outcome in patients treated with thermochemotherapy. The technology is not approved yet in the United States.

The benefit of intravesical electromotive drug administration (EMDA) of mitomycin has been recently reported from a prospective randomized clinical trial in

the setting of a preoperative instillation prior to TURBT. The trial randomized 374 patients to mitomycin by passive instillation, EMDA or no mitomycin. EMDA mitomycin was administered preoperatively before the TURBT. Ninety-seven patients of 374 patients had high-grade tumors. The majority of patients had low or intermediate risk tumors based on the EORTC calculator. The disease-free interval for was significantly longer in patients treated with EMDA with 52 months versus 16 and 12 months after postoperative mitomycin or TURBT alone respectively. These effects were sustained when the disease-free interval was stratified by risk category with a significant benefit for multifocal high-risk disease (44).

Doublet intravesical chemotherapy

Lightfoot *et al.* reported the combination of gemcitabine and mitomycin as a sequential treatment retrospective case review (45). Forty-seven patients who were unfit or unwilling to undergo cystectomy were included in the analysis. Thirty-six patients had undergone previous BCG treatment (one or two cycles). The sequential regimen consisted of the instillation of one gram of gemcitabine with subsequent bladder drainage followed by instillation of 40 mg of mitomycin. All patients received a 6-week induction course and 1 year of monthly maintenance treatments. Response rates were directly correlated with the number of prior BCG treatments. Patients who were BCG refractory had a CR rate of 69%. The recurrence-free survival was 50% and 32% after 1 and 2 years of treatment. The retrospective nature and heterogeneous patient population do not allow for management recommendations but the concept of combining treatment modalities with different targets is an excellent opportunity for future drug trials. A new study from the University of Iowa using the doublet gemcitabine and docetaxel was recently reported in Bladder Cancer and found treatment success of 32% at 2 years.

Docetaxel

Docetaxel is a taxane and used as a chemotherapy agent in metastatic prostate and breast cancer. McKiernan *et al.* have previously demonstrated that it can be safely used in intravesical therapy and showed a 56% response rate in patients with BCG refractory disease (46). More recently Barlow *et al.* presented results from a single center analysis of 54 patients who had failed at least one course of prior

BCG with or without interferon (47). After the first induction cycle 59% of patients had a CR to docetaxel. One- and three-year recurrence-free survival rates were 40% and 25% respectively. The analysis did not reveal a benefit for a monthly maintenance schedule for up to 9 months after the first three months control.

Abraxane

A recently published phase II trial evaluated treatment response to nanoparticle albumin-bound paclitaxel (Abraxane®). The 28 patients who were enrolled had recurrent high-grade disease after at least on cycle of prior intravesical treatment BCG + intravesical chemotherapy and refused or were unfit for cystectomy. Nab-paclitaxel was administered weekly for 6 weeks and after CR continued on a monthly maintenance schedule for a total of 6 months. Initial response rate was 35.7% at 6 weeks defined as a negative biopsy and cytology. Recurrence-free survival was 30.6% at 2 years of follow-up (48).

Gene therapy

The basis of gene therapy relies on viral or non-viral delivery systems that can safely transduce the urothelium at high efficiency. Vectors can deliver genes of various sizes depending on the packaging systems. One immunotherapy approach is based on induction of high levels of interferon-alpha endogenously secreted from transduced urothelial and bladder tumor cells. The technique relies on utilization of an adenoviral vector adenovirus (Ad)-IFN-α encoding for a secreted gene product of interferon-alpha and therefore bypassing the requirement to transduce every tumor cell (49). Syn3 is used as an excipient to overcome the lack of the coxsackie and adenovirus receptor (CAR), which is frequently absent in urothelial cancer. An ongoing phase II clinical trial is evaluating this treatment with the primary endpoint of prevention of high-grade recurrence in a BCG refractory patient population (NCT01687244). This trial has completed accrual and if positive will be followed with a single arm Phase II registration trial.

The adenovirus vector CG0070 is a replication competent adenovirus, which stimulates production of GM-CSF, is currently studied in a phase II clinical trial (NCT01438112). The treatment concept is based on enhancing the immune response by stimulating dendritic and effector cells.

Targeted therapies

The Cancer Genome Atlas Project has reported a comprehensive integrated analysis of genomic data from muscle-invasive bladder tumors (50). Twelve percent of bladder tumors had FGFR3 mutations, which affected kinase-activating sites. Activating point mutations of FGFR3 occur in up to 80% of patients with noninvasive tumors suggesting this as a rationale target. A current phase II trial of the receptor tyrosine kinase inhibitor dovitinib is directed at patients with BCG-refractory NMIBC with FGFR3 mutations or overexpression (NCT01732107).

Over 40% of urothelial cancers of the bladder have been shown to have alterations in the PI3K/AKT/mTOR pathway (51). The mTOR pathway acts downstream of the PI3K pathway and regulates metabolism of the cell promoting growth and cell proliferation. The mTOR inhibitor everolimus is being evaluated in a phase I/II trial in combination with intravesical gemcitabine for the management of patients with BCG refractory bladder cancer (NCT01259063).

Trial design for registration trials

In 2013 the AUA and the FDA held a workshop on trial design for patients with non-muscle-invasive bladder. There was consensus that clinical trials should include high-grade papillary disease and CIS. The meeting also addressed the challenges of defining standardized endpoints in the setting of NMIBC. Uniformly this should include:

   (I)   Failure to achieve a CR in patients with CIS;
   (II) Recurrence with CIS or papillary high-grade disease.

The panel recommended a definition of a successful treatment as a 40-50% response rate at 6 months and durable response rate of 30% at 18-24 months. Moreover there was consensus that any clinical trial with BCG refractory patients should not be placebo controlled as the panel felt this was unethical due to the aggressive nature of the disease. On the contrary placebo controlled trials may be feasible for low-grade disease or perioperative intravesical chemotherapy instillations (15).

Conclusions

BCG failure is one of the most complex and challenging scenarios in urologic oncology. It is imperative for the clinician to have a clear understanding of the indications for BCG treatment and the definition of BCG failure.

The utilization of drug combinations and device-assisted treatments has the potential to improve treatment response. Inhibition of immune checkpoints and targeted therapy addressing central pathways of cell regulation are the subject of multiple clinical trials and could help to define new treatment algorithms. Patients who have failed BCG treatment are at a high risk for disease progression and a definitive treatment with cystectomy should be offered. The hopefully transient shortage of BCG may become a recurring problem in the future. It is imperative for any urologist to be aware of the potential alternatives and their limitations.

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Footnote

Conflicts of Interest: Seth P. Lerner has affiliations (grants) with Imalux, Photocure, Tengion, and FKD, and serves as an advisory board member/consultant for Genentech, Biocancell, Vaxxion, Theracoat, Dendreon, Sitka, and Nucleix.

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TGF-β mediated DNA methylation in prostate cancer

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Abstract: Almost all tumors harbor a defective negative feedback loop of signaling by transforming growth factor-β (TGF-β). Epigenetic mechanisms of gene regulation, including DNA methylation, are fundamental to normal cellular function and also play a major role in carcinogenesis. Recent evidence demonstrated that TGF-β signaling mediates cancer development and progression. Many key events in TGF-β signaling in cancer included auto-induction of TGF-β1 and increased expression of DNA methyltransferases (DNMTs), suggesting that DNA methylation plays a significant role in cancer development and progression. In this review, we performed an extensive survey of the literature linking TGF-β signaling to DNA methylation in prostate cancer. It appeared that almost all DNA methylated genes detected in prostate cancer are directly or indirectly related to TGF-β signaling. This knowledge has provided a basis for our future directions of prostate cancer research and strategies for prevention and therapy for prostate cancer.

Keywords: TGF-β; DNA methylation; prostate cancer; DNMT; Erk activation; tumor development and progression

Introduction

The underlying mechanism promoting tumor progression has been elusive. Almost all tumors harbor a defective negative feedback loop of signaling by transforming growth factor-β (TGF-β). TGF-β signaling consists of Smad and non-Smad pathways (1). In advanced cancer cells, the non-Smad pathways predominate and progress leading to deregulated signaling cascades (2). This deregulation creates a unique TGF-β mediated tumor microenvironment that sets off a vicious cycle and promotes many of the hallmarks of tumor progression, including sustained angiogenesis, immune system evasion, proliferation, loss of the apoptotic response, epithelial-to-mesenchymal transition (EMT) and metastasis. These combined effects lead to uncontrolled tumor growth and spread, for which we coin the term "TGF-β mediated vicious cycle in tumor progression".

Recent evidence demonstrated that TGF-β mediates aggressive cancer including auto-induction of TGF-β1 and increased expression of DNA methyltransferases (DNMTs) (2,3). This latter observation suggests that the expression of these methylated genes may be an important event in TGF-β mediated tumor progression.

DNA methylation in cancer

Epigenetic changes are characteristic of nearly all malignancies and include changes in DNA methylation, histone modification and altered expression of microRNAs. DNA methylation plays a critical role in cancer development and progression. Alteration of DNA methylation patterns leads to deregulation of gene expression, in the absence of mutation. In the past few years, there has been an explosion in the number of publications in DNA methylation in all types of cancers

(900 papers as of March 2012), including representative publications in prostate cancer (4-7), bladder cancer (8), renal cell carcinoma (9), breast cancer (10), lung cancer (11), ovarian cancer (12), oral cancer (13), pancreatic cancer (14), and other cancers. All tumors that have been examined show changes in DNA methylation, suggesting that this may represent a basic element of cancer biology, which has a significant impact on tumor pathology. Readers are referred to many excellent reviews on the biology of DNA methylation (15-17). This increased interest in the study of DNA methylation has created an opportunity for us to query the relationship between TGF-β signaling and DNA methylation in cancer, which has not been appreciated to date.

Biology of TGF-β signaling

TGF-β is a potent pleiotropic cytokine that regulates mammalian development, differentiation, and homeostasis in essentially all cell types and tissues. Its signaling is mediated through Smad and non-Smad pathways to regulate transcription, translation, microRNA biogenesis, protein synthesis and post-translational modifications (1,18,19). TGF-β binds to the type II TGF-β receptor (TβRII) which recruits and transphosphorylates the type I TGF-β receptor (TβRI) (20). The activated TβRI then phosphorylates Smad2 and Smad3 at the c-terminus. Activated Smad2/3 forms heterooligomers with Smad4 and migrates to the nucleus to regulate transcription. The Smad complexes interact with a myriad of transcriptional co-regulators and other factors to mediate target gene expression or repression (21,22). Smad2/3 also interacts with and regulates microRNA processing. TGF-β also signals through a number of non-Samd pathways, including m-TOR, RhoA, Ras, MAPK, PI3K/AKT, PP2A/p70s6K, and JNK (1,23,24). Finally, a direct action of the activated TβRI can interact with eEF1A1 to block protein synthesis (19). Dysregulation of both Smad and non-Smad pathways is implicated in aberrant TGF-β signaling and its pro-tumorigenic events in advanced cancer (3).

TGF-β signaling and DNA methylation

TGF-β is a key regulator for DNA methylation through an increase in DNMTs expression, especially in cancer (3,12). There exists a differential effect of TGF-β mediated DNMT activities between benign and malignant cells. In benign cells, TGF-β inhibits DNMT expression (25,26). In cancer cells, TGF-β stimulates DNMT expression (3,12). It should be noted that, in light of the importance of both TGF-β signaling

and DNA methylation in tumor progression, the majority of the methylated genes in cancer are relevant to TGF-β signaling (12). This is consistent with our observation that over-expression of TGF-β and/or DNMTs is associated with aggressiveness and poor prognosis in prostate cancer (3,27).

Review of literature

In this review, we will focus our discussion in prostate cancer as an example, because the pattern of DNA methylation is organ specific. We surveyed the recent literature to identify the existing methylated genes in prostate cancer and attempt to determine which ones are mediated by TGF-β signaling. We have identified over 80 genes in which promoters are methylated in prostate cancer. This is a significant increase from 2006, when only 30 genes had been identified (28). Interestingly, the non-Smad pathways of known relevance to TGF-β are more often associated with de novo gene methylation (3,29). In contrast, the Smad-mediated pathways often lead to promoter de-methylation of genes (see below). In *Table 1*, we summarize the known TGF-β relevant genes in which the promoter becomes methylated in prostate cancer. We also identified those which have been known to be induced by TGF-β. Since, in advanced cancer cells, TGF-β induces the activation of Erk, JNK, AKT, and NF-κB (1,3), the above methylated gene have been documented in the literature to be related with one of the above transcription factors, thus are considered as TGF-β relevant.

In addition, there are a few genes that are de-methylated and are mediated through Smad2/3 activation, such as α2 [1] collagen (113), CD133 (26), and maspin (or SFN, 14-3-3 sigma) (41,59,67,114,115). However, a reversal of the methylation status in these genes can be observed in cancer cells when the TGF-β signaling events switched from the Smad pathways to the non-Smad pathways in cancer cells as in the case for maspin (116) and CD133 (117).

Table 2 lists genes that are not currently documented in the literature as TGF-β relevant. However, TGF-β mediates an over-expression of DNMTs in cancer cells, which is responsible for promoter methylation of these genes and. in non-cancer cells, TGF-β down-regulates the expression of DNMTs (25,26).

DNA methylation associated with tumor initiation and progression

A characteristic of DNA methylation in cancer is its

Table 1 Genes with known association with TGF-β that have DNA hypermethylation in prostate cancer

Name	Function	Reference
1. TBRI	TGF-β receptor type I	(30,31)
2. TBRIITGF-β	TGF-β receptor type II	(31,32)
3. cdh13herin	Adhesion molecule, tumor suppressor	(33,34)
4. TTP (tristetrapolin)	Loss of TTP stabilizes c-Myc mRNA	(35)
5. TGFBI (Betaig-h3)	TGF-β induced gene	(36-38)
6. IGFBP3	IGF binding protein 3	(39,40)
7. beta 4-integrin	Promotes focal adhesion	(34)
8. MAL	Promotes cell differentiation	(41,42)
9. SLIT2	Negative regulation of migration	(36,41,43)
10. Bcl2	Involved in apoptosis	(40,41)
11. Caspase 8	Pro-apoptotic gene	(44)
12. EPHA7	Tumor suppressor in prostate cancer	(45-47)
13. BTG3	Tumor suppressor	(48,49)
14. PTGS2	Pro-inflammatory enzyme	(50-52)
15. HIN1 (or SCGB3A1)	Tumor suppressor	(41,53)
16. RASSF1A	Tumor suppressor gene	(54-56)
17. CHD13	Adhesion molecule	(41,57,58)
18. p15, p16, p21, p27, p57	Cell cycle regulators	(57,59-61)
19. RASSF1A	Pro-apoptotic, negative Ras effector	(41,62)
20. TWIST1	Suppressor of E-cadherin	(41)
21. FHIT	Induces apoptosis though Bak	(63,64)
22. SOCS3	Negative regulator of cytokine	(65,66)
23. TIMP-2, TIMP-3	Inhibitors of metalloproteinase	(67-69)
24. PITX2	Activator of cyclin D2	(41,70-72)
25. DcR1, DcR2	Fail to induced apoptosis through TRAIL	(73,74)
26. GLIPR1 (or RTVP-1)	p53 target gene	(75,76)
27. MGMT	DNA repair gene	(77-81)
28. DKK3 (SFRP1)	Wnt antagonist	(82,83)
29. RUNX3	Tumor suppressor	(84-86)
30. CAV-1	Tumor suppressor	(87,88)
31. Clusterin	Apoptotic protein	(89-91)
32. TFPI2 (PP5, MSPI)	A potent inhibitor of matrix-metalloproteinases	(92,93)
33. SOX7	Suppressor of β-catenin	(94,95)
34. SLC5A8	Tumor suppressor	(96,97)
35. SLC18A2 (or VMAT2)	Affects apoptosis and migration	(98,99)
36. LPL	Tumor suppressor gene	(100,101)
37. HRK (or ATF-2)	Proapoptosis	(102,103)
38. INHBB	Inhibin betaB	(104,105)
39. ID4	Inhibitor of DNA binding	(41,106-108)
40. FYN	Promotes proliferation and motility	(109,110)
41. HPP1 (TMEFF2)	TGF-β signal pathway	(73,84)
42. RRAD	Ras-related GTPases	(111,112)
43. DRM/Gremlin	Down-regulated in Mos-transformed cells	(73,84)

Table 2 Methylated genes in prostate cancer whose regulation by TGF-β is not yet known

Name	Function	Reference
1. HLAa	HLA class-I antigen	(41)
2. ERβ	Estrogen receptor	(67)
3. ERα	Estrogen reeptor	(67)
4. AR	Androgen receptor	(67)
5. RARβ	Tumor suppressor	(67)
6. DAPK1	Regulate cell death	(118)
7. MDR1	Multi-drug resistant gene	(41,119)
8. APC	Antagonist of Wnt	(41,119-121)
9. CD44	Cell migration and adhesion	(52,57)
10. MCAM (MUC18, CD146)	In advanced PCa	(41,122)
11. TIG1	Retinoic acid receptor responder	(41,123)
12. THRB	Thyroid hormone receptor B	(41)
13. Laminin-5	Role in adhesion and motility	(124)
14. WIF1	Wnt inhibitory factor	(125-127)
15. TSLC1	Tumor suppressor	(128)
16. RIZ1	Rb-interacting zinc finger gene 1	(73,129)
17. Cyclin D2 (or CCND2)	Regulate cell cycle	(54,67,130)
18. GSTP1	Cell detoxification	(4,7,121,131)
19. PDLIM4	Actin binding protein, tumor suppressor	(41,132)
20. Sprouty1	negative regulators of MAPK/PI3K	(133)
21. ZNF331	Tumor suppressor	(134)
22. TMS1(ASC, PYCARD)	Induces apoptosis by caspase	(57,73,135)
23. GPX3	Anti-oxidant	(82,119)
24. NKX2.5	Repress calreticulin expression	(41)
25. NKX3.1	Promotes normal differentiation	(136)
26. DPYS	Sensitivity to 5-FU	(41,137)
27. ENDRB	Endothelin receptor type B	(5,41)
28. CADM2	Cell adhesion molecule	(138)
29. XAF1	Interference with caspase inhibition of XIAP	(139-141)
30. CRBP1	Cellular retinol binding protein, promotes apoptosis	(73,142)
31. FAS (TNFRSF6, APT1, CD95/Apo-1)	Induces apoptosis	(143)
32. RPRM	Inhibits Cdc2-cyclin b1 activity	(73,123)
33. GSTM1	Detoxification	(82)
34. EPB41L3	Erythrocyte membrane protein band 4.1-like 3	(28)
35. SCTR	Gene encoding the secretin receptor	(105)
36. SOCS1	Negative regulator of cytokine	(73,84)
37. HIC	Tumor suppressor	(79,81)

heterogeneity. Despite of this variation, some trends can be discerned. We rationalize that genes that are wildly methylated are likely involved during early stages of tumor development, such as GSTP-1 (4), which may be used for the early detection of prostate cancer. Many investigators have used specific methylation pattern for prediction of cancer progression. However, during progression of prostate cancer, the tumor becomes increasingly heterogeneous, it will be difficult to pinpoint which genes are methylated that can be used as a prognostic marker and such efforts have been met with mixed results (144). It is reasonable to assume that as tumors progress, there will be more genes that undergo promoter methylation and demethylation. Therefore, the development of a rapid analysis of DNA methylation profile make it possible to follow the methylation patterns which may be used as an indication of disease progression.

Conclusions and future directions

Based on the present review, it is apparent that TGF-β signaling and DNA methylation are two important events in prostate cancer development and progression. In tumor progression, the deregulated TGF-β signaling mediates an increase in the number of genes undergoing DNA hypermethylation. These genes are generally associated with prevention of apoptosis, promotion of proliferation, facilitation of cell migration and evasion of the immune surveillance, resulting in tumor progression. In the era of personalized medicine, it becomes more important that we clearly define which genes are affected by TGF-β signaling and which genes are promoter hypermethylated during prostate cancer progression. Recent reports point out that some dietary and lifestyle interventions in cancer patients are mainly mediated through a reduction in DNA methylation (125,145,146), while others may lead to both gains and losses (147). It is possible that these dietary and lifestyle factors may be mediated at least partly through a normalization of the vicious cycle of TGF-β signaling in cancer microenvironment (148).

Acknowledgements

Footnote

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146

Lee et al. TGF-β mediated DNA methylation in prostate cancer

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Somatostatin receptors over-expression in castration resistant prostate cancer detected by PET/CT: preliminary report of in six patients

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Abstract: Prostate cancer (PC) is usually characterized by an excellent prognosis, largely due to little biological aggressiveness and the power of hormonal deprivation therapy. In spite of these favorable characteristics, however, a significant quota of patients does not respond to androgen deprivation therapy (ADT) and develop a progressive disease. Castration-resistant prostate cancer (CRPC) is defined by disease progression in spite of ADT. This progression may show any combination of a rise in serum prostate-specific antigen (PSA), clinical and radiological progression of pre-existing disease, and appearance of new metastases. This event is a striking change in the clinical scenario, since the power of treatment for CRPC patients with distant metastases is very limited. Somatostatin is a hormone produced by neuroendocrine cells. Its distant effects are mediated by the binding to five specific receptors, which are the most striking parameter for neuroendocrine. Various synthetic somatostatin agonists able to bind to the receptors have been synthesized during the past two decades for diagnostic and therapeutic purposes. Octreotide, the most popular of these, is widely used to treat patients affected by neuroendocrine tumors. A number of researches carried out in the past evaluated the possible neuroendocrine differentiation (NED) of PC cells in the castration resistant phase. If proved, the presence of a specific class of receptor on cell's surfaces should give a potentially biological target to be used for therapy. However, these studies led to contradictory results. Aim of our phase III diagnostic trial was to study "*in vivo*" the over-expression of somatostatin receptors (SSTRs) in CRPC patients by PET/CT after the administration of the somatostatin analog [^{68}Ga-DOTANOC,1-Nal(3)]-octreotide labeled with ^{68}Ga. Every area of increased uptake corresponding to a metastasis detected with other methods was considered as SSTRs expressing. False positivity to SSTRs expression was considered those localizations with a suspicious uptake not confirmed by other radiologic procedures. On the other hand, metastatic lesions lacking the radiopharmaceutical's uptake were considered not SSTRs expressing metastases. The preliminary results in 6 of the 67 patients scheduled by our phase III trial showed metastases with a variable SSTRs expression in 2 patients.

Keywords: Castration resistant prostate cancer (CRPC); neuroendocrine differentiation (NED); somatostatin receptors (SSTRs); ^{68}Ga-DOTANOC; PET/CT

Introduction

The meaning of neuroendocrine differentiation (NED) and somatostatin receptors (SSTRs) expression in castration resistant prostate cancer (CRPC) (1,2) has still not an established meaning (3-5). On the other hand, the possible presence of SSTRs on CRPC cell surface should provide a good therapeutic chance in patients with few treatment options to the oncologist. Serum chromogranin A (CgA) test has been the main surrogate parameter to define the SSTRs positivity. However, serum CgA raise in serum suffers of a low specificity (6-8), and moreover, it is not synonymous of SSTR expression (9). In summary, the raise of CgA does not guarantee the SSTR expression. Thus, a reliable and easy to use method to study *in vivo* SSTR presence should help significantly.

^{68}Ga-DOTANOC is a radiopharmaceutical analog for SSTR2, SSTR3 and SSTR5, usually employed to image the neuroendocrine carcinomas (NET) with PET/CT. Aim of our phase IIIA trial to the study of CRPC is to detect their SSTRs overexpression by [68]GA-DOTANOC PET/CT. Here we present the preliminary results of the first six patients recruited.

Material and methods

Six patients with CRPC were enrolled in this phase III trial (EUDRA CT number 2010-021026-35) granted by Regione Lombardia. The local Ethical Committee approved the trial design. All the patients gave their written informed consent before the enrollment in the study.

CRPC was defined a rise in serum prostate-specific antigen (PSA) and/or progression of pre-existing disease and/or appearance of new metastases despite androgen deprivation therapy (ADT). Basically, our recruitment criteria encompassed: (I) asymptomatic non-metastatic CRPC; (II) asymptomatic metastatic CRPC with prior treatments; (III) symptomatic, metastatic CRPC with prior treatments. For the first group of patients, the PSA was in unremitting raise for more than three consecutive evaluations during ADT. No measurable lesions, KPS >80%, life expectancy >3 months, good hematologic parameters and a wash-out time from the last chemotherapy of at least one month were requested. The main exclusion criterion was age (less than 18 and more than 85 years old).

One week after the patients were declared eligible for the trial, they were admitted to our hospital.

PET/CT was carried one hour after the i.v. administration of nearly 185 MBq of ^{68}Ga-DOTANOC, synthesized following the procedures reported in the literature (10). PET/CT scan was carried out with a Siemens Biograph 6 PET/CT scanner (Siemens Healthcare, Italia), and the acquisition parameters for the CT were: kV =130; effective mAs =70; maximum reconstructed width =5 mm without overlap; pitch 1.5 mm; standard reconstruction algorithm. PET was performed from the lower thighs, with 6 bed positions (3 min per bed) and reconstructed using standard algorithms provided by Siemens.

Following our trial's specifications, only one ^{68}Ga-DOTANOC PET/CT examination was carried out in these patients.

Results

The main clinical characteristics of the six patients at diagnosis of PC are summarized in *Table 1*. The main clinical characteristics of the six patients at the onset of castration resistance are summarized in *Table 2*.

The five patients with bone metastases had diffuse multiple localization patterns at CT and bone scan. One of these had also an impressive lung involvement with

Table 1 Main clinical characteristics of the patients enrolled in our study at diagnosis

Patients	Age	Gleason Score	PSA	M+	Surgery	EBRT	Adjuvant therapy
1	47	4+3	870	Bone	Yes	No	ADT, bisphosphonate
2	61	4+5	2.3	None	No	Yes	None
3	61	4+4	5.9	None	No	Yes	ADT, bisphosphonate
4	71	4+5	24	Bone	No	No	ADT, bisphosphonate
5	59	4+4	3.4	Bone	No	No	ADT, bisphosphonate
6	55	4+4	407	Bone	No	No	ADT, bisphosphonate

PSA, prostate-specific antigen; M+, synchronous metastases; EBRT, external-beam radiation therapy; ADT, androgen deprivation therapy.

Table 2 Main clinical characteristics of the patients enrolled in our study at castration resistance onset

Patients	Age	Recruitment criterion	PSA (ng/mL)	CgA (ng/mL)	68GA-DOTANOC uptake
1	49	Multiple bone metastases	5	289	Bone
2	61	Multiple bone metastases	20	13	Negative
3	61	Raising PSA	21	NA	Negative
4	71	Multiple bone metastases	246	NA	Negative
5	59	Bone metastases + lung lymphangitis	4	1, 5	Bone, lung
6	55	Multiple bone metastases	5	2,400	Negative
PSA, prostate-specific antigen; CgA, chromogranin A.					
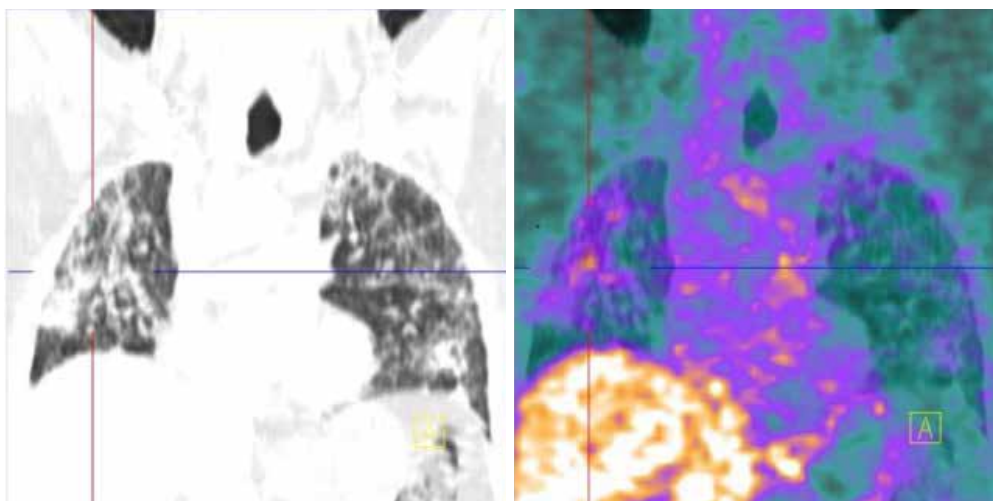
Figure 1 Coronal view of a ^{68}Ga-DOTANOC PET/CT scan of patient 5. Irregular shaped areas of mild increase uptake are clearly visible in both lungs corresponding to septal thickening due to lymphangitic spread of the disease (localizer).

lymphangitic carcinomatosis at CT scan.

^{68}Ga-DOTANOC PET/CT was positive in two patients. In patient 5 some areas of uptake were detected in both lungs in areas of irregular septal thickening, consistent with the lymphangitic spread (*Figure 1*) and in bone metastases previously evidenced by CT scan.

The second patient positive to ^{68}Ga-DOTANOC PET/CT (patient 1) had multiple bone metastases detected at the diagnosis carried out one year before. The examination showed multiple areas of radiopharmaceutical's uptake (*Figure 2*).

Figure 3 shows the negative pattern of ^{68}Ga-DOTANOC uptake in patient 3, and compared it with the findings of ^{18}F-Choline PET/CT. This patient was the only patient without already known parenchymal metastases at the enrollment. His recent pathological anamnesis clued of a hormonal recurrence. ^{18}F-Choline PET/CT carried

out to restage him detected a focal uptake in the left side of the prostate (*Figure 3A*). However, this finding was not considered diagnostic due to the occasional and unpredictable uptake of the radiopharmaceutical in non prostatectomized patients, Thus, the nodule was not studied by biopsy and a wait and see strategy was decided. Two months later the patient was recruited due to the continuous raise of PSA in spite of ADT. ^{68}Ga-DOTANOC PET/CT did not disclose any uptake in the whole of body. More in detail, no specific uptake in the area corresponding to the previous ^{18}F-Choline PET/CT was detected (*Figure 3B*). One more time, a wait and see strategy was adopted. The ^{18}F-Choline PET/CT carried out four months later, confirmed and reinforced the finding of the first similar examination showing an increased uptake in the left side of the prostate gland (*Figure 3C*). This final evidence was considered suffices for the diagnosis, no biopsy was decided
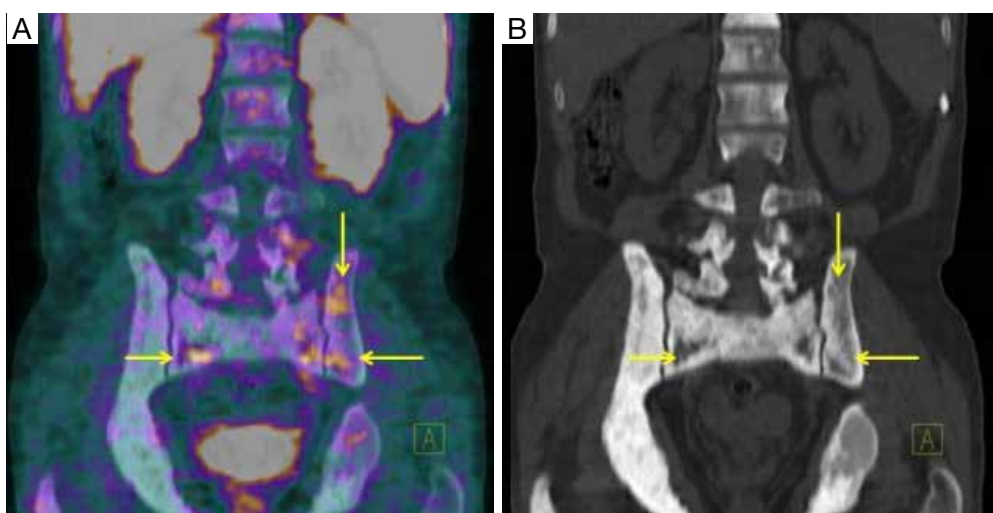
Figure 2 Coronal view of a ^{68}Ga-DOTANOC PET/CT scan in patient 1. Areas of increase uptake (yellow arrows) are clearly visible on fused PET/CT (A) in the sacrum and left iliac wing, corresponding to skeletal spread with a prevalently lytic component on co-registered CT (B). The high uptake of the kidneys is illustrative of the SUV scale which needs to be stressed to highlight the low SSTRs expression of the metastases.
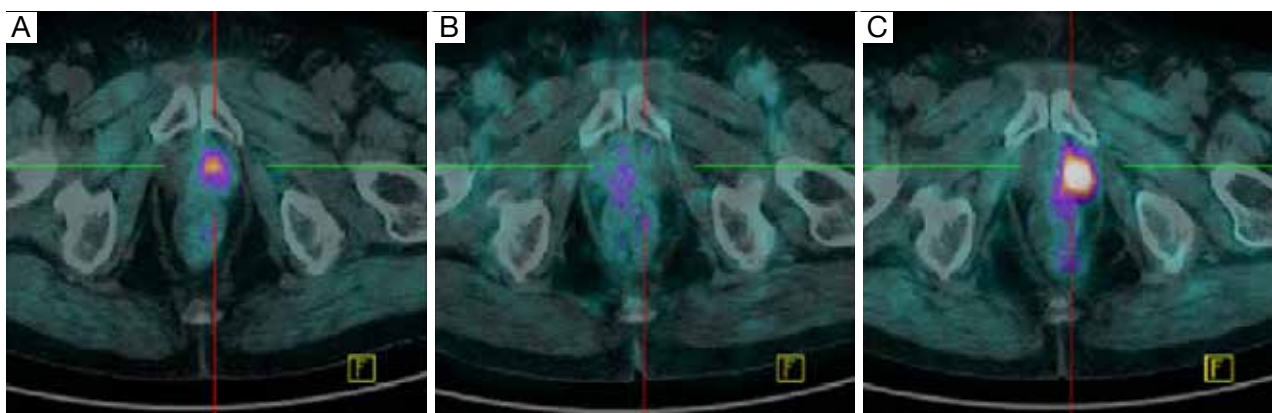
Figure 3 Transaxial prostate view of a ^{18}F-Choline (A, at diagnosis), ^{68}Ga-DOTANOC (B), and ^{18}F-Choline (C, at follow up) PET/CT (patient 3). The follow-up ^{18}F-Choline PET/CT shows an increase of the uptake compared to the same examination carried out at diagnosis, confirming the site of relapse. No uptake is present in ^{68}Ga-DOTANOC PET/CT.

and the patient started abiraterone acetate treatment. Therefore, in this case ^{68}Ga-DOTANOC PET/CT did not disclosed SSTRs in the relapse.

Discussion

The presence of SSTR in PC is still not completely understood. Some studies suggests that SSTR2 are overexpressed in CRPC (11,12) whereas others disagree with this finding (13-15). These contradictory results

perhaps reflect the pattern of receptor expression, which are probably different in the primary compared to the metastatic disease.

The main incentive to study and quantify SSTRs in CRPC is to evaluate the possibility to use them as a therapeutic target with somatostatin analogs. Therefore, we did not pursue a diagnostic objective i.e., we did not look for metastases. Indeed, their presence was one of the inclusion criteria of our trial. In this regard, the terms true positive or false positive lose their emphasis. Indeed, of the

pivotal point of our examination shifted from the detection of possible, but still not proved, metastases (typical of the "pure" diagnostic approach) towards the description of the receptorial panel of the widespread secondary. In conclusion, the question to answer with ^{68}Ga-DOTANOC is: do these neoplasms overexpress SSTRs? And in how many of the metastases show significant SSTRs expression? Could this information be worth for therapeutic purposes?

Some researchers treated patients affected by CRPC on the base of serum CgA level taken as a surrogate marker of SSTRs expressions. However, serum CgA elevation is not a synonymous of this biological behavior (16-18). A possible comment is this approach, "blind" to the effective expression of the receptors, may partly explain the poor response to somatostatin analogs.

Nuclear Medicine procedures with gamma emitting radiopharmaceuticals have been occasionally employed in the past to detect SSTR overexpression in PC (19,20). In the last few years, newer PET/CT radiopharmaceuticals have been synthesized. These are ^{68}Ga-DOTATOC, ^{68}Ga-DOTANOC and ^{68}Ga-DOTATATE, three almost similar compounds with only slight differences in chemical structure and receptor's affinity. In 2010, the first study to evidence SSTRs overexpression in CRPC with ^{68}Ga-DOTATOC, revealed a weak uptake of the metastases. The researcher concluded suggesting the use of a radiopharmaceutical with different affinity for SSTRs (21). ^{68}Ga-DOTANOC differs with ^{68}Ga-DOTATOC in the amino acidic sequences. This change results in different receptors affinity, i.e., [68]GA-DOTANOC binds to SSTR types 2, 3 and 5, whereas ^{68}Ga-DOTATOC lacks of affinity for SSTR3. Thus, if some neoplasms overexpress SSTR3 in a significant quota, they may be imaged with ^{68}Ga-DOTANOC but not by ^{68}Ga-DOTATOC.

Our case series showed ^{68}Ga-DOTANOC uptake in two patients with skeletal and in lung metastases. The possible criticism is that the real correspondence between SSTRs expression evidenced by [68]GA-DOTANOC and real presence of SSTRs on cell surface may be reached only with tissue samples. In our opinion, however, the clinical, radiologic and biochemical scenario of our patients give strong evidences about the metastatic nature of skeletal and pulmonary changes. Moreover, the proposal to biopsy a suspected skeletal metastasis in a plural-treated patient in sharp clinical, laboratory and radiological disease progression should be criticized from the ethical point of view. Indeed, a biopsy to test SSTRs expression could be justified only if it results in a variation of the treatment, which is the aim of our study. The same criticism could hamper the definition of a control population. Our regulatory rules are intransigent in avoiding unnecessary radiation exposure in patients and healthy population. However, the experience of [68]GA-DOTANOC biodistribution in patients affected by in neuroendocrine tumors provided us useful information to define that skeletal and lung uptakes were abnormal. It goes without saying that the study of organs involved in ^{68}Ga-DOTANOC clearance (liver, kidneys) or physiologic uptake (spleen) is not possible. On the other hand, these anatomic districts are not preferential sites of CRPC metastatization. Finally, it must be stressed that unlike ^{18}F-FDG, ^{68}Ga-DOTANOC is not a "metabolic" tracer. Thus, its uptake is far more dependent by SSTRs overexpression than by the blood flow.

Unfortunately, [68]GA-DOTANOC uptake in CRPC (SUV mean 1.57) is scant if compared to neuroendocrine tumors. Probably this finding comes from they are not naïve neuroendocrine neoplasms thus process to express receptors is not fully accomplished.

We are unable to assess the prognostic significance of SSTRs overexpression. Surely, the paucity of their number hampers the use of similar radiolabeled compounds for treatment, as it comes for neuroendocrine tumors. Indeed, this kind of treatment calls for a higher tumor to background ratio to balance renal and hematological toxicity. Nevertheless, the clinical relevance of SSTRs overexpression should not be unacknowledged, particularly in those patients in which a significant amount of receptor is detected. The hypothesis to add somatostatin analogs to the usual therapeutic schedules as a complement to other pharmaceuticals could be considered especially in light of its low toxicity. The hart of the cultural leap is to start thinking this examination like a bridge between the diagnosis and therapy. In this setting, [68]GA-DOTANOC PET/CT may play a pivotal role.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Prostate cancer magnetic resonance imaging (MRI): multidisciplinary standpoint

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Abstract: Prostate cancer is the most common cancer diagnosed in men and a leading cause of death. Accurate assessment is a prerequisite for optimal clinical management and therapy selection of prostate cancer. There are several parameters and nomograms to differentiate between patients with clinically insignificant disease and patients in need of treatment. Magnetic resonance imaging (MRI) is a technique which provides more detailed anatomical images due to high spatial resolution, superior contrast resolution, and multiplanar capability. State-of-the-art MRI techniques, such as diffusion weighted imaging (DWI), MR spectroscopic imaging (MRSI), dynamic contrast enhanced MRI (DCE-MRI), improve interpretation of prostate cancer imaging. In this article, we review the major role of MRI in the advanced management of prostate cancer to noninvasively improve tumor staging, biologic potential, treatment planning, therapy response, local recurrence, and to guide target biopsy for clinical suspected cancer with previous negative biopsy. Finally, future challenges and opportunities in prostate cancer management in the area of functional MRI are discussed as well.

Keywords: Prostate cancer; magnetic resonance imaging; management; treatment

Introduction

It was in 1853 that John Adams first described a case of prostate cancer (Pca) in a 59-year-old male patient, and this disease was considered rare because of the short life expectancy at the time. In 2012, there were expected to be about 239,000 new cases diagnosed with Pca and about 30,000 Pca deaths (1). Nowadays, it is the most common noncutaneous cancer and the second/third leading cause of cancer death in men in the United States and European Community (1-3). The management and imaging in Pca remains a big challenge.

The main diagnostic biomarker for Pca is prostate-specific antigen (PSA). PSA test was approved by the U.S. Food and Drug Administration (FDA) in 1986 to monitor the disease status (4). However, a PSA test has some drawbacks. It is not capable of differentiating between Pca, benign prostatic hyperplasia (BPH), and chronic prostatitis, particularly when serum PSA level is lower than 10 ng/mL. This method indeed produces over-diagnosis of clinically insignificant cancers. Thus, in 2012, the U.S. Preventive Services Task Force (USPSTF) recommended against PSA-based screening all men for Pca (5). Transrectal Ultrasound (TRUS)-guided prostate biopsy has become a standard method to obtain

specimen for histopathological examination. Positive results of biopsy of the prostate confirm clinical suspicion of Pca, but they provide limited information on extent and differentiation of Pca. Furthermore, prostate biopsy without evidence of Pca does not rule out its presence (6).

To replace somewhat arbitrary combinations of individual variables, there is a need for instruments to aid patients and their physicians in treatment decision. Using algorithms that incorporate multiple variables, the nomograms have been developed to give a prediction of the pathologic stage, the probability of freedom from disease recurrence. The Partin staging nomogram (also called the "Partin tables"), which is based on serum PSA value, clinical stage, Gleason score, and was first published in 1993 and was updated in 1997 and again in 2001 to predict the pathological stage at radical prostatectomy. Other nomograms, such as Kattan's nomograms, have been developed to predict stage, recurrence, or biologic potential (7). As an important advance in accurate prediction for clinical medicine, the nomograms allow calculation of the continuous probability of a particular trend and tend to outperform both expert clinicians and risk grouping. The nomograms are widely used for individual patient counseling and important decision-making. However, the nomograms are limited by the lack of results from imaging studies and digital rectal examination (DRE)-based clinical staging. Thus, despite the high predictive ability and the cost-effectiveness of the nomograms, there is still some room for improved accuracy of prediction.

MRI is a good imaging modality of choice in Pca detection, localization, and staging (8-10). The interpretation of Pca on T2-weighted MR imaging (T2WI) can be affected by false-positive findings such as prostatitis, postbiopsy hemorrhage, and fibrosis (11,12). To improve the diagnostic accuracy of Pca imaging, functional MR imaging (fMRI) techniques have been applied, such as diffusion-weighted MR imaging (DWI) (13-15), proton (1H) MR spectroscopic imaging (MRSI) (16-18), and dynamic contrast-enhanced MR imaging (DCE-MRI) (19-21).

DWI has quickly evolved to become one of the most relevant sequences for imaging Pca. In tumor, the increased cellularity and associated loss of ductal morphology result in a smaller extracellular space, the restriction of water diffusion and a corresponding reduction in ADC values (22). A recent meta-analysis demonstrated the sensitivity and specificity of DWI combined with T2WI to range from 65% to 84% and 77% to 87%, respectively (23). MRSI identifies Pca by an increased ratio of choline plus polyamines plus creatine to citrate (24). As a result of increased energy metabolism, the citrate level is reduced in tumor. Owing to a high phospholipid cell membrane turnover the choline level is elevated in proliferating malignant tissue (25). DCE-MR imaging relies on tumor neoangiogenesis for Pca detection. In malignant tumour, the number of vessels (microvascular density) is increased in comparison with the surrounding normal tissue, leading to greater relative tumoral enhancement (26).

This review addresses the major role of MRI in the advanced management of Pca to improve cancer staging noninvasively, biologic potential, treatment planning, therapy response, local recurrence, and to guide target biopsy for clinically suspected cancer with previous negative biopsy, and discusses the future prospects of MRI in Pca management from a multidisciplinary standpoint.

Prostate cancer staging

The staging of Pca is based on tumor, node and metastasis (TNM) staging. The latest modification was made in 2010 by the American Joint Committee on Cancer (AJCC). The 2010 revised TNM system, shown in *Table 1*, is clinically useful and precisely stratifies newly diagnosed cancer (27). The most important advantage is distinguishing between patients with pathologically organ-confined Pca (pT2) from those with non-confined Pca (pT3-4). As is well known, once the tumor extends outside the prostate, the chances of cure are substantially diminished (28,29).

Detection of OCPC (pT2)

Clinicians must distinguish between patients with pathologically organ-confined prostate cancer (OCPC) (pT2) and those with non-organ-confined prostate cancer (pT3-4). T2 tumors are subclassified as T2a (less than one-half of one lobe involved) (*Figure 1*), T2b (more than one-half of one lobe involved), and T2c (bilateral involvement). After radical prostatectomy (RP), patients with OCPC have an excellent prognosis, as more than 90% of them are free from biochemical recurrence in the period of 5 years (30).

One study of *Wang et al.* demonstrated that MR findings contributed significant incremental value to the Partin tables in predicting OCPC. The contribution of MR findings was significant in all risk groups but was greatest in the intermediate- and high-risk groups. Overall, in the prediction of OCPC, the area under the ROC curve (AUC) for the staging nomograms was 0.80, while the AUC for

Table 1 Prostate tumor node metastasis (TNM) staging (American Joint Committee on Cancer, 7th ed. 2010)

Evaluation of the (primary) tumor (T)

  Clinical

    TX: can not evaluate primary tumor

    T0: no evidence of primary tumor

    T1: clinically inapparent tumor neither palpable nor visible by imaging

    T1a: tumor was incidentally found in less than 5% of prostate tissue resected

    T1b: tumor was incidentally found in more than 5% of prostate tissue resected

    T1c: tumor was found in a needle biopsy performed because of elevated serum PSA

    T2: tumor confined within prostate[1]

    T2a: the tumor is in half or less than half of one of the prostate gland's 2 lobes

    T2b: the tumor is in more than half of one lobe, but not both

    T2c: the tumor is in both lobes

    T3: the tumor has spread through the prostatic capsule (if it is only part-way through, it is still T2)

    T3a: the tumor has spread through the capsule on one or both sides

    T3b: the tumor has invaded one or both seminal vesicles

    T4: the tumor has invaded adjacent structures other than seminal vesicles (e.g. external sphincter, rectum, bladder, levator muscles, and/or pelvic wall)

  Pathologic (pT)[2]

    pT2: organ confined

    pT2a: unilateral, one-half of one side or less

    pT2b: unilateral, involving more than one-half of side but not both sides

    pT2c: bilateral disease

    pT3: extraprostatic extension

    pT3a: extraprostatic extension or microscopic invasion of bladder neck

    pT3b: seminal vesicles invasion

    pT4: Invasion of rectum, levator muscles, and/or pelvic wall

Evaluation of the regional lymph nodes (N)

  (p)NX: regional lymph nodes were not assessed (sampled)

  (p)N0: there has been no spread to the regional lymph nodes

  (p)N1: there has been spread to the regional lymph nodes

Evaluation of distant metastasis (M)

  M0: there is no distant metastasis

  M1: there is distant metastasis

  M1a: the cancer has spread to lymph nodes beyond the regional ones

  M1b: the cancer has spread to bone

  M1c: the cancer has spread to other sites (regardless of bone involvement)

[1]Tumor found in one or both lobes by needle biopsy, but not palpable or reliably visible by imaging, is classified as T1c; [2]There is no pathologic T1 classification
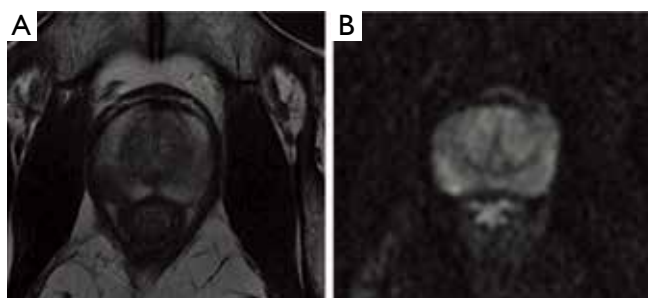
Figure 1 3T magnetic resonance (MR) images of organ-confined prostate cancer (OCPC) in a 59-year-old man with a Gleason score of 3+4 and a PSA level of 12.09 ng/mL. A. Transverse 3 mm-thick MR (4580/105) image shows a low-signal-intensity lesion in the right lobe of the prostate; B. Transverse 3 mm-thick DWI (3500/73, b-value of 1,000 s/mm^2) clearly demonstrates a focal high intensity area, a fining indicative of increase diffusion

the staging nomograms plus MR findings was 0.88. In the combined endorectal MRI-MRSI group, the AUC were 0.81 for the staging nomograms and 0.90 for the staging nomograms plus MR findings (31).

Detection of extracapsular extension (ECE) (pT3a)

ECE of Pca is associated with increased risk of a positive surgical margin, which in turn influences postoperative biochemical recurrence after radical prostatectomy (32). On T2-weighted MRI, criteria for detecting ECE include at least one of the following: irregular capsular bulge or edge retraction, disruption of the prostatic capsule, extension into the periprostatic fat, broad contact with the capsule (>12 mm), obliteration of the rectoprostatic angle, or asymmetry of the neurovascular bundles (*Figures 2,3*) (33).

A study of 32 patients demonstrated the mean sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for assessment of ECE with the combined DCE and T2WI 3 Tesla MRI system using an endorectal coil were 86%, 95%, 90%, and 93%, respectively (34). Bloch *et al.* (35) analysed the value of DCE combined with T2WI at 3 Tesla scanner for determining ECE of Pca, and found that the overall sensitivity, specificity, PPV and NPV for ECE were 75%, 92%, 79% and 91%, respectively. 3 Tesla MRI of the prostate combining DCE and T2WI is an accurate pretherapeutic staging tool for assessment of ECE in clinical practice. In a study using MRI with combining transaxial and coronal plane images using picture and communication systems (PACS) cross-referencing to

Figure 2 3T magnetic resonance (MR) images of extracapsular extension (ECE) of prostate cancer in a 57-year-old man with a Gleason score of 4+4 and a PSA level of 13.85 ng/mL. A and B. Transverse 3 mm-thick MR (4580/105) image, and coronal 3 mm-thick MR (2990/86) image show a hypointense tumor with extraprostatic extension in the left peripheral zone of the prostate; C. Transverse 3 mm-thick DWI (3500/73, b-value of 1,000 s/mm^2) shows intense, increased signal (restricted diffusion) throughout the mass; D. The disease was clinically staged as T4 prostate cancer and confirmed by pathology

facilitate the diagnosis of ECE, Wang and colleagues (36) showed that sensitivity and specificity for ECE with MRI alone and with cross-referencing were 43% and 94% and 57% and 100% for reviewer 1 and 40% and 93% and 59% and 98% for reviewer 2, respectively. The weighted Kappa was 0.56 with MRI alone and 0.76 with cross-referencing, indicating good interobserver agreement.

Detection of seminal vesicle invasion (SVI) (pT3b)

SVI is considered an important marker of tumor progression and connected with increased risk of lymph node invasion, local tumor recurrence. On MRI T2WI direct signs of SVI are contiguous low-signal intensity (SI) tumor extension from base of the gland to seminal vesicles, focal low-SI within the seminal vesicles disruption or loss of the normal structure of the seminal vesicles, non-visualization or enlarged of the ejaculatory ducts, obliteration of seminal vesicle angle and decreased conspicuity of seminal vesicles (*Figure 4*).

Figure 3 3T magnetic resonance (MR) images of extracapsular extension (ECE) of prostate cancer in a 69-year-old man with a Gleason score of 4+3 and a PSA level of 40.64 ng/mL. A. Transverse 3 mm-thick MR (4580/105) image shows a hypointense tumor with extraprostatic extension in the left peripheral zone of the prostate; B. Transverse 3 mm-thick DWI (3500/73, b-value of 1,000 s/mm^2) shows intense, increased signal (restricted diffusion) throughout the mass; C. A color-coded wash-out map shows a focal area of wash-out in the location of cancer; D. The disease was clinically staged as T4 prostate cancer and confirmed by pathology

Figure 4 3T magnetic resonance (MR) images of seminal vesicle invasion (SVI) of prostate cancer in a 69-year-old man with a Gleason score of 4+4 and a PSA level of 49.7 ng/mL. A and B. Transverse 3 mm-thick MR (4580/105) image and sagittal 3 mm-thick MR (3088/70) image show a hypointense tumor with bilateral seminal vesicle invasion in the apex of the prostate; B. Transverse 3 mm-thick DWI (3500/73, b-value of 1,000 s/mm^2) shows intense, increased signal (restricted diffusion) throughout the mass; D. The disease was clinically staged as T4 prostate cancer and confirmed by pathology

A study of 45 consecutive patients demonstrating the endorectal MRI following radiation therapy can help identify tumor sites and depict ECE and SVI with reasonable accuracy in patients with recurrent Pca (37). The AUC values for prediction of SVI were 0.76 (95% CI: 0.62, 0.90) for reader 1 and 0.70 (95% CI: 0.56, 0.85) for reader 2. The Kappa statistics used to assess interobserver agreement were fair (0.45, 0.47 for tumor location, SVI, respectively).

A study investigated 154 consecutive patients who underwent endorectal MRI before surgery. MRI sensitivity, specificity, PPV, NPV, overall accuracy resulted in respectively 0.88, 0.98, 0.82, 0.99 and 0.97 for SVI (38). Nepple *et al.* evaluated the accuracy of endorectal MRI compared with subsequent pathology specimen from prostatectomy. PPV, NPV, sensitivity, specificity of MRI were 93%, 75%, 94%, 38%, 99% for SVI. Endorectal MRI in the evaluation of high-risk Pca was moderately accurate

for SVI (39).

A study of 1,161 consecutive patients demonstrated that endorectal coil MRI had limited clinical value in preoperatively detecting SVI (40). In evaluating SVI, sensitivity and specificity were 33% and 89%, respectively. The PPV of MRI to assess SVI was 50% in both, with a NPV of 63%.

The addition of DWI to MRI has been shown to significantly increase staging accuracy for the less inexperienced readers and thus reduce interobserver variability (41). A study of 30 patients demonstrated significant improvement in the prediction of SVI for the less experienced readers. Interobserver agreement showed a substantial agreement (Kappa =0.613) for T2WI, and a substantial agreement (Kappa =0.737) for T2WI with DWI (41). In 2009, a study of Ren *et al.* showed that T2WI combined with DWI demonstrated significantly higher accuracy than T2WI alone in the detection of SVI (42).
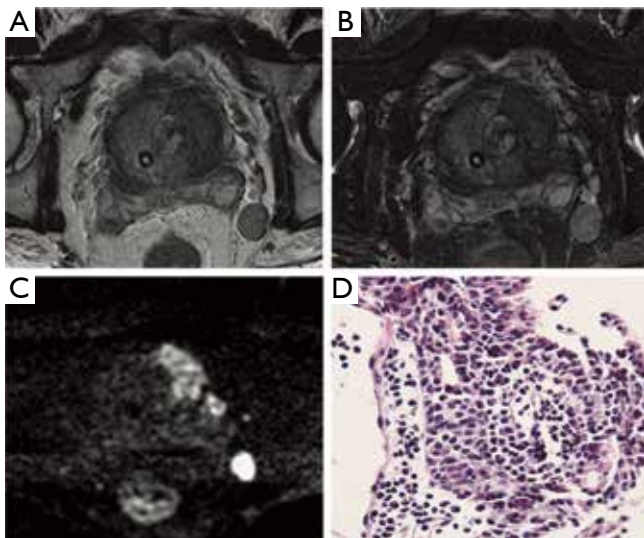
Figure 5 3T MR images of prostate cancer in a 72-year-old man with a Gleason score of 4+5 and a PSA level of 23.89 ng/mL. A and B. Transverse 3 mm-thick T2WI (4580/105) and transverse 3 mm-thick T2 weighted fat-saturated MR imaging (4580/105) show a hypointense tumor with extraprostatic extension in the left transitional and peripheral zone of the prostate, and intermediate SI bulky adenopathy with short-axis dimensions of >8 mm is present in left external iliac and obturator distributions; C. Transverse 3 mm-thick DWI (3500/73, b-value of 1,000 s/mm^2) shows intense, increased signal (restricted diffusion) throughout the mass; D. The disease was clinically staged as T3M1 prostate cancer and confirmed by pathology

Detection of LNM

Regarding the lymph node metastasis (LNM), 70% of them are too small (<8 mm) to be evaluated using MRI, so conventional size criteria may underestimate the extent of nodal disease. A meta-analysis reported that MRI demonstrated equally poor performance in the detection of LNM from Pca with a sensitivity of around 30% (43). For this reason, recently two other MR techniques have been developed: MR lymphography (MRL) [which uses a lymph node-specific contrast agent called ultrasmall superparamagnetic particles of iron oxide (USPIO)] and DWI-MRI (*Figure 5*).

In 1998, Bellin *et al.* reported on the initial clinical experience with MRL and found a perfect sensitivity of 100% at 80% specificity (44). In another prospective study with 334 lymph nodes in 80 patients, sensitivity and specificity were 90.5% and 97.8%, respectively (45). More

recently, it has been shown that MRL is significantly more accurate than multidetector-row CT (46), and that in 41% of PCa patients MRL can detect LNM outside the surgical area of routine pelvic lymph node dissection (46). Although these results are very promising, MRL has not yet become available for clinical use due to the lack of an U.S. Food and Drug Administration (FDA) -approved lymph node-specific contrast agent.

The added value of DWI compared to USPIO-MRL did not improve diagnostic accuracy, but rather reduced significantly reading time for detecting pelvic LNM (47). However, one study also reported a good accuracy based on ADC value alone, with a sensitivity of 86.0% and a specificity of 85.3% (48).

A study of 411 consecutive patients demonstrated that MRI was an independent statistically significant predictor of LNM (P=0.002), with PPV and NPV value of 50% and 96.36%, respectively. On multivariate analysis, prediction of lymph node status using the model that included all MRI variables (ECE, SVI, and LNM) along with the Partin table results had also a significantly greater AUC than the univariate model that included only MRI LNM findings (AUC =0.892 *vs.* 0.633, respectively, P<0.01) (49).

Prostate cancer biologic potential

The Gleason scoring system remained one of the most powerful prognostic predictors in Pca for nearly 50 years after its initial description (50). It was endorsed as the primary staging system for Pca by the College of American Pathologists, the Armed Forces Institute of Pathology Fascicle on Prostate Cancer, the Association of Directors of Anatomic and Surgical Pathology, and the World Health Organization (WHO) (51).

Gleason grade has been associated with biochemical failure, local recurrences, and distant metastases such as skeletal and LNM after prostatectomy or radiation therapy (52-54). Since Gleason scores of 3+4, or lower, are associated with lower disease progression rates, and Gleason scores of 4+3, or higher, are associated with higher disease progression rates (55), a differentiating between both is meaningful.

Several studies reporting an association of Gleason staging with MRI are a great quantity, especially with DWI a significant negative correlation between Gleason score and ADC values been found (56,57). Furthermore, choline plus creatine-to-citrate ratios determined by using MRSI have also been correlated with Gleason grade (58,59). Wang *et al.*
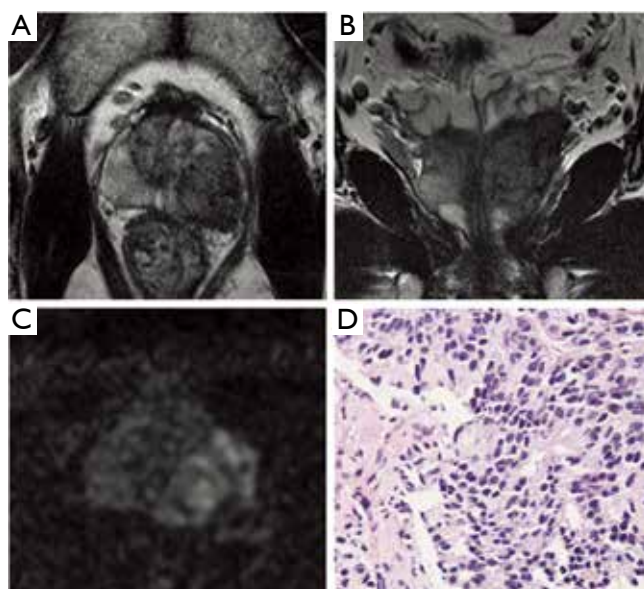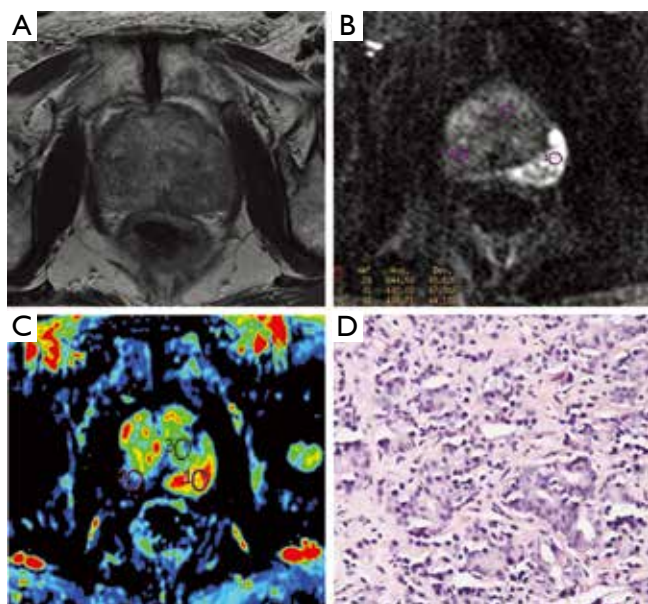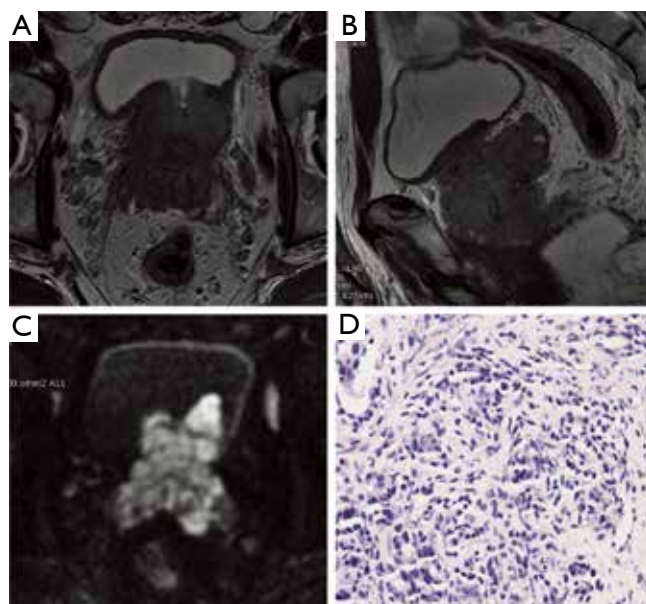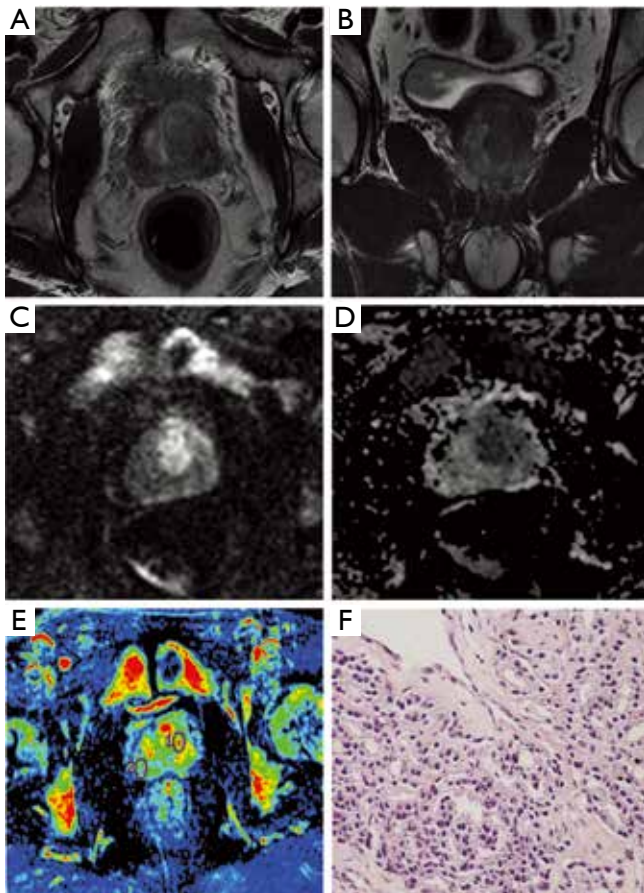
**Figure 6** 3T magnetic resonance (MR) images of extracapsular extension (ECE) of prostate cancer in a 56-year-old man with a Gleason score of 4+5 and a PSA level of 40.64 ng/mL. A and B. Transverse 3 mm-thick MR (4580/105) image and coronal 3 mm-thick MR (2990/86) image show a hypointense tumor with extraprostatic extension in the left transitional and peripheral zone of the prostate; C. Transverse 3 mm-thick DWI (3500/73, b-value of 1,000 s/mm^2) shows intense, increased signal (restricted diffusion) throughout the mass; D. An ADC map shows a focal hypointense lesion extending outside the capsule; E. A color-coded wash-out map shows a focal area of wash-out in the location of cancer and left pulic bone metastases; F. The disease was clinically staged as T3 prostate cancer and confirmed by pathology

even reported the correlation of SI of Pca on T2WI with Gleason grade and found that SI evaluation on T2WI may facilitate noninvasive assessment of Pca aggressiveness (60).

Treatment planning

There are several therapeutic options including pelvic lymph node dissection (PLND), external beam radiotherapy (EBRT), radical prostatectomy (RP), androgen deprivation therapy (ADT), brachytherapy, cryosurgery, hyperthermia, and chemotherapy. Monotherapy or combination therapy is performed based on the TNM staging and clinical symptoms of the cancer. Good treatment strategies require a very careful evaluation of an individual prognosis to avoid inappropriate therapy induced morbidity or treatment failure. It is imperative that all tools available are used for different patients so that cancer is controlled.

RP is well established as a definitive treatment option in the management of localized Pca. The goal of this procedure is to achieve excellent oncologic control with negative surgical margins while preserving urinary continence and erectile function. A nerve-sparing radical prostatectomy preserves the neurovascular bundle (NVB) running along the posterior-lateral aspect of the prostate. This procedure is the standard of care for men with a low preoperative risk of extraprostatic diseases who wish to retain erectile function, and is also associated with improved urinary continence (61-64). The primary risk of nerve sparing is a positive surgical margin in a patient with organ-confined or extraprostatic extension (65,66). As such, accurate preoperative staging is very important for guiding treatment, and imaging techniques could provide a significant contribution.

Therapy response

Early selection of patients who are most likely to benefit from chemotherapy or radiotherapy may prevent the risk of toxicity in non-responding patients with prostate tumor. Early response to chemotherapy is monitored with DWI especially in bone metastases, as well as significant changes in perfusion due to tumor vascularity and extraction coefficient derived from DCE-MRI (*Figure 6*).

Foltz *et al.* found regional and temporal changes in ADC and T2 relaxation during radiation therapy (RT) in patients with low and intermediate risk localized Pca (67). A study of Franiel *et al.* showed statistically significant changes in perfusion and extraction coefficient parameters derived from DCE-MRI in monitoring the tissue changes to percutaneous intensity-modulated radiotherapy of Pca (68).

A study also demonstrated that after ADT, there was a significant reduction in all DCE-MRI parameters measured in tumor regions of interest (K^{trans}, K$_{ep}$, V$_p$). ADC values significantly decreased in areas of normal-appearing peripheral zone. As MRI provided dynamic information

that was helpful in therapy response, their findings suggested that DCE as a marker of angiogenesis may help demonstrate ADT resistance and DWI may be more accurate in determining presence of tumor cell death versus residual tumor (69).

Tumor recurrence

Approximately 25% to 30% of patients who underwent RP will develop local or systemic recurrent diseases (70,71). Biochemical failure (i.e., a rising serum PSA in the absence of demonstrable metastases) is widely accepted as an appropriate end point for defining treatment failure in men with localized Pca. The serum PSA is routinely used to monitor disease recurrence after definitive therapy because biochemical recurrence antedates metastatic disease progression and Pca-specific mortality by an average of 10 years, respectively (72-74). Biochemical recurrence-free probability after salvage radical prostatectomy at 5 years ranged from 37% to 55% and the estimated cancer-specific survival at 10 years ranged from 70% to 83% (75).

Diagnosis of recurrence of Pca remains challenging by imaging, especially in the early stage. At present, serial serum PSA test plays the important role in the assessment of recurrence and progression of Pca after initial radical treatment (76).

The current consensus considers a PSA increase over a threshold of 0.2 ng/mL as the cutoff that necessitates further evaluation (77). The main role of imaging would be to identify the patients with local recurrence who would potentially benefit from salvage radiotherapy. Detecting the site of recurrence is difficult, mainly because of the absence of any signs or symptoms in the early stage (78). A critical diagnostic dilemma for the evaluation of patients with biochemical failure is to differentiate between patients who only have local recurrence and those who have metastatic spread. At this point, diagnostic imaging strategies are able to provide crucial information toward differentiating local recurrence versus metastatic spread and in helping plan further therapeutic interventions.

To guide target biopsy for clinically suspected cancer in patients with negative biopsy Cancer suspicious regions (CSRs) seen on multiparametric MRI can be targeted for biopsy. This can be done by either performing a TRUS-guided biopsy or a MR-guided biopsy.

TRUS-guided prostate biopsy is the gold standard for the diagnosis of Pca. When applied as a sextant biopsy in patients with a total PSA value ranging from 4-10 ng/mL,

this approach has a sensitivity of 39-52% and a specificity of 81-82% (79). Yet, about 20% of Pca are not detected at the first biopsy. When the first biopsy is negative, a repeat biopsy may be recommended, which has a cancer detection rate between 20% to 35% (80-82).

MRI-guided prostate biopsy is a diagnostic option for patients with CSRs, this technology has gained growing importance in the diagnosis of Pca. The capability of combining MR imaging with techniques to simultaneously perform a targeted biopsy of the prostate is of particular interest to urologists.

Several studies have already demonstrated this technology improved cancerous detection rate in subjects with an elevated PSA and repetitive negative TRUS-guided biopsies (11,83,84). In a study of 54 patients with elevated PSA and negative biopsies, MRI had a sensitivity of 83% and a PPV of 50% for detection of Pca. A study of 92 patients concluded that for patients with elevated PSA and 2 previous negative biopsies, a negative MRI can rule out cancer and avoid subsequent biopsies (85).

In a study of 68 patients with repeat negative TRUS-guided prostate biopsies, the tumor detection rate of 3 Tesla MRI-guided biopsy was 59% (40 of 68 cases) using a median of 4 cores (86). In a study of 96 patients with TRUS-negative results, the sensitivity, specificity, PPV and NPV of MRI-guided core biopsies for Pca detection were 95.8%, 95.5%, 95.8% and 99.5% to 95.5% (87).

MR-compatible robots for transrectal prostate biopsy are being developed. Preliminary results found in phantom and patient feasibility studies are promising (88-90). In future studies, robotics could also play an important role in guiding focal treatment of PCa. But before robot-assisted MRI guided focal therapy can be realized, further extensive research needs to be done.

Future prospects

Although functional MR system for staging Pca on 1.5 Tesla is commercially available and is becoming more widely used, 3 Tesla MR scanners offer improvements in both spatial and temporal resolution and in speed. Increasing static magnetic field strength, B_0, from 1.5 Tesla to 3 Tesla will result in a theoretical doubling of the signal-to-noise ratio (SNR). The increase in SNR results in an increase in spatial and temporal resolution and a decrease in the acquisition time (91). However, a disadvantage of 3 Tesla is the increased susceptibility effect in comparison with 1.5 Tesla due to the higher field inhomogeneity as

well as the chemical shift effect, which are directly related to magnetic field strength (92).

Conclusions

The increasing incidence of Pca, which is the most frequently diagnosed malignancy in the Western male population (1), poses an increasing burden on health care. MRI is able to provide detailed anatomical images due to high spatial resolution, superior contrast resolution and multiplanar capability (93). MRI noninvasively improves cancer staging, biologic potential and treatment planning, monitors antitumor therapy and local recurrence, and guides target biopsy for clinically suspected cancer with previous negative biopsy. State-of-the-art techniques, such as DWI, MRSI, DCE-MRI, high-field strength scanner, image postprocessing and PACS improved interpretation of Pca images. To interpret these studies accurately, there is still a need for multi-institutional studies to standardize functional MRI techniques and interpretation criteria.

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Footnote

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The current and future role of magnetic resonance imaging in prostate cancer detection and management

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Purpose: Accurate detection of clinically significant prostate cancer (PC) and correct risk attribution are essential to individually counsel men with PC. Multiparametric MRI (mpMRI) facilitates correct localization of index lesions within the prostate and MRI-targeted prostate biopsy (TPB) helps to avoid the shortcomings of conventional biopsy such as false-negative results or underdiagnosis of aggressive PC. In this review we summarize the different sequences of mpMRI, characterize the possibilities of incorporating MRI in the biopsy workflow and outline the performance of targeted and systematic cores in significant cancer detection. Furthermore, we outline the potential of MRI in patients undergoing active surveillance (AS) and in the pre-operative setting.

Materials and methods: An electronic MEDLINE/PubMed search up to February 2015 was performed. English language articles were reviewed for inclusion ability and data were extracted, analyzed and summarized.

Results: Targeted biopsies significantly outperform conventional systematic biopsies in the detection of significant PC and are not inferior when compared to transperineal saturation biopsies. MpMRI can detect index lesions in app. 90% of cases as compared to prostatectomy specimen. The diagnostic performance of biparametric MRI (T2w + DWI) is not inferior to mpMRI, offering options to diminish cost- and time-consumption. Since app 10% of significant lesions are still MRI-invisible, systematic cores seem to be necessary. In-bore biopsy and MRI/TRUS-fusion-guided biopsy tend to be superior techniques compared to cognitive fusion. In AS, mpMRI avoids underdetection of significant PC and confirms low-risk disease accurately. In higher-risk disease, pre-surgical MRI can change the clinically-based surgical plan in up to a third of cases.

Conclusions: mpMRI and targeted biopsies are able to detect significant PC accurately and mitigate insignificant PC detection. As long as the negative predictive value (NPV) is still imperfect, systematic cores should not be omitted for optimal staging of disease. The potential to correctly classify aggressiveness of disease in AS patients and to guide and plan prostatectomy is evolving.

Keywords: Prostate cancer (PC); magnetic resonance imaging (MRI); multiparametric MRI (mpMRI); MRI/TRUS-fusion-guided biopsy; staging; targeted prostate biopsy (TPB); systematic biopsy (SB); radical prostatectomy (RP); PIRADS v1.0; PIRADS v2.0

172

Radtke et al. Future of MRI in PC

Introduction

Prostate cancer (PC) is the most common noncutaneous malignancy in men in Western countries (1). In 2011, around 900,000 new cases and among 250,000 deaths were recorded worldwide (1). According the European Association of Urology guidelines an elevated prostate specific antigen (PSA)-level should trigger an extended 12-core systematic TRUS-guided biopsy, which is endorsed as the optimal biopsy method (2). This diagnostic strategy is based on random sampling and is largely operator dependent. Consecutively, this biopsy technique is subject to sampling error and provides poor characterization of PC aggressiveness (3). The main shortcomings of the 12-core TRUS biopsy technique are failure to detect clinically significant cancer and imprecise PC risk stratification (undersampling) and detection of small low-risk clinically insignificant cancers (overdetection) (4,5). This diagnostic uncertainty can lead to repeat biopsy, delayed detection of significant disease or disease overtreatment. The need of precise tumor detection and staging increases in the context of recent trends of active surveillance (AS) and focal therapy.

Since its first usage in 1983, magnetic resonance imaging (MRI) is increasingly used for PC diagnosis because of growing availability and multiparametric imaging, combining anatomic and functional data (6). A number of studies confirm the diagnostic reliability of multiparametric MRI (mpMRI) for PC detection (4,7). In the past, widespread acceptance of mpMRI suffered from a lack of standardized diagnostic criteria for reporting of results, leading to a substantial variability in interpretation (8). To standardize the evaluation and reporting of prostate MRI, the European Society of Urogenital Radiology (ESUR) published guidelines based on an expert consensus in 2012, termed the Prostate Imaging Reporting and Data System (PIRADS) (9). This guideline provides explicit and standardized criteria for Likert-scoring of multiparametric sequences [T2w, diffusion-weighted imaging (DWI), dynamic contrast enhanced imaging (DCE) and MR-spectroscopy] (9). Since then, the PIRADS score has been externally validated with a good accuracy, suggesting that this 5-point-Likert scoring allows to detect PC accurately (10-13). The T2w, DWI and DCE sequences have been maturing as being most accurate for PC detection, whereas the use of MR-spectroscopy has mostly been abandoned.

The accuracy of mpMRI and PIRADS scoring has not only been established for biopsy specimen, but also for histopathologic correlation using prostatectomy specimen. In the pre-PIRADS era, Isebaert *et al.* found a sensitivity of 58.5% for PC detection (14). Recent publications demonstrate detection rates of significant PC between 80-96% for MRI compared to whole-mount sections (15-17).

MRI-targeted biopsies (TB) can be taken by various approaches. Visual estimation (VE) allows the adaptation of TB in clinical practice without costs for new equipment, but lacks real-time feedback regarding accuracy. The effectiveness and accuracy of VE biopsy vary among studies. Haffner *et al.* detected significant PC in 43% of men using TB and missed only 5.2% of significant PC as compared to standard biopsy (18). In addition, Kasivisvanathan *et al.* reported non-inferiority of VE TB as compared to transperineal mapping (19). However, the performance of VE TB is strongly experience dependent, limiting the wide-spread of this approach (3). In-bore MRI guided biopsy is an alternative, offering a cancer detection rate (CDR) of 41% and finding mostly clinically significant PC (20). These results have been corroborated by recent studies, demonstrating that in-bore MRI guided biopsy is a feasible technique, requiring fewer cores to detect similar rates of significant cancer and a median detection rate for all PC around 42% (21,22). However, the use of in-bore TB alone might be critical as several studies demonstrate that app. 10% of significant tumors are MRI-invisible (12,13,23,24). MRI/TRUS-fusion-guided biopsies with software-registration potentially overcome limitations of cognitive fusion through reproducible methods of identifying MRI lesions on ultrasound (25). The utility of TB versus systematic biopsy (SB) has recently been established in a large cohort that has been analysed according to standards formulated by an international consensus meeting (26,27). Siddiqui *et al.* published the results of 1,003 patients undergoing MRI-TB of MRI-visible lesions in addition to standard 12-core biopsy (26). In their study, TB detected significantly more (30%, P<0.001) high-risk PC and 17% fewer low-risk PC (P=0.002) compared to SB (26). However, TB alone missed 8.1% of intermediate- and high-risk PC compared to the combination of TB and SB. Moreover, when compared to prostatectomy specimen the negative predictive value of TB to exclude significant disease was still imperfect (70%). Furthermore, when TB were compared to a different reference test (24-core transperineal SB), 20.9% of significant PC were detected by TB alone, whereas 12.8% were missed by TB alone (12). Overall, no statistically significant difference in significant PC detection occurred between both approaches (12). In conclusion, for optimal staging both TB and SB should be taken to detect significant PC accurately, echoing other
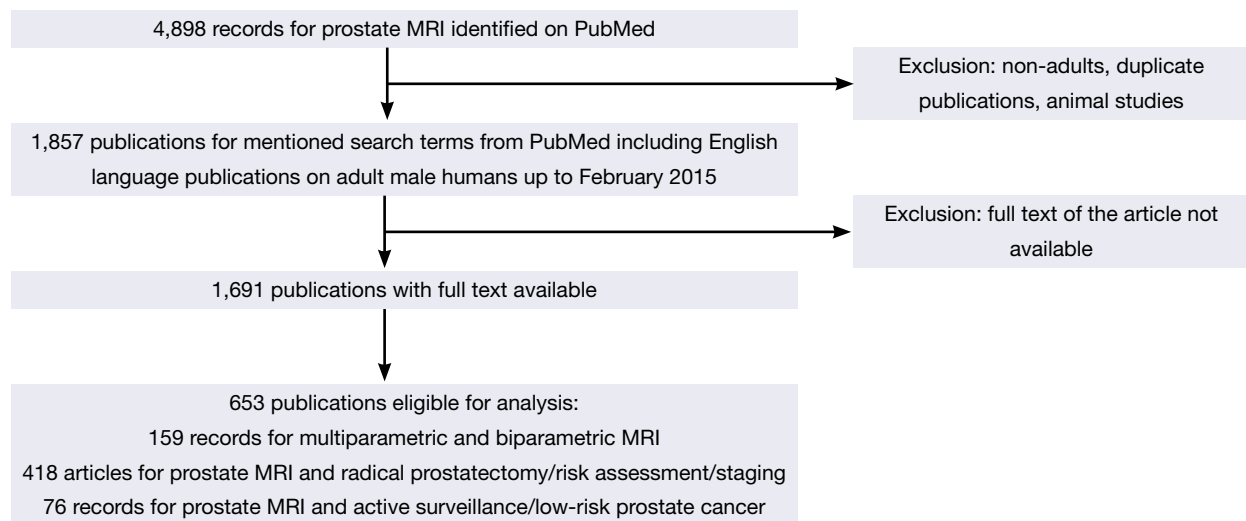
Figure 1 Study flowchart with inclusion and exclusion criteria for manuscripts into analysis. MRI, magnetic resonance imaging.

recent publications (12,24).

MpMRI also has the potential to predict extracapsular extension (ECE) on radical prostatectomy (RP) (28). Somford *et al.* and Marcus *et al.* have demonstrated promising negative (NPV) and positive predictive values (PPVs) of ECE and the possibility of changing the surgical strategy (28,29).

In this review we evaluate the role of MRI in the pre-biopsy setting and the utility to predict RP outcome. Moreover we describe the different MRI sequences and biopsy techniques.

Materials and methods

We searched MEDLINE/PubMed for English language manuscripts published up to February 2015 using the following search terms: MRI, multiparametric MRI, biparametric MRI, MRI-guided, MRI-targeted, image-guided, MRI-ultrasound fusion, cognitive, prostate, PC, biopsy, detection, AS, risk assessment, risk stratification, PIRADS, NMR, cancer detection, visual estimation and extraprostatic extension (EPE). Non-English articles were excluded from analysis. Inclusion criteria were male gender, adult and availability of full text. Overall, 653 publications were included. Data were extracted and analyzed.

The literature search and study pre-selection is graphically displayed in *Figure 1*.

Results

Limitations of the contemporary systematic biopsy technique

False-negative biopsy
Standard 12-core transrectal biopsies need optimal spatial distribution for tumor detection and are consequently subject to sampling error. Undersampling occurs in up to 30-80% of patients with significant PC (4,26,30). Especially cancers with small volume or PC located in the transition zone (TZ) and anterior prostate are difficult to detect by random 12-core transrectal approaches (31,32). Additionally larger glands are subject to greater risk of false-negative biopsy (30). To overcome this sampling error, multiple repeat biopsies are often performed. However, these can result in overdetection of indolent cancers that may not have caused harm (3).

Incorrect risk stratification
The undersampling of the prostate during systematic transrectal procedures can lead to incorrect risk stratification of PC. More strict definitions of PC do not only include the Gleason score (GS) (≥3+4 or ≥4+3), but also the lesion volume (33,34). Random biopsies risk inadequate lesion sampling, as the cancer core length is significantly decreased compared to TB (35,36). This may reveal a small length of tumor in a core with a low GS, when in fact a significant lesion may occur adjacent to the biopsy location (3,37).

174

Radtke et al. Future of MRI in PC

Another risk of conventional TRUS-guided biopsy is upgrading. Dinh *et al.* recently analyzed a SEER-database cohort of 10,273 patients, and found an upgrading of 44% from clinically low-risk PC in biopsy to GS ≥7 in RP specimen (38). Shaw *et al.* also found an upgrading of 50.2% from low-risk PC to intermediate- and high-risk PC in RP specimen analyzing 848 patients (39).

Detection of clinically insignificant disease

Approximately 60% of men over age of 80 years harbor clinically insignificant PC without suffering from it at autopsy studies (40). These clinically insignificant PC are often identified by chance during a SB and may contribute to overdetection and overtreatment of indolent PC (5,41,42).

Risk of infection

Although the TRUS-biopsy approach is the standard diagnostic approach for over 20 years, significant side-effects and morbidity are rising, especially post-biopsy infections (43). In a national Swedish cohort of 51,321 men, Lundström *et al.* showed an infection rate of 6% after transrectal biopsy (44). Prevention and prophylaxis from infections caused by rectal milieu is especially important, since the frequency of Escherichia coli resistant to fluoroquinolone increased from 11% in 2006 to 13% in 2011 (44,45). Additionally, Feliciano *et al.* described not only a fluorquinolone resistance of Escherichia coli, but also to gentamicin (22% of cases), trimethoprim/sulfamethoxazole in 44%, piperacillin in 72% and ampicillin in 94% (46). Furthermore Cohen *et al.* described an initial fluoroquinolone resistance of Escherichia coli in 24.4% of cases in an initial biopsy cohort of 416 men (47).

Transperineal approach

The transperineal approach is an alternative to the transrectal entry path, causing less risk of infection. Additionally, the anterior prostate is easier to access. Furthermore, transperineal mapping biopsy specimen undergo significantly less upgrading on RP specimen (8% versus 52% in a publication of Crawford *et al.*) (48). One disadvantage is that transperineal biopsies are more painful. Thus, anesthesia is needed, especially in case of saturation or mapping biopsies averting the wide-spread use of this approach.

Extended systematic and saturation biopsies

The debate on the optimal number of biopsy core samples that should be taken is still open. Ploussard *et al.*

have demonstrated that an increase from 12 to 21 cores significantly increases the detection rate of significant PC (49). Transperineal mapping biopsy (TPMB) aims for optimal staging and detection of all significant PC by using an external grid of 5 mm (50,51). Lecornet *et al.* have shown that TPMB detects nearly all significant PC lesions above 0.5 mL (52). However, TPMB is significantly more invasive than SB leading to urinary retention and the potential for oversampling of clinically insignificant PC, which often results in overtreatment. Valerio *et al.* report that, beside an accurate index lesion detection, insignificant PC was detected in up to 42.9% of TPMB (53). Additionally, the prostate is mobile, deformable and swells during biopsy, so real-time sampling errors in vivo might still occur (25). Thus, Ukimura *et al.* conclude that the specific clinical indication for TPMB, remains under debate (25).

Prostate mpMRI for PC detection and localization

The first application of prostate MRI was published by Hricak *et al.* in 1983 (54). Since then, the field strength of MRI increased from 0.35 Tesla (T) to 3T and standardized multiparametric sequences (T2w, DWI and DCE) were established. MRI and TB detect more clinically significant PC compared to standard 12-core TRUS-biopsy (4,26,37,55). To establish a standardized MRI technique and a quantitative structured reporting system, the ESUR promoted the ESUR guidelines in 2012 (9). In 2015, a revised PIRADS version was published by the American College of Radiologists (56). The imaging techniques are described in the following sections.

T2-weighted imaging

T2w MR images have high spatial resolution and clearly define the prostate's zonal anatomy (*Figure 2*) (59). PC in the peripheral zone often appear as a low signal intensity area on T2w (9). The degree of intensity decrease differs with the GS, with higher GS components showing lower signal intensities (60). However, T2w imaging can result in false positive findings, as low-signal intensity also occurs in acute and chronic prostatitis, atrophy, scars, post-irradiation, hyperplasia and post-biopsy hemorrhage (3). Because of the heterogeneous appearance of benign prostate hyperplasia with both, increased and decreased signal intensity, PC in the TZ can be difficult to distinguish from benign tissue (61,62). Morphologic features such as homogeneously low signal intensity, irregular edges of the suspicious lesion, invasion into the urethra or the anterior fibromuscular stroma
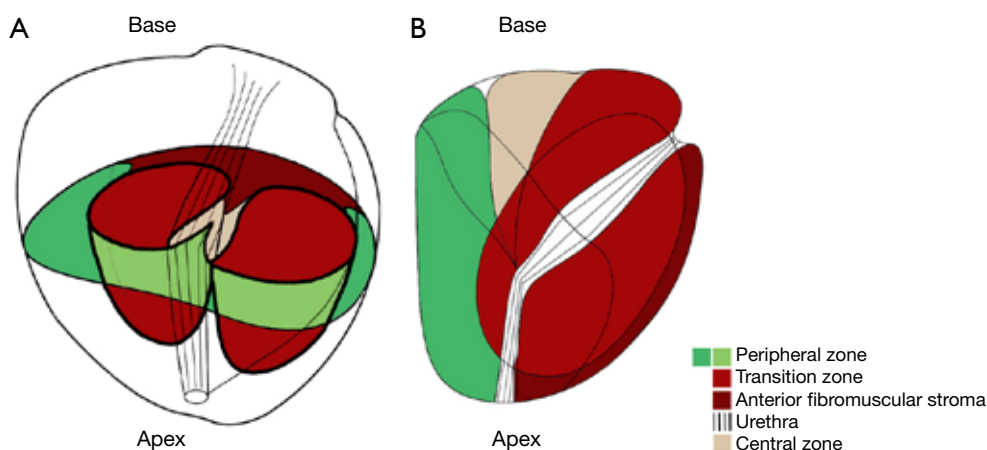
Figure 2 Scheme of prostate zones. Transversal (A) and sagittal (B) scheme of prostate zones, according to McNeal *et al.* and adapted from Bouyé *et al.* (57,58). The dark red colored area represents the anterior fibromuscular stroma and the bright red colored area the TZ of the prostate (57,58). The peripheral zone is colored in green, the central zone in beige. TZ, transition zone.

(AFMS), and lenticular shape are helpful for detection of TZ tumors (63). Moreover, the updated PIRADS guidelines state that T2w Imaging is the dominant sequence for the TZ [*Table 1* (see part A) and *Table 2*] (56).

Diffusion-weighted imaging (DWI)

DWI is based on Brownian motion and measures random motion of water molecules. The strength of the gradient that determines the degree of diffusion-weighting is reflected by the sequence's b value. Performing DWI with multiple b values, it is possible to compute the apparent diffusion coefficient (ADC) based on the signal intensity measured at each b value image to quantify the restriction of water diffusion. B values of 0, 100 and 800-1,000 s/mm^2 are recommended (9). For ADC calculation, the highest b value should be 1,000 s/mm^2 (9). The utility of higher b values up to 2,000 s/mm^2 is under debate (64,65). On ADC maps, PC frequently shows low ADC values and an inverse correlation exists between quantitative ADC values and the GS (65-67). While ADC does correlate with GS, the confidence intervals are widely overlapping, limiting the ability to use ADC as a surrogate of GS (3,67).

Limitations of DWI include low signal-to-noise ratio and image distortion, both of which become more problematic at higher b values (3). Nonetheless, DWI is a widely available technique, and given its association with tumor aggressiveness, it may prove to be the primary sequence for tumor detection and characterization, especially in the peripheral zone, were it is recommended as the dominant sequence [*Table 1* (see part B) and *Table 2*] (56,67).

Dynamic contrast-enhanced imaging and role of biparametric MRI

Dynamic contrast-enhanced MRI consists of a series of fast T1w-sequences covering the prostate, before and after a bolus injection of gadolinium chelate (9). It is the most common imaging method for evaluation of tumor vascularity (68). As many other malignancies, PC often demonstrate early enhancement compared to normal tissue (69). However, kinetics of PC enhancement can be variable and heterogeneous (56). Recent guidelines recommend to include DCE not to miss some small significant PC (56). If focal lesions are found, T2w and DWI images should be carefully interrogated for corresponding abnormalities (56). At present, the additional value of DCE is discussed controversially. Some publications state that addition of DCE and/or DW imaging to T2-weighted MRI significantly improved sensitivity from 63% to 79-81% in the peripheral zone, while maintaining a stable specificity (7). Yoshizako *et al.* demonstrated that the combined use of DWI, DCE, and T2-weighted MRI increased the accuracy in detection of TZ cancer compared to T2w alone, from 64% to 79% (70). The PIRADS 2015 guidelines still recommend the use of DCE, whereas Rosenkrantz *et al.*, Hoeks *et al.*, Rais-Bahrami *et al.* and Schimmöller *et al.* postulate that additional DCE did not improve the detection and localization accuracy of significant PC in all zones and especially in the TZ (56,62,71-73). When performed, DCE is positive when there is enhancement that is focal, earlier or contemporaneous with enhancement of adjacent normal prostatic tissue and usually corresponding

Table 1 PIRADS scoring for (A) T2w imaging, (B) DWI and (C) DCE imaging, according to the 2015 version of the American College of Radiologists (58)

Score	PZ or TZ
A: T2-weighted imaging	
1	PZ: uniform hyperintens signal intensity (normal);
	TZ: homogeneous intermediate signal intensity (normal)
2	PZ: linear or wedge-shaped hypointensity or diffuse mild hypointensity, usually indistinct margin;
	TZ: circumscribed hypointense or heterogeneous encapsulated nodules
3	PZ: heterogeneous signal intensity or non-circumscribed, rounded, moderate hypointensity Includes others that do not qualify as 2, 4 or 5;
	TZ: heterogeneous signal intensity with obscured margins. Includes others that do not qualify as 2, 4 or 5
4	PZ: circumscribed, homogenous moderate hypointense focus/mass confined to prostate and <1.5 cm in greatest dimension;
	TZ: lenticular or non-circumscribed, homogeneous moderately hypointense, and <1.5 cm in greatest dimension
5	PZ: same as 4 but ≥1.5 cm in greatest dimension or definite extraprostatic extension/invasive behaviour;
	TZ: Same as 4 but ≥1.5 cm in greatest dimension or definite extraprostatic extension/invasive behaviour
B: Diffusion-weighted imaging	
1	No abnormality (i.e., normal) on ADC and high b value DWI
2	Indistinct hypointense on ADC
3	Focal mildly/moderately hypointense on ADC and isointense/mildly hyperintense on high b value DWI
4	Focal markedly hypointense on ADC and markedly hyperintense on high b value DWI; <1.5 cm in greatest dimension
5	Same as 4 but ≥1.5 cm in greatest dimension or definite extraprostatic extension (EPE)/invasive behaviour
C: Dynamic contrast enhanced imaging	
–	No early enhancement, or;
	diffuse enhancement not corresponding to a focal finding on T2 and/or DWI or;
	focal enhancement corresponding to a lesion demonstrating features of BPH on T2w
+	focal, and; earlier than or contemporaneously with enhancement of adjacent normal prostatic tissues, and;
	corresponds to suspicious finding on T2w and/or DWI

PIRADS, Prostate Imaging Reporting and Data System; PZ, peripheral zone; TZ, transition zone; ADC, apparent diffusion coefficient; DWI, diffusion weighted imaging.

Table 2 Dominant sequence distribution to PZ and TZ for T2w Imaging and DWI

PZ				TZ				Assessment without adequate DWI (PZ and TZ)				Assessment without adequate DCE (TZ)			
DWI	T2w	DCE	PIRADS	T2w	DWI	DCE	PIRADS	T2w	DWI	DCE	PIRADS	T2w	DWI	DCE	PIRADS
1	Any	Any	1	1	Any	Any	1	1	x	Any	1	1	Any	x	1
2	Any	Any	2	2	Any	Any	2	2	x	Any	2	2	Any	x	2
3	Any	–	3	3	≤4	Any	3	3	x	–	3	3	≤4	x	3
		+	4		5	Any	4		x	+	4		5	x	4
4	Any	Any	4	4	Any	Any	4	4	x	Any	4	4	Any	x	4
5	Any	Any	5	5	Any	Any	5	5	x	Any	5	5	Any	x	5

Any indicates 1-5. PZ, peripheral zone; TZ, transition zone; DWI, diffusion weighted imaging; DCE, dynamic contrast enhanced imaging sequence; PIRADS, Prostate Imaging Reporting and Data System.
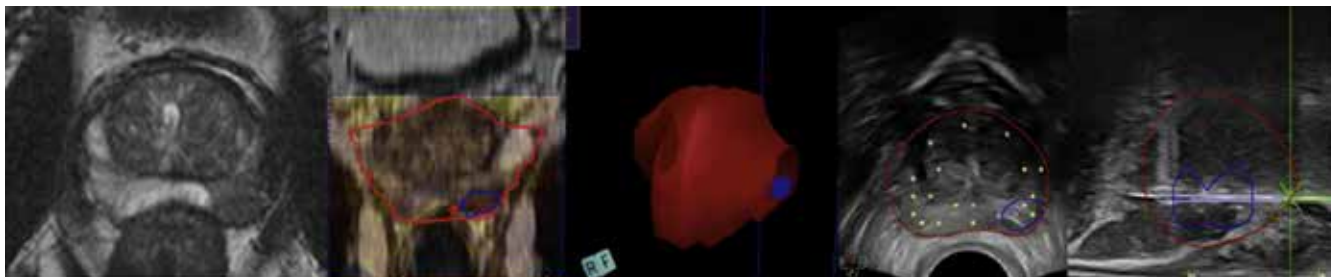
Figure 3 Example of MRI/TRUS-fusion guided biopsy using the MedCom system. MRI-targeted biopsy with ultrasound guidance and software registration. Series showing (from left to right): T2-weighted image showing a low-intensity lesion in the left peripheral zone, delineation of the target volume on the T2-weighted image, a three-dimensional model of prostate volume and target volume, registration of MR volume to ultrasound image, and the biopsy needle within the target volume on the ultrasound image. MRI, magnetic resonance imaging; TRUS, transrectal ultrasound.

to suspicious findings on T2w and/or DWI [*Table 1* (see part C) and *Table 2*] (56).

Technique of MRI targeted biopsy

In general, TB can be performed as direct in-bore MRI-guided biopsy, as VE biopsy or as fusion-biopsy with software-registration. Fusion-guided biopsies consist of co-registering pre-acquired MRI data with real-time US with the use of software and computing of the probe location and can be performed using elastic fusion systems (e.g., Koelis Urostation, Eigen Artemis) or rigid fusion systems (e.g., Philips Uronav, MedCom BiopSee; *Figure 3*).

Visual estimation

VE allows adaptation of TB in clinical practice without significant upfront cost, but carries a significant learning curve and lacks real-time feedback regarding accuracy (3,19,74). MRI and TRUS images are superimposed by a cognitive overlay of TRUS and MR images during biopsy, using a printed document or by displaying MR images on the screen of a workstation located in the TRUS room, adjacent to the TRUS platform (19). The physician aims the target lesion with knowledge of lesion localization on MRI. Several publications analyzed the value of cognitively performed TB. Lawrentschuk *et al.* detected a higher performance of cognitive TB over random cores, in particular in anterior lesions (75). Haffner *et al.* compared, in a retrospective study, results of TB with those of 12 random biopsies in 555 patients. A TB strategy alone would have necessitated only a mean of 3.8 cores per patient and avoided unnecessary biopsies in 38% of patients with a normal MRI, while avoiding the diagnosis

of insignificant cancer detected by random biopsies in 13% cases (18). In this study, 13 significant cancers were missed with TB alone and 12 significant cancers were missed with the standard approach (18). In another study, Puech *et al.* found that MRI prior to biopsy improved CDR which raised from 59% by 12-core SB to 65% by cognitive TB. With regard to significant cancer (Cancer core length >3 mm on any core and GS >3+3) CDR was 67% for TB and 52% for conventional biopsies (35). Labanaris *et al.* showed that TB allow an exact match of biopsy and surgical GS in 90% and concluded that MRI should be performed prior to biopsy to solve underestimation of GS by SB (76). Kasivisvanathan *et al.* detected a statistically not significantly lower CDR of TB (57% cancer detecion rate) compared to a strict reference-test of TPMB (62% CDR) (19). Wysock *et al.* published that VE was slightly inferior to MRI/TRUS-fusion biopsy for all PC (CDR 20.3% *vs.* 32.0%, P=0.1374) and for significant PC (CDR 15.1% *vs.* 26.7%) (74). In conclusion, the currently published studies show an improved accuracy and cancer detection compared to conventional TRUS-biopsy.

In-bore MRI-guided biopsy

The in-bore biopsy approach has the advantages of accurate depiction of needle placement, fewer sampled cores, and a low likelihood of missed targets if these are MRI-visible (20). In-bore biopsy is a targeted biopsy directly performed within the MRI tube. It has the disadvantage of increased cost- and time consumption and the inability to routinely sample the remaining gland (20,21). This is in particular important, as MRI misses app. 10% of significant lesions compared to final RP pathology (15,16). Quentin *et al.* demonstrate an excellent significant cancer detection by in-bore TB of 92.2% (21). In the series of Hoeks *et al.*

178

Radtke et al. Future of MRI in PC

265 patients with suspicious lesions on mpMRI with prior negative TRUS-biopsies underwent transrectal in-bore TB, resulting in a CDR of 41% with 87% of these detected cancers found to be clinically significant (20). Multiple studies have corroborated these results, demonstrating that in-bore MRI-guided biopsy is a feasible diagnostic technique in patients with prior negative biopsy, offering a median detection rate of 42%, significantly higher than reported detection rates for repeat SB (22). Pokorny *et al.* demonstrated that an MRI-guided biopsy pathway reduced the diagnosis of low-risk PC by 89.4% and increased the detection of intermediate-risk/high-risk PC by 17.7% (36). However, compared to RP specimen, 14.7% of patients were undergraded by the mpMRI and MRI-guided biopsy approach (36).

MRI/TRUS-fusion-guided biopsy

MR-fusion-guided TB are more often histologically informative and, thus, may overcome the limitations of cognitive TB through reproducible methods for identification of MRI lesions on ultrasound (3,74). Several commercial platforms have become available varying in the method of co-registration and utilizing different hardware platforms for aligning the biopsy with the co-registered image (3,77). MRI/TRUS-fusion-guided biopsy potentially has greater reproducibility due to less operator dependence and by providing real time feedback of actual biopsied locations, compared to VE (3). Disadvantages include higher costs for the software/device, dependence on the software for accuracy, and associated learning curve and operator training (3). Recent publications focused on the detection of PC and of significant disease compared to conventional SB or TPMB as reference-test (4,12,15,26,36,37,78,79). Using the Uronav system and conventional 12-core TRUS-biopsy as reference-test, Siddiqui *et al.* recently demonstrated in a cohort of >1,000 patients that TB diagnosed 30% more high-risk cancers versus standard biopsy (P<0.001) and 17% fewer low-risk cancers (P=0.002) using primary Gleason pattern four as significance level (26). Salami *et al.* and Rastinehad *et al.* used GS ≥3+4 as significant cancer and published that 14.3% to 20.9% of significant PC were detected by TB alone and missed by standard TRUS approach (37,78). Moreover, upgrading from insignificant to significant PC by MRI/TRUS-fusion guided biopsy occurred in 23.5% (37). On the other hand, 4/105 significant PC were missed by MRI/TRUS-fusion guided biopsy (37). Kuru *et al.* and Radtke *et al.* from our group analyzed the detection accuracy of TB compared to

a transperineal saturation biopsy as reference-test (12,79). TB detected significantly more PC than SB on per-core analysis (30% *vs.* 8.2%) (79). Analyzing the detection rates of TB versus transperineal SB, TB alone did not lead to a significantly lower detection of significant PC, defined as GS ≥3+4 (P=0.711) (12). At the same time, TB alone avoided overdiagnosis of 43.8% of low-grade tumors (12). Only applying TB in man with suspicious MRI (PIRADS score ≥2) may reduce both, cost and overdetection of low-risk PC, but would have underdiagnosed 11 patients with GS ≥3+4 PC (14.6%) (12). Using RP as reference test, Baco *et al.* demonstrated that 98% of index tumors, defined as the highest GS or biggest volume in case of multifocality with equal GS, were diagnosed by MRI and that the correct location was diagnosed in 98% by MRI/TRUS-fusion guided TB, using the Koelis Urostation (*Figure 4*) (15). However, in the larger prostatectomy cohort of the Siddiqui publication, the negative predictive value (NPV) of TB to exclude significant disease was only 70%.

Comparative studies of different targeted biopsy approaches

Only a few studies have compared the CDR between different targeting techniques and the results are controversial (5,35,74). In a study comparing VE with two MRI/TRUS-fusion devices, Delongchamps *et al.* reported that cognitive fusion-biopsy was not significantly better than SB, while both software co-registration devices tested (Esaote/MyLabTMTwice and Koelis/Urostation) significantly increased CDR compared to SB using conditional logistic regression analysis in a cohort of 391 patients (5). Wysock *et al.* compared MRI/TRUS-fusion-guided biopsies using the Eigen/Artemis system versus VE targeting in a prospective study including 125 men with suspicious lesions (74). They found that MRI/TRUS-fusion-guided biopsies had a slightly improved CDR compared to VE for all cancers (32% *vs.* 26.7%, P=0.1374) and for GS ≥3+4 (20.3% *vs.* 15.1%, P=0.0523). Puech *et al.* observed no difference in the CDR of PC for rigid software co-registration using MedCom Navigator compared to cognitive fusion TB (53% *vs.* 47%) (35). Additionally, no differences were detected for cancer positivity in the subgroups of posterior (46 of 79, 58%), anterior (33 of 79, 42%), or smallest (25 of 79, 32%) MR imaging targets (35).

Targeted versus systematic SB

Several publications investigated the detection accuracy
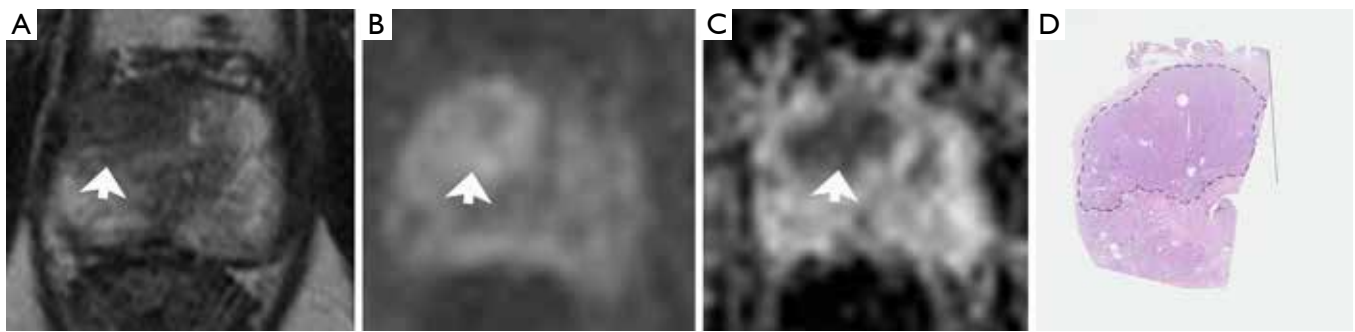
Figure 4 Accuracy of MRI and MRI-targeted biopsy compared to RP specimen. Results of prebiopsy MRI (T2w and DWI is presented) of a 68-year-old male with PSA level 8.1 ng/mL. One negative transrectal prostate biopsy was performed two years before. MRI detected a hypointense area in the right TZon T2w imaging (A, arrow), affecting the AFMS and the anterior horn of the right peripheral zone. Signal intensity was increased on Diffusion-weighted imaging on b value 1,000 sec/mm^2 (B, arrow) with a low signal intensity on ADC map (C, arrow). On biopsy specimen, a GS 4+3 PC occurred in three targeted cores. The patient underwent RP. RP specimen detected a GS 4+3 pT2c PC in the TZ, affecting the AFMS and anterior horn of peripheral zone with negative surgical margins (D, black line). GS, gleason score; DWI, diffusion-weighted imaging; MRI, magnetic resonance imaging; ADC, apparent diffusion coefficient.

of TB and SB alone or in combination (12,26,37,78). As described above, Siddiqui *et al.* from the National Institutes of Health (NIH) analyzed the value of TB *vs.* 12-core TRUS-biopsy in a cohort of 1003 men (26). Additional standard biopsy diagnosed 22% more PC , but 83% of these cases were low-risk PC, while only 5% were high-risk PC (26). The number needed to biopsy with SB in addition to TB to detect one high-risk PC was 200 men (26). Rastinehad *et al.* detected an upgrading from insignificant to significant PC by MRI/TRUS-fusion guided biopsy versus SB in 23.5% (37). On the other hand, only 3.8% of significant PC (defined as GS ≥3+4) were missed by MRI/TRUS-fusion guided biopsy (37). Le *et al.* reported that 17% were diagnosed with GS ≥3+4 PC by 12-core random biopsy alone, whereas 36% of GS ≥3+4 PC were exclusively detected by TB (24).

When MRI/TRUS-fusion guided biopsies are compared to transperineal saturation biopsies, exclusive detection by fusion-guided biopsy occurred in 20.9% of GS ≥3+4 PC and on the other hand in 12.8% by SB alone (12). Valerio *et al.* published in a systematic review of 14 publications, that MRI/TRUS-fusion biopsies detected a median of 9.1% additional clinically significant cancers (range, 5-16.2%) that were missed by standard biopsy alone (53). In contrast, standard biopsies detected a median of 2.1% (range, 0-12.4%) additional clinically significant cancers that were missed by MRI/TRUS-fusion biopsies (53). If the standard biopsy is only a TRUS-biopsy approach, the range of significant PC diagnosted exclusively by standard

biopsy stooad at 0-7% (53). In conclusion, Le *et al.* and Radtke *et al.* postulated that the combination of TB and SB represents the reference-standard for cancer detection (12,24). As long as TB miss 3.8-17% of significant PC (according to different definitions of significance), SB should not generally be omitted. Men with suspicion of PC should be counseled and then they may choose if they prefer reduction of overdiagnosis (TB alone) or maximum safety (combination of TB and SB) (12).

Correlation of MRI with surgical pathology

The utility of mpMRI to accurately detect PC and index lesions within the prostate is supported by several studies (15,80-82). An example from our group is given in *Figure 4*. The correlation between histologic lesions and MRI findings is difficult to determine, especially due to the variations between MR sections and prostatectomy slices, and the shrinkage during histopathologic processing of the specimens (80). Correction for this variability has been attempted by using a shrinkage factor, as well as different methods of co-registration between histology and imaging (82-85). However, the tissue shrinkage factor varies among different studies between 1.14 and 1.50 (83). Rosenkrantz *et al.* published one of the first series comparing whole mount sections to mpMRI in 51 patients. They detected a sensitivity and a PPV for an exact match between suspicious lesion on MRI and whole-mount section (belonging to the same region in a 18-sector scheme) in 60.2% and 65.3% of

180

Radtke et al. Future of MRI in PC

patients, respectively (81). Regarding approximate matches (discrepancy of up to one region) sensitivity was 75.9% and PPV was 82.6% (81). Turkbey *et al.* published a sensitivity of 80% for the detection of significant PC using T2w Imaging and a sensitivity of 94% for significant lesions in the PZ (82,86). Rud *et al.* used a biparametric MRI (T2w and DWI) to detect the index lesion on consecutive RP, defined as the tumor with EPE, or highest GS, or the largest tumor volume (TV), in that order of priority, in 199 patients (80). In their study, 92% of index lesions and 70% of all lesions were correctly identified by MRI (80). In lesions with TV above 0.5 mL, 86% of cancers were correctly assessed (80). Only 8% of index lesions and 14% of lesions >0.5 mL were missed by MRI (80). In the PIRADS era, Baco *et al.* analyzed the accuracy of mpMRI and MRI/TRUS-fusion guided biopsy in 135 consecutive patients (15). The location of the index lesion was correctly defined in 95% of patients (15). In the remaining 5%, the index tumor was invisible on MRI, but each had a small TV ≤0.4 mL (15). For the MRI-visible index lesions, targeted biopsy-proven PC showed 100% correspondence with the location of the index lesion in RP specimens (15,56,87). The combination of SB and TB detected the index tumor location correctly in 132/135 (98%) of patients (15). Interestingly, both studies demonstrate that the TV of the index lesion was underestimated by MRI (average underestimation 5.9% in Baco *et al.* without utility of a shrinkage factor and 30% in Rud *et al.* with a shrinkage factor of 15%) (15,80). Delongchamps *et al.* analyzed a cohort of 125 consecutive patients, who underwent mpMRI and both, TB and SB and consecutively RP for localized PC (16). MpMRI missed 10% of significant tumor foci on a per-lesion basis but none of the significant PC on a per-patient basis (16). MRI/TRUS-fusion guided TB missed 6% of their targets, resulting in an underdetection of 4% of significant PC (16). Their results suggest that TB alone performed in patients with a suspicious mpMRI would not leave patients undiagnosed with aggressive tumors (16). However, SB in patients with normal mpMRI but increased PSA should not be omitted (16).

MRI in patients with low-risk cancer—utility among men undergoing AS

Accurate risk stratification of patients undergoing AS versus active treatment is crucial for a sound AS program with high patient safety and to reduce potential morbidities associated with radical treatment (88). The most criticized part of AS is its dependence on the initial biopsy quality, since a

high number of PC are upgraded in GS after RP (39,89). This cumulates in rather low treatment-free survival rates, although a recently published large cohort study, including 819 patients harboring low-risk PC, has demonstrated excellent results in 10-year (98.1%) and 15-year (94.3%) PC-specific survival.

Accurate and safe stratification means to correctly rule in low-risk disease on the one hand, and to rule out significant PC on the other hand. With regards to AS candidacy, Vargas *et al.* demonstrated that mpMRI can predict upstaging in re-biopsies of AS patients in up to 98% (90,91). Similarly, the utility of MRI/transrectal ultrasound (TRUS)-fusion biopsies in AS cohorts has been demonstrated with encouraging results. Hu *et al.* have shown an upgrading in GS, core involvement and TV of 36% compared to 12-core-TRUS-biopsy (92). Best detection accuracy was demonstrated for the combination of TB and SB, as TB alone led to underdetection of 10% of significant PC (92).

A recently published systematic review focused on mpMRI in AS (93). Schoots *et al.* found an overall reclassification rate of 33% according to PRIAS criteria when TB are used after initial SB (93). In AS follow-up, mpMRI using PIRADS scoring has the potential to rule out significant PC. Mullins *et al.*, Vargas *et al.* and Da Rosa *et al.* showed a NPV of above 90% for a pristine MRI to rule out significant PC (90,94,95).

Thus, Schoots *et al.* conclude that MRI can detect clinically significant disease in one third to half of men at the start of surveillance and in the follow-up course (93). However, at the moment no robust data are available to support the use of MRI in place of repeat biopsy to detect progression over time (93).

The role of MRI in risk stratification and prediction of ECE and RP outcome

MpMRI has demonstrated excellent accuracy in index lesion detection, compared to RP specimens described by recent publications. However, risk group stratification for localized PC, as defined by NCCN criteria incorporates serum PSA-level, the GS of biopsy specimen and the clinical T-stage based on digital rectal examination (DRE) and lacks formal mpMRI incorporation (96).

In the 2012 ESUR guidelines a scoring system for extracapsular disease was published, including criteria regarding ECE, seminal vesicle infiltration (SV), adjacent and infiltration of distal sphincter and bladder neck (*Table 3*, *Figure 5*) (9).

Somford *et al.* validated the ESUR scoring system in a

Table 3 ESUR scoring of extraprostatic disease, according to ESUR prostate MR guidelines (9)

Criteria	Findings	Score
Extra-capsular extension	Abutment	1
	Irregularity	3
	Neurovascular bundle thickening	4
	Bulge, loss of capsule	4
	Measurable extra-capsular disease	5
Seminal vesicles	Expansion	1
	Low T2 signal	2
	Filling in of angle	3
	Enhancement and impeded diffusion	4
Distal sphincter	Adjacent tumour	3
	Effacement of low signal sphincter muscle	3
	Abnormal enhancement extending into sphincter	4
Bladder neck	Adjacent tumour	2
	Loss of low T2 signal in bladder muscle	3
	Abnormal enhancement extending into bladder neck	4
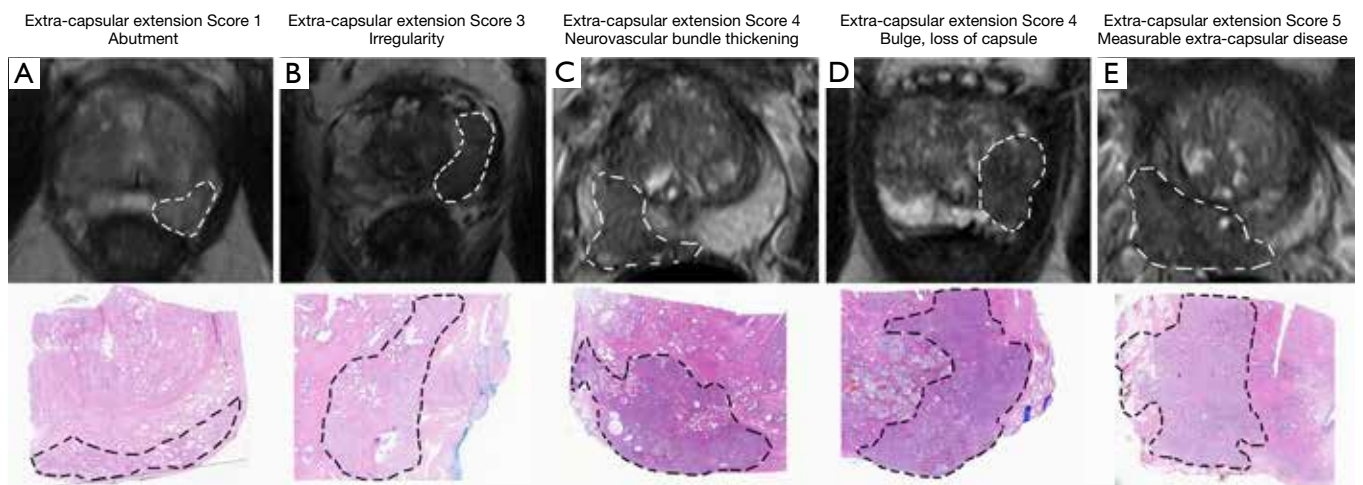
Figure 5 Figures are demonstrating the ESUR ECE-score on mpMRI (T2w Imaging) and histopathologic correlations for every level (9): (A) ESUR extra-capsular extension Score 1—Abutment; (B) ESUR extra-capsular extension Score 3—Irregularity; (C) ESUR extra-capsular extension Score 4—Neurovascular bundle thickening; (D) ESUR extra-capsular extension Score 4—Bulge, loss of capsule; (E) ESUR extra-capsular extension Score 5—Measureable extra-capsular disease. ESUR, the European Society of Urogenital Radiology; ECE, extracapsular extension.

cohort of 183 patients and found a NPV of 87.7% for ECE in patients with low-risk PC and a PPV for ECE in high-risk patients of 88.8% (28). This was slightly higher than an already excellent PPV in the studies of Cornud et al and Rud *et al.* (97,98). Marcus *et al.* investigated the impact of preoperative MRI on NCCN risk group classification in a cohort of 71 patients and found that 16.9% of patients were upstaged by MRI, mostly (83.3% of these subgroups) from intermediate- to high-risk (29). Additionally, the treatment regime was changed in 8.5% due to presurgical MRI (29). McClure *et al.* focused on differences between the initial surgical plan, according to D'Amico risk stratification, and the performed RP with knowledge of the presurgical MRI (99). They found a change in the initial surgical plan in 27% of patients, analyzing a cohort of 104 consecutive men (99). In their study, the surgical plan was changed to a nerve-sparing

technique in 61% and to a non-nerve-sparing in 39% (99). Wang *et al.* and Sala *et al.* from Memorial-Sloan-Kettering Cancer Center developed a score for ECE and seminal vesicle invasion that is analogous to the PIRADS score published by the ESUR (100,101). They found an AUC of 0.76 for MRI to predict seminal vesicle invasion. When the MRI score was compared to a Kattan nomogram, the combination of both significantly increased the AUC (AUC 0.86 versus AUC 0.80 for Kattan nomogram, P<0.05) (100). Sala *et al.* reported an AUC of 0.87 for prediction of ECE in a cohort of 45 patients how underwent salvage RP (101).

In contrast to clinical parameters like NCCN criteria, prostate MRI offers localized staging and allows the surgeon to sculpt the extent of PC and possible ECE (102). Another decision-making tool is maximum capsule contact length on MRI. Baco *et al.* analyzed the predictive value of MRI-determined tumor contact length to the capsule and found a correlation between ECE and tumor contact length of r=0.839 (P<0.001) using Spearman's regression (103). Based on ROC curve analysis, the best threshold of MRI determined tumor contact length was 20 mm (103).

In conclusion, MR imaging can potentially improve the accuracy of the surgeon's decision to resect or preserve the neurovascular bundle in patients undergoing RP (102).

Conclusions

MpMRI represents a potential tool to overcome limitations of conventional TRUS-biopsy. The established PIRADS scores make mpMRI generalizable and reproducible. Compared to the gold-standard of RP, MRI detects app. 90% of significant index lesions correctly, but TV is currently underestimated. Several techniques of TB are available and the optimal method has not yet been established. The encouraging results of in-bore TB and MRI/TRUS-fusion guided biopsies may outperform VE. TB detect significantly more significant PC compared to SB and are non-inferior compared to TPMB as reference test. However, as long as TB miss around 5-15% of significant PC (according to different definitions of significance), SB should not be omitted, especially in patients without previous biopsy. Among patients under AS, mpMRI helps to confirm AS eligibility, by correct prediction of upstaging in re-biopsies of AS patients and by accurately ruling-out significant PC. The future role of mpMRI in the presurgical setting of RP is emerging, as mpMRI can help to change the initial surgical plan, according to clinical decision making, in up to 30% of cases. Before wide incorporation of mpMRI, further

comparative studies, including randomized multicenter studies, and evaluation of cost-effectiveness are necessary, but potential cost-saving approaches like biparametric MRI are in the starting gates. Additionally the role of molecular imaging, e.g., PSMA-PET-CT/MRI, might provide ancillary information on tumor characterization and PC aggressiveness, and its role will be depicted in further publications (104,105).

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Utility of ADC measurement on diffusion-weighted MRI in differentiation of prostate cancer, normal prostate and prostatitis

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Abstract: To determine the utility of apparent diffusion coefficient (ADC) values in differentiation of prostate cancer from normal prostate parenchyma and prostatitis we obtained ADC values of 50 patients at b 100, 600 and 1,000 s/mm^2 diffusion gradients. The ADC values of prostate cancer group were significantly lower than normal prostate and prostatitis group at b 600 and 1,000 s/mm^2 gradients. The ADC values at high diffusion gradients may be used in differentiation prostate cancer from normal prostate and prostatitis.

Keywords: Diffusion-weighted MR imaging; prostate cancer; apparent diffusion coefficient (ADC); prostatitis

Introduction

Prostate cancer is most frequently encountered tumor of men (1). The prognosis of prostate cancer mostly depends on early diagnosis. Rectal examination, measurement of prostate specific antigen (PSA) and transrectal ultrasound (TRUS) have been used to detect prostate cancer at early phase.

Diffusion weighted magnetic resonance imaging (DWMRI) is based on the molecular diffusion of water molecules in biological tissues (2). Apparent diffusion coefficient (ADC) value is a quantitative parameter of DWMRI representing water diffusion in extracellular and extravascular space and capillary perfusion (3). ADC values have been shown to be decreased in various malignancies of different organs due to hypercellularity (4-7). Recent studies concluded that ADC measurement on DWMRI can differentiate malignant prostate lesions from benign prostatic tissue (8-11).

In this study, our aim was to investigate the role of ADC measurement in differentiation between prostate cancer, normal prostate parenchyma and prostatitis at low (b 100 s/mm^2), intermediate (b 600 s/mm^2) and high (b 1,000 s/mm^2) diffusion gradients.

Materials and methods

Patient group

Fifty patients (age range, 50-85 years old; mean age, 67 years old) presented with the suspicion of prostate cancer according to abnormal digital rectal examination and increased PSA levels were included in this study. Dynamic contrast enhanced MRI and DWMRI followed by TRUS guided biopsy were performed in all patients.

MRI protocol

Dynamic contrast enhanced MRI and DWMRI of patients were performed with 1.5 T GE Signa Hispeed Excite MR System (General Electric, Milwaukee, WI). MRI examinations were obtained with body coil in the supine position. In all MRI examinations prostate was localized in the center of the 4-channel TORSO or spine coil. The MRI protocol included axial and coronal T1- and T2-weighted images, axial and coronal T1-weighted images after intravenous contrast agent administration and DW images obtained at b 100, 600 and 1,000 s/mm^2 gradients. The parameters of DWMRI examinations were as follows:

Table 1 Mean ADC* values ($\times 10^{-3}$ mm^2/s) of group I, II and III patients at b 100, b 600 and b 1000 gradients**

Groups	Number of patients	b 100 ADC values	b 600 ADC values	b 1000 ADC values
Group I	11	2.34±0.05	1.72±0.03	1.47±0.02
Group II	9	2.30± 0.05	1.72±0.02	1.49±0.02
Group III	30	2.27±0.06	1.58±0.03	1.37±0.02

*ADC, $\times 10^{-3}$ mm^2/s; **b gradients, s/mm^2

matrix, 128×128; NEX, 1.0; FOV, 20; slice thickness, 5 mm; slice gap, 0; diffusion direction, all directions; TR, 8,000 ms; TE, 80 ms; mean region of interests (ROI), 45 mm^2. ADC maps obtained from DW images at b 100, b 600 and b 1,000 s/mm^2 gradients.

Analysis of the MR images and ADC measurement

ADC measurements of prostate were done on 3 separate levels (apical gland, midgland and basal gland) in the prostate of the patients. Twelve quadrants visualized as suspicious on T2-weighted images on each three gland level was used to measure ADC value constituting a total of 36 ADC value measurement at all three diffusion gradients in each patient. Twelve quadrant measurements were intended to be obtained from the localizations that biopsy specimens were obtained. The ADC values were measured by insertion of ROI which have the mean area of 45 mm^2. ADC measurements were performed on color-coded ADC maps automatically after calculating the diffusion difference between each gradient (b 100, 600 and 1,000 s/mm^2) and the b 0 gradient on a workstation (Advantage Windows, software version 2.0, General Electric Medical Systems). Monoexponential method was used in ADC measurements. A minimum mean square error estimator was used in monoexponential method to minimize the mean square error of the fitted ADC values.

Pathologic findings

Prostatectomy specimens were sliced between the surgical boundaries of proximal and distal urethra. Prostatectomy specimens were examined with 5 mm thick slices in accordance with 5 mm thick DW images. By this method, we aimed to make ADC measurement from the points as possible same with the pathological examination localizations.

The ADC values of benign and malignant prostate lesions were compared with the histopathologic results of prostatectomy and biopsy specimens. Patients with benign prostate lesions, prostatitis and prostate cancer were classified as group I, II and III, respectively.

Statistical analysis

Statistical analysis was performed with SPSS (Statistical Package for the Social Sciences) 10.0 for Windows programme. ADC values were defined as mean ± standard deviation. Student t test, and ROC analysis tests were used to compare ADC values of group I, II and III patients at b 100, 600 and 1,000 s/mm^2 gradients. The differences in the ADC values were considered to be statistically significant when the P value was <0.05.

Results

Histopathologic examination of TRUS guided biopsy results of 50 patients revealed 30 adenocarcinoma, 11 normal prostate parenchyma and 9 prostatitis. Radical prostatectomy was performed in 10 patients who were diagnosed as adenocarcinoma while 20 patients with prostate carcinoma were treated with medical treatment and transurethral resection (TUR) of prostate. Patients with prostatitis were treated with drug therapy.

Prostate cancers manifested with intense enhancement at arterial phase and exhibited wash-out at late phase on dynamic contrast enhanced MR images. Early enhancement with heterogeneous appearance and patchy pattern was observed on contrast enhanced MR images in patients with prostatitis. The ADC values obtained in all groups decreased with the increase in diffusion gradients. The distribution of ADC values of normal prostate parenchyma, prostatitis and prostate cancer group at b 100, 600 and 1,000 s/mm^2 gradients are illustrated on *Table 1*.

Mean ADC value of prostate cancer group (group III) was significantly lower than normal prostate parenchyma group (group I) (P=0.001) and prostatitis group (group II) (P=0.001) at b 600 and 1,000 s/mm^2 gradients (*Figures 1-6*). No significant difference was obtained between ADC values

Figure 1 Normal prostate. T2-weighted MR image demonstrates normal prostate parenchyma with hyperintense peripheral zone (arrows)
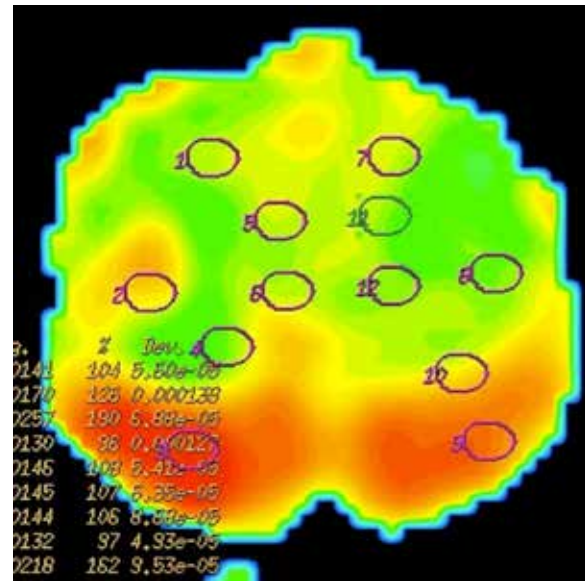
Figure 2 ADC map demonstrates green coloured central zone and red coloured peripheral zone representing restricted and unrestricted diffusion, respectively
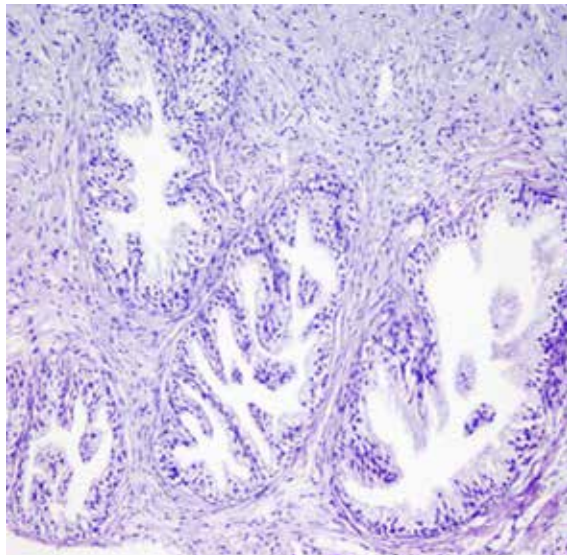
Figure 3 Histopathologic specimen of prostate reveals normal glandular structures
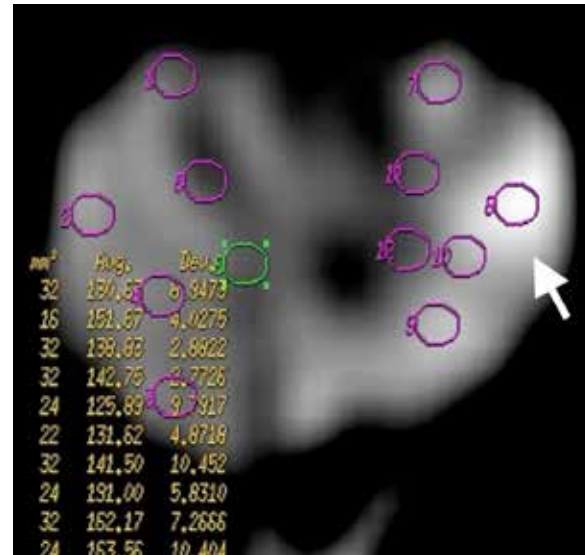
Figure 4 Prostate cancer. DWMRI at b 1,000 s/mm^2 gradient demonstrates increased signal intensity (arrow) in left peripheral zone of prostate representing prostate cancer

of group III patients and group I and group II patients at b 100 s/mm^2 gradient (P=0.72 and P=0.8, respectively). Mean ADC values of group I and II patients were not significantly different at b 100, 600 and 1,000 s/mm^2 gradients (P=0.90, 1 and 0.98, respectively) (*Table 2*).

The results of ROC analysis between ADC values of group I-III and group II-III patients are summarized in *Tables 3,4*, respectively. High sensitivity and low specificity values obtained in ROC analysis of ADC values in differentiation between group I-III patients and group II-III patients.
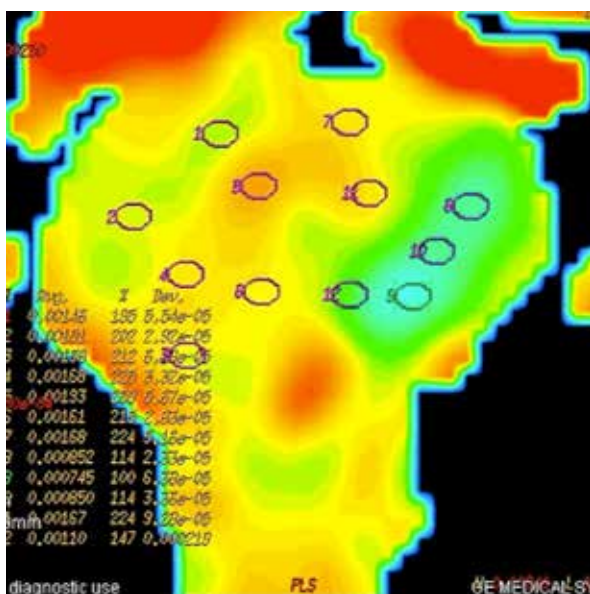
Figure 5 ADC map of DW image. Tumoral lesion appears with green colour representing restriction of diffusion. ROIs are inserted on 12 localizations in this section of prostate
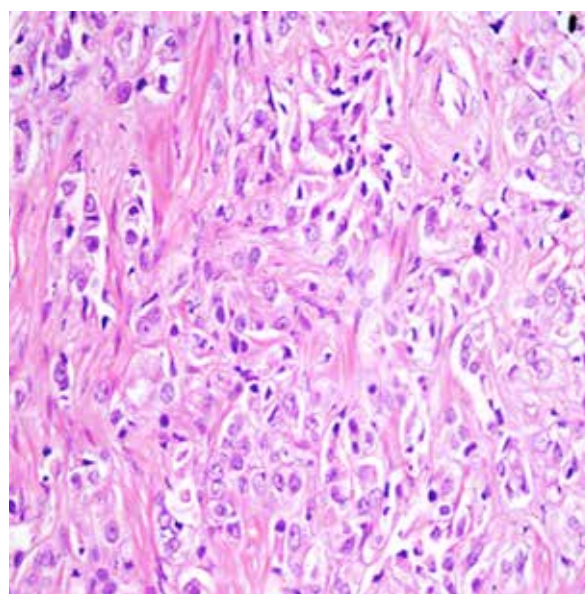
Figure 6 Histopathologic specimen of prostate cancer manifests with hypercellularity and absence of glandular structure

Table 2 The results of comparison between ADC values of group I, II and III patients at b 100, 600 and 1,000 s/mm^2 gradients

Groups	b 100*	b 600*	b 1,000*
Group I-II	P=0.90	P=1	P=0.98
Group I-III	P=0.72	P=0.001	P=0.001
Group II-III	P=0.8	P=0.001	P=0.001

*b gradients, s/mm^2

Discussion

The diagnosis of prostate cancer has been mainly based on TRUS guided biopsy. However TRUS guided biopsy is reported to have 40% false negative rates (12,13). Prostate cancer detection can be improved by imaging prostate with high- resolution T2-weighted scans and dynamic contrast enhanced MRI examination.

DWMRI is an emerging imaging technique that is able to demonstrate signal alterations secondary to restriction of molecular water movement in biological tissues. The ADC value as a quantitative parameter of DWMRI represents the magnitude of molecular movement in biological tissues. The restriction of diffusion results in decreased

ADC values on ADC maps generated from DW images. Since prostate cancer manifests with increased cellularity and altered glandular structure of prostate gland at histopathological examination the utility of DWMRI in the diagnosis of prostate cancer has been investigated before in several studies (8,9,14). These studies yielded significant difference between ADC values of prostate cancer and benign prostate lesions. The ADC values of cancerous lesions have been found lower than normal parenchyma of prostate (9,15). The sensitivity and specificity of DWMRI for prostate cancer detection were reported as 57-93.3% and 57-100%, respectively (16). The results of our study are in concordance with these results since we found significant difference between ADC values of normal prostate parenchyma and prostate cancer at b 600 and 1,000 s/mm^2 gradients. However we found no significant difference between ADC values of prostate cancer and normal prostate parenchyma at b 100 s/mm^2 gradient. The ADC values obtained at low diffusion gradients represent either molecular diffusion and perfusion characteristics of biological tissues. Blood perfusion cause increased ADC values even in the setting of diffusion restriction in the tissue at low diffusion gradients. The absence of significant difference between ADC values of prostate cancer and normal prostate parenchyma at b 100 s/mm^2 gradient may be attributed to perfusion effect of blood flow in the prostate.

Table 3 The results of ROC analysis between ADC* values of normal prostate parenchyma (group I) and prostate cancer (group III) at b 100, b 600 and b 1,000** gradients

	b 100 (ADC threshold value: 1.58)	b 600 (ADC threshold value: 1.52)	b 1,000 (ADC threshold value: 1.33)
AUC	0.527	0.607	0.579
P	0.1047	0.0001	0.0001
Sensitivity	92.93	77.27	71.46
Specificity	15.02	38.58	40.35

*ADC, ×10^{-3} mm^2/s; **b gradients, s/mm^2

Table 4 The results of ROC analysis between ADC* values of prostatitis (group II) and prostate cancer (group III) at b 100, b 600 and b 1,000** gradients

	b 100 (ADC threshold value: 1.61)	b 600 (ADC threshold value: 1.52)	b 1,000 (ADC threshold value: 1.34)
AUC	0.513	0.626	0.630
P	0.4700	0.0001	0.0001
Sensitivity	92.58	82.10	79
Specificity	15.75	38.58	42

*ADC, ×10^{-3} mm^2/s; **b gradients, s/mm^2

Koo *et al.* investigated the sensitivity results of various diffusion gradients (b 300, 700, 1,000 and 2,000 s/mm^2) in predicting prostate cancer localizations at 3 T MRI and they found that b 1,000 s/mm^2 gradient revealed higher sensitivity values (85%) than other diffusion gradients (17).

Prostatitis may present as acute or chronic illness of prostate. Although presenting symptoms of acute and chronic prostatitis are different they may mimic prostate cancer on conventional MRI with the appearance of low signal intensity on T2-weighted images and early enhancement on contrast enhanced MR images. Differentiation between ADC values of normal prostate parenchyma and prostatitis could not be achieved in our study. This may be secondary to inadequate restriction of diffusion in prostatitis. Comparison of ADC values between prostatitis group (group II) and prostate cancer group (group III) yielded significant difference at b 600 and 1,000 s/mm^2 gradients. Absence of difference between ADC values of group II and group III patients at b 100 s/mm^2 gradient may also be attributed to increased ADC values of group III patients secondary to perfusion effect of blood flow. Prostatitis is characterized by increased cellularity consisted of inflammatory cells which may result in diffusion restriction. However, increased perfusion and extracellular fluid resulting from edema in prostatitis may increase ADC values. As a result, we could not achieve to obtain significant difference between ADC values of normal

prostate parenchyma and prostatitis group in our study. The ADC values of prostate cancer, normal prostate tissue and prostatitis were also compared with 3T MRI studies which revealed significantly lower ADC values in prostate cancer than normal prostate tissue and prostatitis (18).

The results of our study revealed that b 600 and 1,000 s/mm^2 gradients were more helpful in differentiation between ADC values of prostate cancers and normal prostate parenchyma. None of three diffusion gradients yielded significant difference in differentiation of normal prostate and prostatitis in all levels of prostate. In our study we found that increased b values representing increased strength of diffusion gradient resulted in decreased sensitivity and increased specificity in differentiation prostate cancer from normal prostate gland and prostatitis (*Tables 3,4*).

This study has some limitations. The main limitation of our study was high possibility of mismatch of DWMRI and histopathologic slices. Shriveling and deformation of prostate specimens after formaldehyde fixation and leaned position of prostate resulting in nonvertical prostate position by urethra on DW images are the major causes of this mismatch (19). This mismatch limits the optimal evaluation of correlation between DW images and histopathological slices. Although b 600 and b 1,000 gradients were helpful in differentiation prostate cancer from normal prostate and prostatitis in our study, ultra-high b values such as b 2,000

was reported to improve the diagnostic performance of MRI with high sensitivity and specificity values (20). We did not measure ADC values of prostate lesions and normal prostate parenchyma at ultra-high *b* values due to decreased signal to noise ratio at ultra-high *b* values on our 1.5 T MRI system. We also did not assess the signal intensity changes in prostate cancer and prostatitis which would be helpful in detection of these lesions. The sensitivity of ADC values in detection of prostate cancer were in concordance with the literature but specificity values were lower than previous reports (*Table 3*) (16). This was attributed to low patient numbers and lack of assessment of signal intensity changes on DW images in our study.

In conclusion, DWMRI with ADC measurement may be used as a complementary imaging method in differentiation of prostate cancer from normal prostate parenchyma and prostatitis at intermediate and high level diffusion gradients.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Near-infrared fluorescence and nuclear imaging and targeting of prostate cancer

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Abstract: Despite advances in the treatment of castration-resistant and bone metastatic prostate cancer (PCa), there is still no clear demonstration that PCa growth and metastases can be unambiguously detected. We review recent advances including our own development of near-infrared fluorescence (NIRF) and near-infrared nuclear (NIRN) imaging approaches. We validated our results in experimental models of PCa bone and soft tissue metastases including PCa colonization at metastatic sites by injecting PCa cells either intratibially or intracardiacally. We describe our experience using noninvasive imaging and targeting modalities to probe PCa tumors grown at metastatic sites, molecular studies to understand the multiple molecular and cellular processes within tumor cells and their interactions with the tumor microenvironment, and targeting tumor growth at metastatic bone site. In this review, current knowledge and emerging technologies based on NIRF and NIRN disciplines will be summarized. Additionally the mechanisms of differential uptake of these agents by normal and cancerous cells will be described.

Keywords: Bone metastasis; heptamethine cyanine dyes; near-infrared fluorescence (NIRF); nuclear imaging; positron emission tomography (PET); prostate cancer (PCa)

Introduction

Prostate cancer (PCa) is a heterogeneous disease with the biological potential to develop an aggressive and lethal phenotype (1). PCa is the most common cancer diagnosed in men in the Western countries but only one of every 8-10 patients diagnosed with PCa will die from this disease (2,3). Thus it is important to develop effective methods not only to diagnose PCa but also to effectively select patients who need to be treated and avoid unnecessary treatment. It is also vital to target tumors that have recurred, escaping initial treatments and developing into metastatic disease. Molecular imaging of PCa using such conventional imaging methodologies as X-ray computed tomography (CT), magnetic resonance imaging (MRI) and ultrasound has been successful for detecting organ-confined or metastatic disease

for disease staging and companion diagnosis and prognosis (4,5). However, these techniques also show the limitations of current cancer-specific imaging and cannot reliably delineate the occurrence, the location, and the biochemical status of cancer and cancer metastases. Positron emission tomography (PET)/single-photon emission computed tomography (SPECT) for nuclear imaging have distinct advantages over conventional imaging, with unique noninvasive properties capable of monitoring the metabolic and molecular characteristics of cancer cells. These approaches utilize short-lived nuclear tracers and acquire signals emanating from the body after administration of imaging agents that target cancer-specific alterations, including glucose, amino acid and fatty acid metabolism, receptor status, cellular proliferation, tumor hypoxia and blood flow (6). Currently, several PET tracers have been applied for clinical imaging of both early-

and late-stage PCa, such as ^{18}F-fluorodeoxyglucose (FDG), choline (^{11}C and ^{18}F labeled), ^{18}F-dihydrotestosterone (FDHT) and sodium ^{18}F-fluoride (NaF). These molecular imaging agents can potentially greatly elevate our ability to diagnose, prognose and monitor treatment responses in PCa patients.

Near-infrared fluorescence (NIRF) imaging agents can potentially increase the sensitivity and specificity of cancer diagnosis because NIRF have low autofluorescence, tissue absorbance, and scatter at NIR wavelengths (700-900 nm) (7). Once the specificity of these agents is established, a number of modifications can be made including the conversion of these agents into effective nuclear imaging probes, or conjugating these agents with effective therapeutic drugs to target cancer metastasis. The NIRF approach shares common physical properties with nuclear imaging techniques, such as the potential use of tracer administration of a contrast agent, which enables the development of combinational use of both techniques for cancer imaging (see below). Most conventional approaches for utilizing NIR dyes in cancer imaging require chemical conjugation of NIR fluorophores with appropriate cancer-targeting moieties such as peptides, antibodies, aptamers, growth factors and metabolic substrates (8-11). However, these approaches are less efficient because of tumor cell heterogeneity. Only a limited number of cancer cells may express the specified cell surface receptors or ligands, and the constant evolution of cancer cells known to occur within a tumor could alter cancer cell surface properties (12,13). The specificity and affinity of targeting ligands can also be altered subtly following chemical conjugation (14). Our group has recently discovered a novel class of NIR heptamethine carbocyanine dyes that can be used as dual imaging and targeting agents, and demonstrated their preferential accumulation and retention in cancer but not normal cells, enabling cancer-specific targeting without the need for chemical conjugation (15,16). These agents greatly fulfill the unmet needs for specific imaging of PCa despite its heterogeneity, which to some extent is inadequately assessed by conventional methods. In this review, we will discuss recent advances in the development of novel NIRF, NIRN and NIRF-derived agents and techniques for imaging and targeting localized and metastatic PCa.

NIRF imaging of prostate cancer

NIRF imaging agents

Indocyanine green (ICG) is a noninvasive NIRF imaging

dye that has been used clinically for more than 50 years for ophthalmic angiography and to determine cardiac output and liver blood flow and function (7,17). This tricarbocynanine dye is also used in cancer patients to map sentinel lymph nodes, for the detection of some tumors due to their enhanced angiogenesis, and for angiography during reconstructive surgery as the only NIRF agent approved by the United States Food and Drug Administration (FDA) (18-21). Most human NIR imaging studies employ ICG within the blood and lymphatic vasculatures. ICG and its derivatives are widely used clinically and show reasonable NIR features (ex/em 760-785/820-840 nm) and the capability to image normal but not cancerous tissues, generating weaker fluorescent properties (i.e., lower extinction coefficients) in comparison to the NIRF described herein (7,17). Several new NIRF agents have been developed including heptamethine carbocyanine dyes. Some of these agents have become commercially available in recent years, such as Cy5.5 (22) and IRDye 800-CW (23), which have been coupled with peptides or antibodies and successfully used for the targeted visualization of neoplastic tumors in animal models. Marshall *et al.*, for example, reported a safety and toxicity study of the NIRF dye IRDye 800CW in rats that showed no evidence of organ toxicity based on the hematologic, clinical chemistry and histopathologic analyses of tissues harvested from the experimental animals (23). These new NIRF agents offer great promise for future clinical applications of NIRF imaging agents.

NIRF imaging of prostate cancer

Conventional application of NIRF agents in PCa imaging use the chemical conjugation of specified NIRF agents to cancer cell-surface targeting moieties, such as peptides and antibodies recognized as tumor-specific biomarkers (19). One such biomarker is prostate-specific antigen (PSA), an androgen receptor (AR) target gene expressed almost exclusively by prostate epithelium. PSA, expressed by both benign and malignant prostate epithelium, potentially reflects active AR signaling activity (24). Since AR-mediated cell signaling pathways are known to determine human PCa initiation and progression, and AR expression and activity are elevated in some castration-resistant PCa (CRPC) (25), many groups have sought to image PCa through the use of PSA as a biomarker. PSA is initially produced as a catalytically active serine protease (free PSA), released subsequently into the perivascular space, by

196

Wu et al. NIFR and nuclear imaging and targeting of PCa

rapid and irreversible conversion to non-catalytic forms (26,27). In a recent study, Ulmert *et al.* described a new approach to specifically image tumor-associated free PSA in multiple preclinical models with [89]Zr-labeled 5A10, a novel radiotracer consisting of a monoclonal antibody that specifically recognizes an epitope adjacent to the catalytic cleft of PSA (28). In line with the same principle, Ho *et al.* developed an enzymatically cleavable peptide sequence labeled with NIR fluorophores (ex/em 740/770 nm), PSA750, which is optically quenched (>95%) and only becomes fluorescent upon cleavage by enzymatically-active PSA, yielding a significantly increased NIR signal from the site where PSA is secreted or deposited (29). Currently, serum PSA levels are widely used clinically as an indicator for primary screening and a biomarker for therapeutic responses, but PSA expression alone cannot distinguish benign from malignant prostate epithelium (30,31). The noninvasive imaging tools developed for measuring tumor-associated PSA expression could more clearly reflect AR-driven changes in PSA expression and could be used to supplement the current clinical PSA test.

Another cell-surface antigen for PCa is prostate-specific membrane antigen (PSMA). PSMA, elevated in CRPC, is a plausible target for imaging probe development (32-34). Radioactive ^{111}In-labeled PSMA antibody has been used as a reagent in SPECT imaging. In addition to being a biomarker of PCa, PSMA has been proposed as a target for image-guided surgery due to its cell surface-expressing characteristics. To better identify prostate tumor margins during surgery, Nakajima *et al.* synthesized an activatable anti-PSMA monoclonal antibody (J591)-NIR fluorophore (ICG) conjugate and tested it in a PC-3 prostate tumor xenograft mouse model (35). Prior to binding to PSMA and cellular internalization, the conjugate yielded little light. However, upon internalization and cleavage, NIR-ICG intensity in PCa was elevated by 18-fold, permitting the detection of PSMA+ PC-3 but not PSMA- PC-3 tumors for up to 10 days after a low-dose (0.25 mg/kg) injection. In another study, Humblet *et al.* synthesized a single nucleophile-containing small molecule specific for the active site of PSMA enzyme that is chemically conjugated to an ICG derivative (10). This conjugate shows high-affinity binding to PSMA in xenograft prostate tumors by NIRF imaging. These NIRF-based PSMA-targeting imaging approaches are reproducible at the cellular level in PCa as well. Liu *et al.* developed a NIRF imaging probe (Cy5.5-CTT-54.2) by chemical conjugation of a Cy5.5 derivative with a potent PSMA inhibitor (CTT-54.2) (36).

The probe displays high potency against PSMA and has demonstrated successful application for specifically labeling PSMA+ LNCaP PCa cells in both 2D and 3D cell culture conditions.

Another approach to developing targeted molecules relies on the aberrant metabolic pathways established by cancer cells. Cancer cells have been observed to exhibit altered metabolism and increased requirements for glucose and glutamine, which is facilitated by the overexpression of glucose transporter proteins (GLUTs) (37-39). FDG is a glucose analog that has been used extensively in cancer detection and therapeutic monitoring in the form of a ^{18}F-FDG probe, detected by PET (40,41). The ^{18}F-FDG probe, however, has several limitations, such as an extremely short half-life for following by positron-emitting nuclides, exhibiting low spatial resolution, being a radioactive compound, and is also abundantly taken up by tissues with high basal metabolic rates, such as the brain. To overcome these intrinsic PET imaging limitations, an NIRF imaging approach has been proposed and developed as a replacement for metabolic imaging using a similar targeting principle. Korotcov *et al.* (42) designed an NIRF probe (cypate) chemically conjugated to one or more glucosamine (GlcN) moieties, a common substrate for all 4 isoforms of GLUTs with higher affinity for GLUT2 than glucose (39,42,43), and demonstrated good uptake of the GlcN-linked NIRF probe in both PC-3 cell culture and live mice. In summary, diverse molecular imaging approaches from different research groups have demonstrated the effectiveness of targeting cell surface-based biomarkers or the metabolic differences between normal and cancer cells for monitoring PCa growth and recognizing the surgical margins of PCa tumors during surgery.

NIRF imaging of metastases in experimental prostate cancer models

Imaging of lymph node metastases

Early-stage PCa develops seminal vesicle invasion and micrometastases to surrounding lymph nodes (LNs). Pelvic LN dissection (PLND) is widely used in the clinic for nodal staging and assessing LN metastases in PCa (44). However, this method is invasive and underestimates LN involvement; 40-50% of patients are found to have metastatic LNs outside the standard resection area (45,46). There is an unmet need for more accurate noninvasive diagnostic techniques. Abnormal lymphatic function has

been associated with a wide spectrum of diseases and is also intimately involved in cancer metastases (7,47). Preclinical cancer studies show apparent dilation and proliferation of tumor-draining lymphatic vessels and tumor-draining lymph node remodeling (47), which offers a targeting opportunity for noninvasive lymphatic imaging with NIRF probes. As the only NIRF agent approved by the FDA, ICG has been used noninvasively in humans to uniquely detect blood and lymphatic vasculatures and used intraoperatively in sentinel LN mapping for visualization of tumor-draining LNs in several types of cancers including PCa (7). Van der Poel *et al.* recently reported an approach integrating ICG with a radioisotope (^{99m}Tc) and nanoparticles for injection into the prostate prior to surgery for improved surgical guidance via multimodal imaging, particularly fluorescence imaging (48). *Ex vivo* analysis further revealed a strong correlation between the radioactive and fluorescent content in the excised LNs. Similar detection outcomes in the percentages of PCa metastases to LNs by NIRF imaging (63%) and μPET/CT (64%) were also reported by Hall *et al.*, further confirmed by pathological examination (49). Alternatively, targeting PCa biomarker molecules as a conventional approach facilitates NIRF detection of LN metastases. Cai *et al.* (50) synthesized NIRF dye (Alexa Fluor 680) conjugated BBN[7-14]NH_2 peptides that target gastrin-releasing peptide receptors (GRPRs), which showed high densities on the cell membranes of prostatic intraepithelial neoplasia (PIN), primary PCa and invasive prostatic carcinomas with predominately negative expression in normal prostate tissue and benign prostatic hyperplasia (BPH) (51,52), in an orthotopic PC-3 xenograft mouse model. Within 2-hour post-injection, the conjugate reached the highest binding specificity and affinity in GRPR+ cancer *in vivo*, and LN and peritoneal metastases were detected by NIRF imaging, which was later confirmed by histopathology. These studies across different groups suggest the promising future clinical utility of NIRF imaging in PCa staging and laparoscopic LN dissection, which would be boosted by improved imaging devices with better signal capture from deep tissue in the near future.

Imaging of bone metastases

PCa patients develop lethal bone metastases in the late stage of disease progression. Current clinical examinations of PCa bone metastases rely on PET/SPECT/CT scans. While potential limitations in the use of these radiotracers in

PET and SPECT imaging including exposure to radiation, difficulty in synthesizing radioactive tracers, and short interval of tracer retention in tumors, they are nevertheless preferred in patients due to the variability of tumor depth in patients. NIRF imaging, however, offers companion diagnostic and prognostic value by having a potentially better safety profile, lower cost and desirable sensitivity for the diagnosis of cancer metastasis. Recent advances have been made in multiple NIRF imaging approaches to the visualization of bone metastases utilizing different targeting strategies. We previously synthesized a NIR quantum dot (QD) probe (QD800) conjugated to an anti-PSMA antibody and demonstrated the specific recognition of C4-2B xenografts residing in mouse tibia by this NIRF conjugate using the IVIS Imaging System (53). The maximal light emission was detected 30 minutes after intravenous injection of QD800 conjugate in mice. In addition to the conventional chemical conjugation method, we were also able to detect metastatic PCa cells in either bone metastases or in the precursor state as circulating tumor cells (CTCs) by a novel class of heptamethine carbocyanine dyes (see below) in an orthotopic ARCaP$_M$ xenograft mouse model (16).

Novel heptamethine carbocyanine fluorescence dye-based imaging of prostate cancer

Heptamethine carbocyanine fluorescence imaging agents

We recently discovered a novel class of NIRF heptamethine carbocyanine dyes, IR-783 and MHI-148, which is an effective cancer-specific imaging agent. These agents show preferential uptake and retention in cancer but not normal cells (15,16). By conjugating chemotherapeutic agents with these dyes, we observed tumor-specific cell kill without cytotoxicity in host mice, suggesting the potential use of these carbocyanine dyes as carriers for cancer-specific targeting by small molecules (see below). The advantages of this new class of NIRF as imaging agents are: (I) they have relatively low molecular weights that facilitate their effective uptake into both localized and metastatic cancers; (II) they can be synthesized in pure form and are stable upon storage; (III) they are taken up by many different types of cancer cells, including circulating or disseminated tumor cells and cancer tissues regardless of their cell-surface properties and their plasticity; and (IV) they have the potential of recognizing live versus dead cells and therefore can be used for follow-up in patients subjected to treatment by hormonal, radiation and chemotherapeutic agents. We

found that these dyes can be retained in established PCa cell lines (C4-2, PC-3 and ARCaP$_M$) with the dyes enriched in the mitochondria and lysosomes, but not in normal prostatic epithelial and fibroblast cells. In an orthotopic ARCaP$_M$ xenograft mouse model receiving intraperitoneal injection of low dose of IR-783 (10 nmol/20 g), the NIRF signals were specifically detected in the primary tumor and its associated bone metastases within 24 hours by fluorescence optical imaging. Similar targeting was also found in spontaneously developed prostate and colon tumors in the TRAMP PCa and ApcMin/+ colon cancer mouse models, respectively (16). Recently, we extended these studies to demonstrate successful detection of dye uptake in freshly harvested human PCa tissue xenografts as well as CTCs using these novel NIRF agents. Additionally this novel class of NIRF showed no systemic toxicity when mice were given a 100-fold excess of the imaging dose of NIRF.

Near-infrared nuclear imaging of prostate cancer using novel heptamethine carbocyanine dyes conjugated to ^{64}Cu as PET probes

Although early detection of PCa by blood tests for elevated levels of PSA has led to early treatment and a reduction in death rates, PSA level alone does not distinguish between PCa and normal conditions that cause elevated PSA (30,31). Because PCa can be a very slow-growing cancer, even confirmation of PCa cells in a biopsy gives no indication whether an active disease will progress within the individual's lifetime. As a result many patients receive painful repeated needle biopsies when PSA is found to be elevated. Successful management of prostate cancer requires early detection, appropriate risk assessment, and optimal treatment to avoid the development of CRPC with potential of lethal progression (54). Nuclear imaging is an attractive modality for the detection and characterization of disease because it is non-invasive, quantitative, provides dynamic real-time data, and allows the diagnosis and follow-up of patients undergoing therapy (6). Whether the development of new nuclear imaging probes could offer the opportunity of differentiating indolent from aggressive prostate tumors remains untested.

Different radionuclide-based imaging agents for planar, PET and SPECT imaging are currently used in the clinic with some under development for PCa. Clinical agents include the bone agent methylene diphosphonate (MDP, ^{99m}Tc labeled), the metabolic agent ^{18}F-FDG, and receptor targeted radiolabeled monoclonal antibodies including the PSMA-based ProstaScint. Agents in development for PCa

include acetate (^{11}C labeled), choline (^{11}C and ^{18}F labeled), 1-aminocyclobutane-1-carboxylic acid (^{11}C and ^{18}F labeled), radiolabeled AR binding compounds, radiolabeled peptides and small molecules for receptors overexpressed in PCa or PCa-associated tumor neovasculature. Despite a variety of probes using nuclear imaging modalities, neither the detection of minimal disease nor the prediction of indolent versus aggressive PCa has been accomplished. A simple, accurate method for localizing cancer within the prostate for focal therapy also remains elusive.

As part of our extensive search for agents that might have cancer-specific uptake in PCa, we reported the discovery of a new class of heptamethine carbocyanine dyes that allow detection of human and mouse tumors with a high degree of sensitivity and specificity (15,16). To further improve the sensitivity and clinical utility of this class of carbocyanine dyes for deep-tissue detection of tumors, we modified the dye by conjugating it with a positron-emitting radionuclide (see below) and tested its feasibility in cultured human PCa cells and metastatic prostate tumors in mice.

We synthesized a PET/NIRF probe PC-1001 by conjugating MHI-148 with a DOTA chelator and subsequent chelating with ^{64}Cu for independent PET and fluorescence imaging (*Figure 1*). This two-component probe is the first example of novel tumor-specific fluorescent dye with both targeting and detection properties in one component and a second component (^{64}Cu-DOTA) with the capability to perform nuclear imaging. In contrast to all other multimodal probes reported to date, the tumor-targeting component is separate from the detection component and thus needs a minimum of three components. The NIRF imaging modality has the merits of simplicity, convenience, and high throughput. The NIRF property of the probe simplifies the early stages of its development for *in vitro* and *in vivo* optimization of parameters and its validity prior to final live animal PET imaging. NIRF alone has inherent shortcomings such as its low resolution and non-quantitative nature. The sensitivity and resolution of NIRF imaging is severely influenced by position and depth of the imaging probes in the body. The positron emitting property of the conjugated probe can overcome the shortcomings of NIRF and provide high sensitivity and deep-tissue spatial resolution for initial detection of primary tumors and their metastatic lesions. Tumor size can then be monitored over time with NIRF imaging. Our recent results showed successful PC-1001/NIRF image of a mouse with a metastatic RANKL-overexpressing LNCaP tumor demonstrating two superficial tumors in the mouse (55)
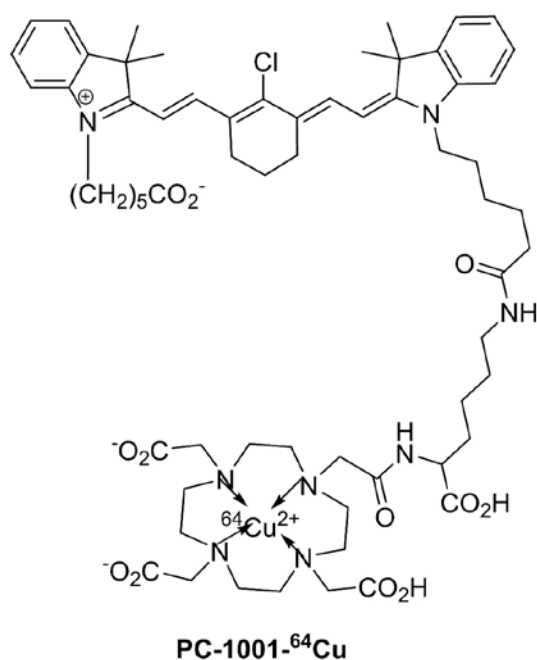
Figure 1 The chemical structure of a PET/NIRF probe, PC-1001-^{64}Cu.

Figure 2 Dual-modality NIRF/PET imaging of prostate tumors in a mouse model. A SCID mouse was intratibially inoculated with RANKL-overexpressing LNCaP tumors (arrows). Twenty-four hours after PC-1001-^{64}Cu (~17 MBq) was i.p injected, NIRF and PET scans were performed with a Caliper Spectrum System and a Focus-120 microPET scanner, respectively. (A) NIRF (ex/em 745/820 nm) image shows two superficial tumor spots; (B) Coronal slice of PET image reveals three isolated tumors. Similar results were obtained using the intracardiac injection of cancer cells to study cancer bone and soft tissue metastases in which more tumors were detected by PET than bioluminescence or NIRF staining, providing further evidence that PET has the advantage of detecting deep-seeded cancer metastases.

(*Figure 2A*), while the PC-1001/PET image of the same mouse revealed an extra tumor in a deep location (*Figure 2B*).

The dual-modality PC-1001 molecular imaging probe described above has demonstrated its applicability for tumor detection and quantitative image analysis in a metastatic PCa mouse model. The PC-1001 probe is accumulated specifically in cancerous tissue with good contrast to normal tissue. This probe could be useful in assisting the evaluation of anti-cancer therapies, anti-cancer drug discovery, and cancer-related biological studies. Further biological and toxicological evaluation of this imaging agent is ongoing, with the aim of advancing into clinical trials. Other laboratories have also designed dual-modality PCa imaging probes. In a recent study, Ghosh *et al*. designed a multimodality chelation scaffold (MMC) that combined a radiometal chelating agent (^{64}Cu) and NIRF dye (a IRDye 800CW derivative) (56). Using MMC-immunoconjugate to target an epithelial cell adhesion molecule (EpCAM), which shows elevated expression with PCa biochemical recurrence and correlation with Gleason scores (57,58), multimodal imaging studies indicated higher tumor accumulation of the dual-labeled conjugate compared to either single-labeled agent in a PC-3 tumor-bearing mouse model. Another example is EphB4, a key member of the Eph receptors overexpressed in numerous tumor types including PCa (59-61),

which has been developed as a promising imaging target. Zhang *et al*. reported the visualization of EphB4+ PC-3M PCa xenografts with an EphB4-binding peptide (TNYL-RAW)-nanoparticle conjugate dually labeled with NIRF fluorophores (Cy7) and a radioisotope ^{111}In, with both NIRF and NIRN imaging (62). The high accumulation of dually labeled peptide in PC-3M tumor could be significantly reduced after co-injection with an excess amount of unlabeled peptide, suggesting the specificity of this imaging probe for recognizing EphB4 receptor. These reports support the promise of dual labeling imaging approaches for improved sensitivity and depth of imaging compared to NIRF imaging alone.

Novel heptamethine carbocyanine drug conjugates for targeting castration-resistant and bone metastatic prostate cancer

Because CRPC is considered as the most advanced and lethal form of PCa, we synthesized a number of

200

Wu et al. NIFR and nuclear imaging and targeting of PCa
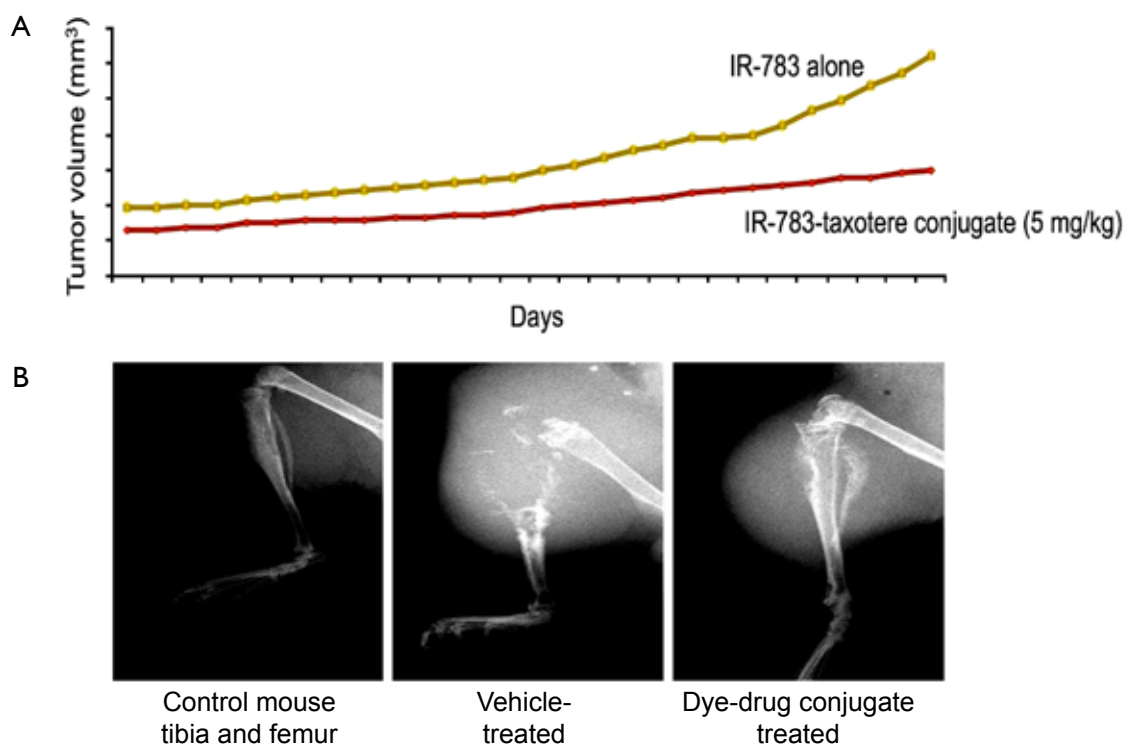
Figure 3 IR-783-docetaxel conjugate reduced prostate tumor growth in mouse skeleton. Nude mice were intratibially inoculated with osteolytic human PC-3 tumors followed by treatment with IR-783-docetaxel conjugate (5 mg/kg). (A) Tumor growth was determined by measuring tumor volume; (B) Bone lesions assessed by X-ray, showed improvement upon IR-783-docetaxel treatment. Note IR-783-docetaxel treated mouse has improved bone lesions when compared to mouse injected with vehicle.

heptamethine carbocyanine drug conjugates to target metastatic PCa more efficiently. The basic principle of these studies is to use heptamethine carbocyanine dyes as the drug carriers. They will be covalently conjugated to drugs through a linker composed of either an ester or a peptide bond. Upon uptake, these dye-drug conjugates accumulate exclusively in tumor tissues. Results using IR-783-docetaxel conjugate showed metastatic PCa tumor shrinkage in mouse tibia (*Figure 3*). While mice treated with this dye-drug conjugate showed no visible toxicity or reduced body weights, control mice treated with docetaxel alone had dramatically reduced body weights and over 50% mortality. These encouraging results have been shown to be repeatable using other dye-drug conjugates. Future safety studies are necessary before moving this concept into the clinic.

Mechanisms of uptake of heptamethine carbocyanine dyes by prostate cancer cells

We have studied the underlying mechanisms by which heptamethine carbocyanine dyes are taken up specifically into cancer but not normal cells. We investigated the effects of tumor hypoxia, a common condition found in a wide range of cancer cells or solid tumors, on the uptake of heptamethine cyanines into cancer cells. In our unpublished studies (Wu and Shao *et al.* 2013), unlike ICG that has a relatively low value of tumor-to-background ratio at 1.4-1.7 (63), measured at 24 hours after the administration of the dye into tumor-bearing mice, we showed that this ratio increased to 9.1 in tumors when MHI-148 was used, which was further enhanced by 2-fold when cells were maintained under hypoxic conditions. We found that a superfamily of organic anion carrier transporters, named organic anion-transporting peptides (OATPs), plays key roles accounting for the differential uptake of these dyes into cancer but not normal cells. We conducted microarray analyses and later confirmed by qRT-PCR, western blots and immunohistochemistry that specific isoforms of OATPs might be responsible for cancer-specific dye uptake. Currently there are 11 known human OATPs

classified into 6 families and subfamilies on the basis of their amino acid sequence homologies, which facilitate the transport of a large number of substrates, including organic acids, drugs and hormones into cells in a highly substrate- and pathophysiologic-dependent manner (64). The dye uptake and retention in cancer cells can be blocked completely by several competitive inhibitors of OATPs, such as bromosulfophthalein (16). Increased expression of select OATPs, such as OATP1B3 and OATP2B1, by either aberrant gene regulation or genetic variation, has been reported in clinical PCa, particularly during progression to a CRPC state (64-67). Notably, OATP1B3 also serves as a testosterone transporter (68,69), and its transporting activity may be further exacerbated by low levels of testosterone in CRPC (67,70,71). In addition, we and another group dissected a direct regulatory mechanism of OATP1B3 expression by hypoxia through HIF1α in PCa cells, which provides a functional link among different mediators that enhance dye uptake. Specific accumulation of dye and dye-drug conjugates in cancer cells can also be attributed to the high-affinity binding of this class of dyes once they enter into cells to interact with nucleic acids and proteins, which warrants further investigation.

Conclusions and future perspectives

We demonstrated the specific uptake and retention of a novel class of NIRF imaging agents, heptamethine carbocyanine dyes that can be used for imaging solid tumors and CTCs freshly harvested from human patients. This new class of NIRF imaging agents has been successfully tested as dual NIRF and NIRN agents using PET/SPECT to detect PCa bone and soft tissue metastases in experimental models. By further conjugating this class of NIRF/NIRN agents with cancer therapeutic drugs, we found that they can serve as drug carriers for the safe delivery of chemotherapies to experimental tumors. We found also that the differential uptake of this class of negatively charged carbocyanine dyes into cancer but not normal cells was largely due to the presence of specific isoforms of OATPs, coupled with specific metabolisms regulated by hypoxia and mitochondrial membrane potentials and the physical chemical reactions of this class of dyes when in close contact with nucleic acids and proteins in cancer cells. While promising data have accumulated thus far, crucial evaluation of the PK, PD and toxicity of the dyes and dye-drug conjugates are needed before this group of novel compounds can be moved into the clinic for improved cancer diagnosis, prognosis and treatment.

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Standard cystectomy fits all: truth or myth?

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Abstract: Radical cystectomy (RC) with pelvic lymph node dissection (PLND) followed by urinary diversion is the treatment of choice for muscle-invasive bladder cancer (BC) and non-invasive BC refractory to transurethral resection of the bladder (TUR-B) and/or intravesical instillation therapies. Since the morbidity and possible mortality of this surgery are relevant, care must be taken in the preoperative selection of patients for the various organ-sparing procedures (e.g., bladder-sparing, nerve sparing, seminal vesicle sparing) and various types of urinary diversion. The patient's performance status and comorbidities, along with individual tumor characteristics, determine possible surgical steps during RC. This individualized approach to RC in each patient can maximize oncological safety and minimize avoidable side effects, rendering 'standard' cystectomy a surgery of the past.

Keywords: Personalized cystectomy; bladder cancer (BC); urinary diversion; pelvic lymph node dissection (PLND); nerve sparing; seminal vesicle sparing

Introduction

Bladder cancer (BC) is a significant global health threat (1) with more than 30,000 deaths per year (2). It is also one of the most costly cancers from diagnosis to death (3). Pelvic lymph node dissection (PLND) and radical cystectomy (RC) followed by urinary diversion is established as the gold-standard treatment for BC invading the bladder muscle (MIBC) and for non-muscle-invasive BC refractory to transurethral resection of the bladder (TUR-B) and/or to intravesical instillation therapies such as bacillus Calmette-Guérin (BCG) and mitomycin (4-6). Removal of the bladder, however, leads to significant perioperative and postoperative morbidity, with a 90-day complication rate as high as 50% to 70%, and thus can negatively impact the patient's quality of life (7-9). Performance status (10), biological age, and preexisting comorbidities (best assessed by the Charlson Comorbidity Index (10,11) are important factors affecting the choice of treatment and the risk of complications after surgery. An individualized approach to therapy is therefore necessary in each patient to maximize oncological safety and minimize the risk of avoidable side effects. The keys to achieving these goals are paying close attention to patient selection before surgery and to specific details during surgery, and providing meticulous postoperative care, instruction, and follow-up to help patients cope with their new urinary diversion. Prior to RC, therefore, several factors must be considered in order to maximize each patient's benefit and to reduce potential harm from this demanding surgery.

Is bladder sparing a viable alternative to RC in MIBC?

The goal of bladder preservation in MIBC is to avoid morbidity and potential mortality in RC without compromising oncological outcomes. Several bladder-sparing options exist: TUR-B alone, partial cystectomy, radiotherapy, chemotherapy alone, or multi-modality therapy. Among these options, trimodal therapy involving maximal TUR-B followed by cisplatin-based chemotherapy and radiotherapy is the most widely accepted strategy (12). While bladder sparing is the only option in patients who are unfit for surgery due to medical comorbidities [with poor 4-year overall survival (OS) rates of 30-42% (13-15)], its value in medically operable patients has not yet been

206

Roth and Thalmann. Personalized cystectomy for bladder cancer

defined due to the lack of randomized controlled trials comparing RC with bladder-sparing therapies. However, the reported 5-year cancer-specific survival (CSS) rates of 50% to 60% (16-18) and 5-year OS rates of 36% to 52% (18-21) are poor when compared to the cystectomy outcomes in centers of excellence reporting 5-year CSS of 83.5% (22) and 5-year OS of 68% (23). Moreover, patients fit for surgery and treated by bladder preservation are rigorously selected, with exclusion criteria such as hydronephrosis, carcinoma in situ (CIS), or impossibility to perform a maximally safe TUR-B, and thus are positively selected compared with the population undergoing primary RC (12). Bladder sparing in medically operable patients, therefore, does not seem to be an equivalent treatment option. In fact, bladder-sparing procedures may only delay RC. Since studies have shown worse survival rates if RC is delayed for more than 3 months (unless the delay was for neoadjuvant chemotherapy) (24,25), prolongation of the interval between diagnosis and RC due to attempted bladder sparing may negatively impact treatment outcome.

Does anesthesia impact outcomes after RC?

One of the key factors affecting optimal outcome following RC is individualized optimization of anesthesia aimed at reducing blood loss, lowering postoperative complications, and improving functional results of orthotopic bladder substitution (26-29). These goals can be achieved mainly through the use of continuous administration of norepinephrine peri- and postoperatively, thus facilitating restrictive deferred intraoperative fluid management (30,31). Additionally, thoracic epidural analgesia leads to a need for minimal opioids peri- and postoperatively, thus accelerating recovery of bowel function and postoperative recovery (29) and reducing postoperative catabolism (32), pulmonary complications due to better diaphragmatic function (27), postoperative stress/inflammatory response, and cardiovascular morbidity in high-risk patients (28,33). It is important, however, to screen patients for contraindications for thoracic epidural analgesia, such as bleeding disorders or anticoagulation, since the possible complications (e.g., neuraxial hematoma and abscess) can be serious, inflicting permanent harm such as paraplegia. If a patient has contraindications for thoracic epidural analgesia, less effective alternatives such as preperitoneal or transversus abdominis plane blocks can be considered (34). In conclusion, individualized anesthesia is part of personalized cystectomy and its importance should not be underestimated.

Is pelvic lymphadenectomy mandatory—and how extensive should it be?

To draw the conclusions first: any kind of PLND is better than none, an extended PLND is better than a limited, but a super-extended has no benefit over an extended PLND (35). Already in 1982 DG Skinner reported that a meticulous PLND can make a difference, namely by decreasing the rate of local recurrence and even achieving a cure in some lymph node (LN)-positive patients (36). However, it is the patient with limited, in most cases microscopic, involvement of a few LNs who has the best chance of long-term survival (36).

Although recent dynamic LN mapping studies revealed that lymphatic drainage of the bladder is complex and individually coined, PLND should be performed bilaterally because cross-over lymphatic drainage is common (40%) (37,38). Roth *et al.* (37) provide strong evidence that a limited PLND (encompassing only the external iliac region and obturator fossa) removes only about 50% of all primary lymphatic landing sites compared to a 90% nodal clearance rate with an extended PLND (up to the mid-upper third of the common iliac vessels and including the areas medial and lateral to the internal iliac vessels). This finding was confirmed by a survival analysis in a cohort of 668 patients operated at two academic urology centers (39). The use of an extended PLND resulted in a more than twofold better 5-year recurrence-free survival rate for patients with ≤ pT3 pN0-2 disease compared to patients in whom the LNs were removed only in a limited field (extended PLND 49%, limited PLND 19%) (39). It could be concluded from this that the higher the proximal template (i.e., the more extended the PLND), the better the outcome. But is it really worthwhile to remove the remaining 8% to 10% of potential lymphatic landing sites located cephalad to the mid-upper third of the common iliac vessels (37)? In fact, a super extended PLND did not show a survival benefit compared to an extended PLND (40) —most probably because the occurrence of positive LNs higher than the endopelvic region is characteristic of systemic disease which cannot be cured by more extended surgery. A superextended PLND is, however, associated with higher morbidity, especially due to the possible harm to sympathetic nerve fibers crossing the bifurcation of the aorta. The mandatory extent of PLND has further been investigated by Roth *et al.* (38). In another dynamic mapping study they could show that the lateral bladder wall does not have lymphatic drainage to the contralateral internal iliac region, which is—on the medial side—another potential spot for harm to the autonomic nerves (38). The latter observation

was confirmed by a patho-anatomical study by Kiss *et al.* (41), who did not find metastases in contralateral internal iliac lymph nodes in unilateral tumors of the lateral bladder wall. Therefore, the location of the tumor within the bladder appears to influence the extent of lymphatic drainage, thus underscoring the need to individually modify the extent of PLND as part of RC.

Should nerve sparing be attempted?

Already in 1982, Walsh and Donker (42) pointed out that preservation of the dorsolateral neurovascular bundle during prostatectomy is important to avoid erectile dysfunction and improve postoperative continence. The significant aggressiveness of BC together with the decades-long limited knowledge regarding the relevant basic neurofunctional anatomy may account for the failure to further evaluate nerve sparing procedures during RC. Autonomic nerves comprised of sympathetic and parasympathetic fibers pass the pelvic plexus (43,44). The parasympathetic system is thought to be responsible for relaxation of the proximal urethra and erectile function, while the sympathetic system controls the tonus of the urethra at rest (42,45-48). Autonomic denervation has been shown to affect the proximal part of the sphincter plexus, causing intrinsic sphincter deficiency (49). Clinical data showing a positive influence of nerve-sparing prostatectomy on erectile function and urinary continence accord with intraoperative findings after electrical stimulation (50). These findings have finally found application in attempts at nerve-sparing during RC as a means of maintaining or improving urinary continence in patients with orthotopic bladder substitution (51). Autonomic nerve sparing, however, not only improves the chance of continence after orthotopic bladder substitution, but also has a positive effect on postoperative erectile function (52,53). While the benefit of autonomic nerve sparing for continence in orthotopic bladder substitution may be greater in the elderly due to their generally weaker urethral sphincter complex, it appears to be even more efficient if performed bilaterally. However, individual factors such as the extent and location of the BC within the bladder, and thus the feasibility of nerve sparing from an oncological point of view, determine if unilateral (on the side contralateral to the tumor) or even bilateral nerve sparing may be attempted.

Should seminal vesicles be preserved?

In men, the role of seminal vesicles in sexual behavior has not yet been documented. However, recent results of mouse experiments suggest that seminal vesicles have a significant effect on the sexual activity of male mice (54). Together with clinical observations that men report stronger sexual desire after seminal vesicle-sparing RC than after removal of the seminal vesicles, this finding raises the question whether seminal vesicle removal at RC should be mandatory. Several reports on simultaneous postero-inferior prostate capsule, vasa deferentia, and seminal vesicle preservation in RC with orthotopic bladder substitution found a postoperative improvement in sexual function and urinary continence (55,56). Minimizing surgical dissection in this manner reduces potential harm to the autonomic nerves, especially the autonomic nerve fibers of the pelvic plexus. The long-term oncological and functional results in these reports (55,56) were excellent (daytime continence and potency rates of up to 95%) and clearly superior to previously reported outcome data from a large series of men undergoing ileal bladder substitution following RC, in which 22% reported having erections without and another 15% with medical assistance (57). However, a meta-analysis of seven prostate-sparing RC series comprising 306 patients with organ-confined ($\leq$ pT2) BC found a systemic recurrence rate twice as high as for standard RC (58). There is controversy therefore regarding the oncological safety of prostate- and/or seminal vesicle-sparing RC. The controversy is even greater because the rate of concurrent prostate cancer and/or occult transitional cell carcinoma (TCC) of the prostate is as high as 48% (59-62), which is especially concerning since local recurrence of BC is lethal in most patients. BC location in the bladder neck or trigone as well as CIS were found to be associated with occult TCC in the prostate (60). As a consequence of the high prevalence of occult prostatic malignancies, RC with removal of the entire prostate but sparing of the seminal vesicles (together with the prostate capsule adjacent to the neurovascular bundle) was introduced. Recently published data show good oncological control and favorable functional outcomes (63). However, maximal local cancer control requires a restrictive selection of patients; patients should not have BC on the side ipsilateral to where seminal vesicle sparing is attempted, or on the trigone/bladder neck. Furthermore, prostate resection biopsies must be free of TCC. For BC located solely in the anterior bladder wall, bilateral seminal vesicle sparing is recommended.

In conclusion, seminal vesicle sparing is an increasingly popular option for individualizing RC to maximally improve postoperative functional outcomes and oncological safety,

208

Roth and Thalmann. Personalized cystectomy for bladder cancer

especially in patients with a strong desire to preserve libido and potency and with favorable tumor characteristics.

What type of urinary diversion—continent or incontinent?

Careful selection of patients is crucial for successful urinary diversion of whatever type (incontinent, continent cutaneous, orthotopic, rectosigmoid). Age is not a factor affecting whether a diversion can be done or not (64). The choice of urinary diversion depends mainly on performance status and preexisting comorbidities (65). All types of orthotopic bladder substitution by intestinal segment require careful patient selection. Preoperative biopsies from the distal prostatic urethra (male) or the bladder neck (female) should be negative except for CIS, which can be treated postoperatively by BCG instillations (66). Evidence of BC in these biopsies would prohibit sparing of the urinary sphincter from an oncological point of view, which is indispensable in continent orthotopic urinary diversions. Additionally, candidates for orthotopic substitution should be continent, physically and mentally able to adapt to and function with an orthotopic bladder substitute, and must be willing and able to participate in an active postoperative reeducation program and adhere to a strict follow-up regimen (57). A glomerular filtration rate of at least 50 mL/min is mandatory for continent reservoirs since the kidneys must compensate the metabolic acidosis following incorporation of bowel in the urinary tract (67). Candidates for a continent urinary diversion should also have normal liver function (risk of hyperammonemia if the reservoir becomes infected), and should not have undergone any previous major bowel resection in the ileocoecal area (risk of vitamin B12 deficiency) (67-69).

While the ileal conduit and cutaneous diversions are established options for urinary diversion (although both have considerable complication rates), orthotopic bladder substitution is now commonly used in both sexes (65,70,71). The type of urinary diversion does not affect oncological outcome (65). Therefore, factors such as patient comorbidities and tumor characteristics determine the type of urinary diversion, which in turn greatly influences how RC is performed (e.g., nerve sparing, seminal vesicle preservation).

Open or (robot-assisted) laparoscopic cystectomy?

Robot-assisted RC has emerged as an alternative to open RC based on its potential to reduce blood loss, the

transfusion rate, and the need for postoperative analgesia, and patients' quicker recovery of bowel function (72-74). Since the first experience with robot-assisted RC was reported in 2003 (75), a number of investigators have reported case series (76-79). However, the absence of long-term oncological and functional outcome data, and a possible selection bias in laparoscopic and robot-assisted laparoscopic RC series make it difficult to compare open versus laparoscopic and robot-assisted laparoscopic RC. Although feasible, laparoscopic and robot-assisted laparoscopic RC is thus still considered experimental (65). Open RC therefore remains the gold standard treatment for MIBC and for non-muscle-invasive BC refractory to TUR-B and/or intravesical instillation therapies due to the thorough characterization of long-term oncological outcomes (80).

Conclusions

Every patient who needs RC must be offered the best possible cancer surgery. This surgery, however, should not be the cause of unnecessary comorbidities. For this reason, each patient should be carefully assessed prior to RC with regard to key factors such as performance status, comorbidities, indications for or against specific procedures, and individual tumor characteristics. During RC close attention must be paid to those surgical details which vary from patient to patient, rendering every cystectomy an individual 'work of art'.

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Footnote

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Outlining the limits of partial nephrectomy

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Abstract: Amongst nephron-sparing modalities, partial nephrectomy (PN) is the standard of care in the treatment of renal cell carcinoma (RCC). Despite the increasing utilization of PN, particularly propagated by robot-assisted, minimally invasive approaches for small renal masses (SRMs), the limits of PN appear to be also evolving. In this review, we sought to address the tumour stage beyond which PN may be oncologically perilous. While the evidence supports PN in the treatment of tumours < pT2a, PN may have a role in advanced or metastatic RCC. Other scenarios wherein PN has limited utility are also explored, including anatomical or surgical factors that dictate the difficulty of the case, such as prior renal surgery. Lastly, we discuss the emerging role of molecular biomarkers, specifically epigenetics, to aid in the risk stratification of SRMs and to select tumours optimally suited for PN.

Keywords: Partial nephrectomy (PN); radical nephrectomy (RN); indications; limits

Introduction

Partial nephrectomy (PN) has evolved to become standard of care for small renal masses (SRMs) and select larger tumours that are amenable to nephron-sparing techniques. Early studies of cT1a (≤4 cm) tumours demonstrated that PN compared to radical nephrectomy (RN) was associated with improved overall survival (OS) (1-3). It is nevertheless important to acknowledge that the European Organization for Research and Treatment of Cancer Genito-Urinary (EORTC-GU) noninferiority phase 3 trial 30904 demonstrated improved OS for RN; however, in the renal cell carcinoma (RCC) subgroup, this trend lost significance (4). However, the increased recognition that chronic kidney disease (CKD) significantly impacts medical morbidity (5-8) has led the American Urological Association (AUA) and European Association of Urology (EAU) guidelines to support PN as the procedure of choice for cT1a tumours (9,10).

Laparoscopic PN (LPN) and robot-assisted PN (RAPN) as minimally-invasive alternatives to the traditional open PN (OPN) have seen increased utilization. While these techniques are associated with reduced postoperative pain and shorter lengths of stay all the while maintaining comparable oncologic outcomes to OPN (11,12), the scope of this review is not focused on the minimally invasive limits of PN. In this review, we aim to analyze tumour staging, renal functional, anatomical, and surgical factors to define limitations where PN may represent significant oncological risk or surgical morbidity, tipping the balance in favour of RN.

Tumour staging considerations

Primary tumour size

Nearly 25% of patients with RCC present with underlying CKD (13) and thus also carry a higher risk of cardiovascular comorbidities (14,15). This has led to increased consideration of PN for larger, specifically T2, renal masses (16). The oncological benefit in this setting remains controversial. Compared to RN, studies have reported equivalent recurrence-free, cancer-specific as well as OS rates for PN in lesions 4-7 cm in size (17-21). The boundaries of what is considered feasible for PN have expanded

to also include more complex tumour locations (17). An analysis of the Surveillance, Epidemiology, and End Results (SEER) database from 1998 to 2008 identified no statistically significant difference in 5-year cancer-specific mortality between RN and PN for lesions T2 or greater (P=0.2) (22). Given that tumour complexity may also influence PN selection, Kopp *et al.* utilized the radius, endophyticity, nearness to collecting system, anterior/posterior, and location polarity (RENAL) nephrometry score to compare T2 masses treated by either RN (n=122) or PN (n=80) (23). After a median follow-up of 41.5 months, no significant differences were identified between median RENAL score and 5-year progression-free survival (69.8% *vs*. 79.9% for RN and PN, respectively; P=0.115) or cancer-specific survival (82.5% *vs*. 86.7% for RN and PN, respectively; P=0.407).

The Mayo Clinic's experience of PN for T2, T3a, and T3b lesions was assessed by matching to a reference RN cohort by stage, tumor size, baseline renal function, age, and gender (24). PN, when compared to RN, was not associated with an increased risk of death from all causes (HR 1.11, 95% CI, 0.72-1.71; P=0.642) or RCC-specific mortality (HR 0.80, 95% CI, 0.43-1.50; P=0.489). Further, after a median follow-up of 3.2 years, 15 PN patients (22%) and 69 RN patients (33%) had metastatic disease (HR 0.74, 95% CI, 0.42-1.29; P=0.234). More recently, outcomes of PN on masses ≥7 cm (including 41% >10 cm) were reported by Long *et al.* In this series of 46 patients, the 5- and 10-year overall and RCC-specific survival rates were 94.5% and 70.9%, respectively (25).

Locally advanced tumours

Invasion and development of a thrombus of the renal vein or inferior vena cava (IVC) associated with a renal mass has historically been managed with RN and thrombectomy (26). Utilization of a nephron sparing surgery (NSS) in this situation remains controversial. It is motivated by preserving renal function in patients expected to have adequate life expectancy, given that the 5-year cancer specific survival is 40-65% in patients with locally advanced RCC, particularly with favorable prognostic factors (27). PN may have a role in this setting; however, the data to support this is limited. In one study comparing the oncologic outcomes based on the surgical technique in T2-T3b tumours, 34 patients underwent PN and 567 patients received RN (28). Disease recurrence was observed in four of the 34 PN patients (12%) versus 164 of the 567 (28.9%) in the RN cohort at a median

follow up of 24.2 and 13.2 months, respectively. While this may reflect a significant selection bias, wherein patients receiving PN likely also had more favorable comorbidities, on multivariate analysis, the type of surgical procedure was not an independent predictor of disease recurrence or RCC-specific death. It should be noted that there were tradeoffs in performing PN—namely, a higher procedure-related complication rate in three patients (9%): two had a prolonged urinary fistula, successfully managed with ureteric stenting, and one patient had hemorrhage requiring emergent re-exploration.

Cytoreductive surgery for metastatic RCC

Approximately 17-30% of patients with RCC present with metastatic disease (29). In appropriately selected patients, cytoreductive nephrectomy remains an important consideration, even in the contemporary tyrosine kinase inhibitor era (30). However, in light of the even shorter life expectancy of patients with metastatic RCC than those with locally advanced cancer, the relative benefit of nephron preservation has to be appropriately balanced with the risk of peri-operative morbidity to select candidates with favorable prognosis.

There are limited reports demonstrating the utility of PN in the metastatic setting. Krambeck and colleagues compared 16 patients who underwent cytoreductive PN and compared their results to 404 patients who underwent RN for cytoreduction (31). Of the 16 patients, 12 had a solitary kidney, which is an important imperative consideration with significant quality of life implications. The cancer specific survival rates of these 16 patients at 1, 3, and 5 years were 81%, 49%, and 49%, respectively. The cancer specific survival rates of the 404 patients who underwent RN at 1, 3, and 5 years were 51%, 21%, and 13%, respectively.

The feasibility and prevalence of cytoreductive PN was assessed by Capitanio *et al.* using the SEER cancer registry from 1988 to 2004 to identify 46 patients that received cyroreductive PN. This cohort was compared to a historical control group from 1997 that underwent RN (32). Multivariate analysis demonstrated no statistically significant difference in cancer specific survival between the two groups (HR 1.40; P=0.16). Additionally, Hellanthal and colleagues analyzed the SEER database from 1988 to 2005 and identified 70 patients with metastatic disease that underwent PN (2%). These patients were 0.49 times less likely to die from RCC than those who underwent RN (P<0.001) (33).

Babaian and colleagues examined the MD Anderson Cancer Center's experience with metastatic RCC patients who underwent PN from 1996 to 2011 (29). Of the 33 patients, 22 patients (67%) died from disease at a median follow up of 27 months. Patients that received PN for either a metachronous contralateral renal mass or a renal mass <4 cm had the best OS (61 and 42 months, respectively).

Renal function considerations

The main advantage of PN over RN is nephron preservation, leading to improved postoperative renal function. However, PN is still associated with some functional decline as the procedure inherently excises nephrons adjacent to the tumor and eventual reconstruction is required, which can lead to devascularization. Renal function after PN depends on the three "Qs": quality [baseline glomerular filtration rate (GFR)], quantity (percentage of renal function preserved), and quickness (ischemia time) (34). Many studies have demonstrated the importance of the "quality" factor, viewing the baseline GFR as the determinant of ultimate renal function following PN (35,36). Effective PN focuses on improving the precise excision of the tumor with minimal margins with careful reconstruction to maximize the number of preserved nephrons all the while minimizing the amount of ischemic injury associated with the procedure. The duration of ischemia remains an important surgeon-modifiable factor (37) and novel techniques to reduce it have shown promise (38).

To our knowledge, apart from end stage renal disease, there is no reliable lower-limit GFR threshold beyond which PN should not be attempted. Further, because the nadir GFR can be multifactorial and difficult to predict, the greatest benefit to nephron sparing may be in those with already compromised renal function. Towards this, enucleative and unclamped techniques may have a specific role in optimizing post-operative renal function in these scenarios (39). Further, the majority of data demonstrating that CKD has an increased risk of progression to end-stage renal disease, cardiac morbidity and even death is due to long-standing medical comorbidities such as diabetes and not surgically-induced causes of CKD. Loss of nephrons due to surgical resection may be associated with a decreased likelihood of CKD progression, relative to those with medically induced CKD (40,41). These data support the notion surgically induced CKD is less harmful than medical CKD, and since patients with CKD are at the greatest risk of further renal function decline with surgery, PN should be favored (42).

Nevertheless, PN is not without morbidity and an earlier reporting of the findings from the EORTC-GU noninferiority phase 3 trial 30904 showed an unanticipated OS benefit for RN (4). These level 1 results, although not necessarily reflective of contemporary PN, should continued to be weighed in the decision making process for patients with marginal renal function.

Other limiting factors

The decision to perform a PN has largely been dependent on the location, complexity, and size of the renal mass. There has been increasing adoption of renal nephrometry systems such as the RENAL score, PADUA prediction score, and centrality index (C-index) to assist in determining the complexity of the PN and the likelihood of complications (43,44). While these factors are critical for determining surgical approach, there are additional anatomic and surgical restraints that can dictate the feasibility of PN. The quantity and the quality of the perinephric fat can influence the technical difficulty of a PN. Much time can be allotted to removing adherent perinephric adipose tissue in preparation for a PN. It is this fat and not necessarily body mass index that is more likely to lead to poor surgical exposure during hilar dissection, tumour excision, and renorrhaphy (45). Recently, Davidiuk *et al.* introduced an image-based scoring system, the Mayo Adhesive Probability (MAP), to predict intraoperative adherent perinephric fat, based on posterior perinephric fat thickness and stranding (46). The anticipation of "sticky" fat would allow surgeons to counsel patients on predicted anatomical challenges during PN and the possibility of conversion to RN.

PN in the setting of prior renal surgery represents a potential limit to the application of NSS. In light of the fact recurrences may be related to the multifocality of renal masses, RN has traditionally been viewed as the optimal surgical strategy, however, the goal of nephron preservation has gained increased traction (47). Multiple, single-institutional experiences with PN in the setting of previous renal surgeries have been reported with acceptable peri-operative outcomes despite the challenging nature of these procedures (*Table 1*) (48-54).

Ablative procedures such as radiofrequency ablation and cryotherapy are increasingly utilized in the management of SRMs, particularly in non-surgical candidates (55-57). However, when ablation is unsuccessful and/or recurrence is identified, salvage surgery typically entails RN, although

Table 1 Surgical outcomes of PN after a previous ipsilateral kidney surgery: a literature overview

Study	Technique	Patients (tumors), n	Time from previous surgery, months	Solitary kidney, %	Tumor size, cm	OR time, min	Unclamped, %	Ischemia time, min	EBL, min	Postoperative complications, %
Autorino *et al.* (48)	RAPN	9 [12]	39.36*	33	2*	150*	33	17.5*	150*	22
Jain *et al.* (49)	RAPN	5 [5]	27$	NR	NR	156$	20	14.6$	220$	NR
Turna *et al.* (50)	LPN	25 [25]	79.2$	0	2.5$	180$	0	35.8$	215$	12
Johnson *et al.* (51)	44 OPN, 3 LPN	47 [51]	NR	33	3.5*	450*	39.3	31*	1,800*	43.2
Magera *et al.* (52)	OPN	18 [22]	46.8*	67	1.9*	NR	64	NR	700*	28
Kowalczyk *et al.* (53)	12 OPN, 1 LPN	13 [16]	33.75*	0	3.2*	NR	25*	27*	1,500*	50
Liu *et al.* (54)	OPN	25	99*	100	3.5*	8.5*	52	46*	2,400*	NR

*, median value; $, mean value. PN, partial nephrectomy; RAPN, robot-assisted PN; NR, not recorded; LPN, laparoscopic PN; OPN, open PN.

several series have demonstrated the possibility of PN, albeit representing very challenging surgical scenarios. Zermann *et al.* reviewed the Cleveland Clinic experience with attempted PN after either radiofrequency ablation or cryotherapy (58). In this small series, only two of the ten patients underwent successful PN on account of significant perinephric fibrosis whereas the remainder underwent RN or abortion of the procedure. A series from the National Cancer Institute reported more successful outcomes for PN after radiofrequency ablation (53). Most of these patients had severe fibrosis, but PN was completed in all patients (n=16) and required a prolonged operative time together with a greater risk of transfusion. In this series, there was a moderate increase in the risk of complications such as prolonged urine leak and the need for re-operation. The MD Anderson Cancer Center experience with salvage renal surgery following energy ablation was recently reported (59). Of 14 patients, 11 underwent PN while the remainder underwent planned RN. The procedures were technically difficult with two patients requiring intraoperative transfusions, together with the potential need for aggressive local resection to achieve negative margins (e.g., resection of the psoas muscle). Taken together, these studies suggest that, in the appropriately selected patients, PN is feasible despite being technically demanding.

Future directions

The increased utilization of abdominal imaging has amplified the incidental detection of SRMs. Of the 64,000 new masses in 2012, nearly 74% of them were SRMs (4 cm or less), and a substantial number of them were benign (20-30%) (60). Accurate characterization of these masses is necessary to guide treatment, including potentially avoiding intervention for benign lesions. Radiologic assessment and needle biopsy are currently used to better characterize SRMs, however, both of these approaches have limitations. Radiologic imaging has little value in predicting small renal mass growth whereas needle biopsies of masses smaller than 3 cm have a high false negative rate.

We are currently investigating the utility of DNA methylation markers from tissue obtained from needle biopsies to improve the diagnostic accuracy and gain prognostic information for SRMs. Our preliminary analysis has demonstrated that there are distinct methylation profiles for SRMs based on their histologic pathologies. When data from *ex vivo* needle biopsies is combined with data from The Cancer Genome Atlas (TCGA), the methylation profile of the specific histologic pathology appears to cluster together and can be used to differentiate one from another (61). Further investigations are underway to determine if methylation data provided from needle biopsies can play a role in the clinical management of patients with SRMs by detecting cancer at the early stages, reducing over-diagnosis and false positives, and accurately identifying non-malignant tumors. In addition to potentially avoiding aggressive treatment, a secondary goal is to identify patients that would most benefit from active surveillance.

Conclusions

Demonstration of safety, equivalent oncologic efficacy together with improved renal functional outcomes, has propelled PN as the standard of care for SRMs. There is increasing consideration of PN in the treatment of tumors of greater size, complexity as well as in locally advanced or cytoreductive scenarios. PN may also have a role in technically challenging scenarios of previous renal surgery or following failed renal mass ablation.

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Footnote

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Penile rehabilitation after radical prostatectomy: does it work?

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Context: Erectile dysfunction (ED) represents one of the most common long-term side effects in patients with clinically localized prostate cancer (PCa) undergoing nerve-sparing radical prostatectomy (RP).

Objective: To analyze the role of penile rehabilitation in the recovery of erectile function (EF) after nerve-sparing RP.

Evidence synthesis: Penile rehabilitation is defined as the use of any intervention or combination with the goal not only to achieve erections sufficient for satisfactory sexual intercourses, but also to return EF to preoperative levels. The concept of rehabilitation is based on the implementation of protocols aimed at improving oxygenation, preserving endothelial structure, and preventing smooth muscle structural alterations. Nowadays, the most commonly adopted approaches for penile rehabilitation after nerve-sparing RP are represented by the administration of phosphodiesterase type-5 inhibitors (PDE5-Is), intracorporeal injection therapy, vacuum erection devices (VED), and the combination of these therapies. Several basic science studies support the rational for the adoption of penile rehabilitation protocols. Particularly, rehabilitation, set as early as possible, seems to be better than leaving the erectile tissues unassisted. On the other hand, results from solid prospective randomized trials finally assessing the long-term beneficial effects of PDE5-Is, intracavernosal injections, or VED on EF recovery after surgery are still lacking.

Conclusions: Although preclinical evidences support the rationale for penile rehabilitation after nerve-sparing RP, clinical studies reported conflicting results regarding its efficacy on long-term EF recovery. Nowadays, which is the optimal rehabilitation program still represents a matter of debate.

Keywords: Prostate cancer (PCa); radical prostatectomy (RP); penile rehabilitation; phosphodiesterase type-5 inhibitors (PDE5-Is); sexual function

Introduction

Prostate cancer (PCa) represents one of the most frequently diagnosed malignancies in the United States and Europe (1). Radical prostatectomy (RP) is one of the most commonly adopted therapeutic options in patients with clinically localized PCa (2). Although this surgical approach is associated with excellent long-term oncologic results (3-5), the risk of short- and long-term adverse events is not negligible (5). Particularly, urinary incontinence (UI) and erectile dysfunction (ED) represent long-term sequelae observed in a non-negligible proportion of patients treated with RP. Of note, these side effects are associated with a profound detrimental impact on patient health-related quality of life.

A number of studies reported satisfactory urinary continence recovery rates after surgery (5-13). However, the postoperative recovery of erectile function (EF) still represents a major challenge for patients and physicians. When considering the risk of ED after surgery, several factors should be considered. First, preoperative patient

characteristics play a major role on the subsequent probability of recovering EF after surgery, where younger and healthier individuals have substantially higher recovery rates as compared to their older and sicker counterparts (9,13-18). Second, preoperative EF represents a significant predictor of the subsequent risk of ED after surgery (14-16,19). Indeed, the probability of achieving satisfactory erections after surgery is extremely low in patients with severe ED as measured by the International Index of Erectile Function (IIEF) (14-16,19,20). Moreover, patients with higher preoperative IIEF might represent individuals more motivated to achieve satisfactory erectile and sexual function after surgery (21). Finally, the surgical technique and surgeon experience have a substantial impact on the probability of ED after surgery (20,22-28). In this context, the knowledge of the surgical anatomy, together with continuous refinements in the surgical approaches and the introduction of minimally invasive surgery might have resulted in improved potency outcomes after surgery (23-31). For example, surgical approaches aimed at preserving the neurovascular bundles deputed to erections have been developed over the last decades (23,24,26). Moreover, the better visualization of the surgical field, as well as lower intraoperative bleeding and more precise excision associated with robot-assisted RP might result into improved functional outcomes at long-term follow-up (25).

Although accurate patient selection and improvements in the surgical technique might minimize the risk of ED after surgery, the removal of the prostate leads to the temporary loss of erections. This would, in turn, result into reduced oxygenation, pro-apoptotic, and pro-fibrotic changes in the corpora cavernosa that would finally result in postoperative ED (31-34). In this context, penile rehabilitation after RP has been proposed as a therapeutic option in order to break this vicious circle, promoting erectile tissue preservation and preventing pro-apoptotic and pro-fibrotic alterations in the corpora cavernosa (31,32).

This review aims at analyzing the rationale of penile rehabilitation after RP in patients with clinically localized PCa. Moreover, we sought to comprehensively evaluate basic science and clinical evidences supporting the adoption of penile rehabilitation after RP.

Evidence acquisition

A literature review was performed in September 2014 using the Medline, Embase, and Web of Science databases. The search strategy included the terms *prostate cancer*, *penile*

rehabilitation, *sexual function*, *radical prostatectomy*, *erectile dysfunction*, *phosphodiesterase type-5 inhibitors*, alone or in combination. We limited our search to large population-based retrospective studies and prospective investigations published between January 2005 and September 2014. Cited references from selected articles and from review articles retrieved in our search were also used to identify manuscripts that were not included in the initial search.

Records were considered relevant to this review if they included patients diagnosed with clinically localized PCa. Only studies including patients treated with RP were evaluated. Only studies assessing EF after RP according to validated tools were evaluated. Results coming from prospective multi-institutional trials were preferred over retrospective single-center studies. Case reports, editorials, and letters were excluded during the review process. Additionally, unpublished data or meeting abstracts were excluded because information that is needed to correctly assess the study quality is usually not available in abstracts.

The primary outcome was the recovery of EF after surgery. The definition of EF recovery was the one used by individual studies.

The articles that provided the highest level of evidence were evaluated and selected with the consensus of all the author of this manuscript. A total of 81 articles were reviewed (*Figure 1*).

Evidence synthesis

The definition of penile rehabilitation and its rationale

The pioneering work of Montorsi *et al.* (35) firstly introduced the concept of penile rehabilitation after RP in the year 1997. Nowadays, penile rehabilitation is defined as the use of any intervention or combination with the goal not only to achieve erections sufficient for satisfactory sexual intercourses, but also to return EF to preoperative levels (31). The rationale of penile rehabilitation is strongly linked to the pathophysiology of ED after RP. In healthy men, during erections the penis oxygenation rises from 35-40 to 75-100 mmHg and there is a balance between the flaccid status and erect status (36). Thus, erectile tissues oxygenation is preserved as long as men obtain erections regularly. In patients undergoing RP, neuropraxia occurs due to direct trauma, inflammation, heating, and ischemia affecting the cavernous nerves, even in men treated with nerve-sparing procedures (32,37,38). The chronic absence of erections related to cavernous nerves neuropraxia after
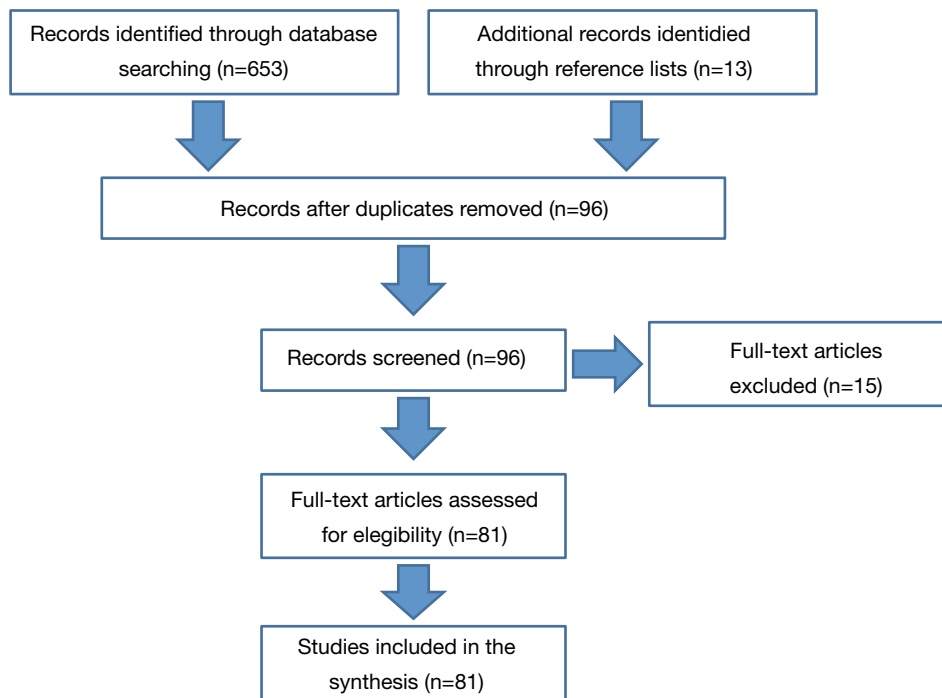
Figure 1 Flow diagram for the identification of the studies included in the literature review.

surgery would result in a state of persisting flaccidity. This, in turn, would lead to fibrogenic cytokine production (e.g., increased expression of TGF-β1, ET-1, NGF, and HIF-1α) and to structural changes in erectile tissues (36,39-41), which might finally result into smooth muscle apoptosis and fibrosis (42). The overexpression of fibrotic tissue would eventually impair the corpora cavernosa elasticity compressive action on subtunical venules, ultimately resulting in postoperative ED (32).

The concept of penile rehabilitation is based on the implementation of therapeutic protocols aimed at improving cavernosal oxygenation, preserving endothelial structure, and finally preventing smooth muscle structural changes (31,36). Nowadays, the most commonly adopted approaches for penile rehabilitation after RP in PCa patients are represented by the administration of PDE5-Is, intracorporeal injection therapy, vacuum erection devices (VED), and the combination of these treatments (31,32).

Phosphodiesterase type-5 inhibitors (PDE5-Is)

The administration of PDE5-Is represents the most commonly performed type of penile rehabilitation after RP, where up to 87% of the participants adopted this treatment strategy (43,44).

Although clinical studies reported conflicting results with regards to the efficacy of rehabilitation protocols based on the administration of PDE5-Is (45-51), preclinical data support the beneficial effects of these molecules (52-64). Indeed, several investigations demonstrated that the chronic administration of PDE5-Is to rats undergoing cavernous nerve injury might decrease erectile tissue fibrosis and apoptosis of smooth muscle (52,53,61-64). In this context, Sildenafil administration has been found to affect the expression of several genes involved in smooth muscle preservation and to reduce oxidative stress (32,56,58). Additionally, the administration of PDE5-Is has been proposed to prevent the degeneration of nervous tissue and stimulate neuroregeneration (61,65). Indeed, an increased amount of nerves has been observed after cavernous nerve injury in rats treated with sildenafil compared to their counterparts left untreated (61). Finally, PDE5-Is might also have a role in endothelial cell preservation, conserving platelet endothelial cell adhesion molecule-1 (CD31) and endothelial Nitric Oxid Synthase (eNOS) expression (54,66). On the other hand, human studies evaluating the morphologic changes to cavernous tissue after the administration of Sildenafil in patients treated with RP reported conflicting results, where neither elastic fibers nor connective tissue content substantially changed compared

222

Gandaglia et al. Penile rehabilitation after radical prostatectomy

to preoperative levels (67,68). However, these investigations are limited by the small number of patients evaluated, by heterogeneity in the surgical technique, and by the lack of a control group.

Taken together, the results of these preclinical studies raised the hypothesis that early administration of PDE5-Is might improve EF recovery after RP and inspired the design of several prospective trials. *Table 1* depicts the characteristics and results of studies evaluating the effectiveness of penile rehabilitation protocols based on the administration of four different PDE5-Is (*Figure 2*).

In their pioneering trial, Padma-Nathan *et al.* (51) randomized 76 patients treated with nerve-sparing RP to sildenafil or placebo nightly for 36 weeks followed by a 8-week drug-free period. Interestingly the authors demonstrated that the return to baseline EF was more marked for men treated with PDE5-Is compared to their counterparts receiving placebo. Moreover, the mean Erectile Function domain of the IIEF (IIEF-EF) was substantially higher in the sildenafil group. Finally, nightly administration of PDE5-Is markedly improved nocturnal penile tumescence and rigidity in patients treated with sildenafil (69). Although this study reported encouraging results and introduced for the first time the concept of penile rehabilitation using PDE5-Is, enrolment ceased early owing to interim analyses showing a lower response rate than expected. Moreover, the lack of a group receiving on-demand dosing limits the applicability of these findings. Under this light, it is worth reporting that a recent randomized trial evaluating patients treated with bilateral nerve-sparing robot-assisted RP failed to show statistically significant differences between patients receiving sildenafil on-demand or nightly at 13-month follow-up (45). However, these results are limited by the small number of patients evaluated (n=100), as well as by the lack of a placebo group and the relatively short follow-up period.

A well-performed randomized controlled trial evaluated the efficacy of penile rehabilitation using vardenafil (50). During a 9-month double-blind period, patients were randomized to placebo, nightly 10 mg vardenafil, and on-demand 10 mg vardenafil. Interestingly, on-demand vardenafil treatment resulted in significantly greater IIEF-EF scores and higher response rates to the Sexual Encounter Profile question 3 [(SEP3); "Did your erection last long enough for you to have successful intercourse?"] than placebo over the entire double-blind treatment period. Patients were then evaluated after an additional 2-month washout period. At this time-point EF recovery was not improved by nightly or on-demand vardenafil compared to placebo (*Figure 3*). Similarly, after a 2-month open-label period no statistically significant differences were observed among treatment groups with respect to IIEF-EF score or SEP3 success rates. Of note, the superiority of the on-demand dosing during the double-blind treatment period might be related to the pharmacokinetic of vardenafil, its onset of action, and the half-life of this drug (70,71). Indeed, patients receiving the drug on-demand might have had the full effect of the treatment when needed, while those in the nightly group had an effect so far as their sexual activity coincided with the administration of vardenafil (32). On the other hand, difficulties to reach a steady state with a single daily administration might limit the efficacy of chronic vardenafil dosing in terms of preservation of erectile tissue after surgery.

When evaluating the efficacy of tadalafil in the penile rehabilitation setting, a randomized controlled study failed to show an improvement in penile length and EF recovery after the administration of 20 mg tadalafil 3 times a week for 6 months (72). However, the small number of patients, short follow-up, and excellent postoperative EF-recovery rates in the placebo group raise some concerns regarding the generalizability of these findings. More recently, a larger study by Montorsi *et al.* (48) evaluated the efficacy tadalafil compared to placebo in the recovery of EF after nerve-sparing RP. Patients were randomized to receive 5 mg tadalafil once daily, 20 mg tadalafil on-demand, or placebo. At the end of the double-blind period (9 months), the IIEF-EF score improvement exceeded the minimally clinically important difference in both tadalafil groups. However, only patients treated with tadalafil once daily had a statistically significant difference in the change in IIEF-EF compared to placebo at this time point. Although the IIEF-EF and SEP-3 improved also during the open-label phase of the study exceeding the minimal clinically important difference for all the groups, no differences were observed between patients treated with tadalafil and placebo after open-label treatment (*Figure 4*). When considering the SEP-3 question, only patients receiving tadalafil once daily had a significant improvement compared to their counterparts receiving placebo at the end of the double-blind period and after open-label treatment. However, no significant differences were observed after 6 weeks drug-free washout. Finally, significantly less shrinkage of penile length was observed in the tadalafil once daily group as compared to placebo at the end of the double-blind period. Concluding, the administration of Tadalafil once daily seems

Table 1 Characteristics of studies evaluating phosphodiesterase type-5 inhibitors (PDE5-Is) in penile rehabilitation protocols

First author and year	Design	Population	Drug	Follow-up	Results
Mulhall et al. 2005	Single center non-randomized trial	Patients with functional preoperative erections treated early postoperatively (within 6 months) with oral sildenafil (n=58) Non responders were switched to intracavernosal injection therapy (n=74)	Sildenafil	18 months	• 64% patients in the rehabilitation group were responding to sildenafil with erections sufficient for sexual intercourse vs. 24% in the no rehabilitation group (P<0.001) • 52% vs. 19% patients in the rehabilitation vs. no rehabilitation group experienced natural erections • 95% vs. 76% patients in the rehabilitation vs. no rehabilitation group responded to intracavernous injection therapy
Padma-Nathan et al. 2008	Multicenter double blind placebo controlled randomized controlled trial	Four weeks after bilateral nerve-sparing radical retropubic prostatectomy, men with normal erectile function before surgery were randomized to double-blind sildenafil [50 (n=23) or 100 mg (n=28)] or placebo (n=25) nightly for 36 weeks, followed by an 8-week drug-free period before assessment of erectile function	Sildenafil	11 months	• Return to baseline erectile function: 27% vs. 4% for patients receiving sildenafil vs. placebo, respectively • RigiScan "responders"—100 mg: 33%, 50 mg: 24%, placebo: 5%
Bannowsky et al. 2008	Single center non randomized study	41 sexually active patients treated with nerve-sparing radical prostatectomy 23 patients with preserved nocturnal erections received sildenafil 25 mg/day at night. A control group of 18 patients was then followed	Sildenafil	12 months	• Sexual Health Inventory for Men (SHIM) score: 14.1 vs. 9.3 in patients treated with sildenafil vs. controls • Erections sufficient for vaginal penetration: 86% vs. 66% for patients treated with sildenafil vs. controls

Table 1 (*continued*)

Table 1 (*continued*)

First author and year	Design	Population	Drug	Follow-up	Results
Montorsi *et al.* 2008	Multicenter double-blind double-dummy randomized controlled trial	A total of 628 men, aged 18-64 yrs, were randomized to treatment. Study design consisted of a 9-mo double-blind treatment period, a 2-mo single-blind washout period, and an optional 2-mo open-label period. Patients received placebo, nightly vardenafil 10 mg, or on-demand vardenafil	Vardenafil	11 months	• On-demand vardenafil treatment resulted in significantly greater IIEF-EF scores and better SEP3 response rates than placebo over the entire double-blind treatment period • No statistically significant differences were observed among treatment groups in the proportion of patients with an IIEF-EF score of ≥22 or in SEP3 success rates after the washout period
Aydogdu *et al.* 2011	Randomized controlled trial	A total of 65 patients underwent bilateral nerve sparing radical prostatectomy. Patients were randomized to control without rehabilitation (group 1) or tadalafil 20 mg 3 times a week for 6 months rehabilitation group (group 2)	Tadalafil	12 months	• In group 1 there was significant decrease in penile measurements at month 3 compared to preoperative measurements • At the 12-month follow-up, there were no differences between stretched penile length in the two groups and no significant differences in erectile function between the two groups
Mulhall *et al.* 2013	Double blind, placebo-controlled randomized study	298 patients aged 18 to 70 years with a history of erectile dysfunction of 6 months or more after bilateral nerve-sparing radical prostatectomy. Patients were randomized to 100 or 200 mg of avanafil or placebo on demand for 12 weeks	Avanafil	12 weeks	• After 12 weeks there were significantly greater increases in SEP2 and SEP3 and change in mean IIEF-EF domain score with 100 and 200 mg avanafil *vs.* placebo (P<0.01) • Following dosing with avanafil 36.4% (28 of 77) of sexual attempts (SEP3) at 15 minutes or less were successful *vs.* 4.5% (2 of 44) for placebo (P<0.01)

Table 1 (*continued*)

Table 1 (*continued*)

First author and year	Design	Population	Drug	Follow-up	Results
Pavlovich et al. 2013	Single-institution, double-blind, randomized controlled study	100 preoperatively potent patients with clinically localized prostate cancer treated with nerve-sparing robot-assisted radical prostatectomy. Patients were randomized to either nightly sildenafil and on-demand placebo (nightly sildenafil group), or on-demand sildenafil and nightly placebo (on-demand sildenafil group; maximum on-demand dose six tablets/month) for 12 months. Patients then underwent a 1-month washout period	Sildenafil	13 months	• No significant differences were observed between treatments (nightly *vs.* on-demand sildenafil) in terms of postoperative IIEF-EF and return to baseline IIEF
Montorsi et al. 2014	Randomized double-blind double-dummy placebo controlled trial	Men ≤68 year of age with prostate cancer (Gleason ≤7) and normal preoperative EF who underwent nerve-sparing RP at 50 centers from nine European countries and Canada (n=423). A 1:1:1 randomization to 9 months of treatment with tadalafil 5 mg once daily, tadalafil 20 mg on demand, or placebo followed by a 6-week drug-free washout and 3-month open-label tadalafil once daily (all patients)	Tadalafil	13.5 months	• 20.9%, 16.9%, and 19.1% of patients in the tadalafil once daily, on demand, and placebo groups, respectively, achieved IIEF EF scores ≥22 after drug-free washout • At the end of double-blind treatment, mean IIEF-EF score improvement significantly exceeded the minimally clinically important difference (MCID: ΔIIEF-EF ≥4) in both tadalafil groups • For SEP-3 (MCID ≥23%), this was the case for tadalafil once daily only • At the end of double-blind treatment, penile length loss was significantly reduced versus placebo in the tadalafil once daily group only (P=0.03)

MCID, minimal clinically important difference; SEP, Sexual Encounter Profile; IIEF-EF, International Index of Erectile Function-Erectile Function domain.

226

Gandaglia et al. Penile rehabilitation after radical prostatectomy

Figure 2 Number of studies assessing the efficacy of PDE5-Is stratified according to the type of drug administered. PDE5-Is, phosphodiesterase type-5 inhibitors.

Figure 3 SEP question 3 patient success rates for the overall double-blind treatment and single-blind washout study periods. SEP, Sexual Encounter Profile. Reproduced with permission from Elsevier (50).

Figure 4 LS mean change in IIEF-EF score over time (error bars present the 95% confidence interval). IIEF-EF, International Index of Erectile Function-Erectile Function domain; LS, least squares; MCID, minimal clinically important difference; MMRM, mixed-effect model for repeated measures; OaD, once a day; PLC, placebo; PRN, on demand; TAD, tadalafil. Reproduced with permission from Elsevier (48).

patients treated with tadalafil once daily had substantially 1.9-fold higher probability of recovering EF after surgery as compared to their counterparts treated with placebo. However, this did not hold true for patients receiving tadalafil on-demand. Of note, the pharmacokinetic profile of tadalafil and his half-life might confer to this molecule the best profile for its use in the rehabilitation setting compared to other PDE5-Is such as sildenafil and vardenafil (70,71,74).

More recently, the efficacy of avanafil in the recovery of EF after RP has also been tested. A randomized trial by Mulhall *et al.* (75) demonstrated that patients receiving 100 or 200 mg avanafil on-demand had substantially higher IIEF-EF and SEP-3 response rates compared to placebo at 12-week follow-up after bilateral nerve-sparing RP. However, the lack of a group of patients receiving avanafil daily precludes a proper generalization of these findings in the penile rehabilitation context.

Taken together, these observations demonstrate that penile rehabilitation might improve postoperative EF in patients treated with nerve-sparing RP for clinically localized PCa (45,48,50,51,69,75). Nonetheless, while basic science studies support the efficacy of PDE5-Is in the preservation of erectile tissues after RP, clinical investigations report contrasting findings. Although chronic administration of tadalafil might represent the best choice in order to prevent alterations to cavernous tissues typical

to be to be more effective than placebo and on-demand dosing in patients with ED following nerve-sparing RP. Although these results were not maintained at the end of the washout period, the administration of tadalafil once daily might have contributed to the preservation of erectile tissue, preventing alterations to the cavernosal integrity as a sequel of neuropraxia typical of patients undergoing RP (36,39-41). A subanalysis performed in the same cohort by Moncada *et al.* (73) recently demonstrated that the administration of tadalafil once daily significantly shortened the time to EF recovery during the 9-month double-blind treatment period. Additionally, at Cox regression analyses

of patients undergoing RP (48,74), the superiority of this treatment over on-demand administration of PDE5-Is is still debated. Nowadays, none of the available randomized controlled trials definitively demonstrated the superiority of daily administration of PDE5-Is compared to the on-demand dosing. Moreover, the beneficial effects of penile rehabilitation protocols using PDE5-Is compared to placebo do not seem to be maintained after the washout period. Nonetheless, basic science and clinical data support the idea that rehabilitation treatments with PDE5-Is are undoubtedly better than leaving the cavernous tissues untreated after nerve-sparing surgery (49,76,77). Further well-designed and well-performed studies with proper patient selection are needed to finally address this issue (74). Indeed, the main limitations of currently available prospective randomized-controlled trials assessing the efficacy of PDE5-Is in the penile rehabilitation setting reside in the relatively short follow-up period, treatment duration and timing of drug administration, type of PDE5-Is chosen, and stringent selection criteria. Patients receiving PDE5-Is in a penile rehabilitation setting should begin treatment as soon as possible and as close to surgery as possible (31,32,49,78,79). Therefore, future randomized trials should include patients treated as early as the removal of the catheter or during the very first months after surgery (80). Moreover, a recent study demonstrated that a 9-month double-blind treatment period was too short to achieve satisfactory EF recovery in the majority of the patients enrolled (73). Therefore, longer treatments could be considered in future studies. Additionally, tadalafil might have the best profile for its use in the penile rehabilitation setting due to his long half-life (70,71,74). Therefore, future studies might focus on this molecule. Finally, patient selection might play a crucial role in the context of prospective randomized-controlled trials assessing the role of PDE5-Is on EF recovery after surgery. Indeed, the inclusion of best candidates for EF recovery (i.e., younger and healthier patients with lower probability of ED after surgery) might limit the effects of PDE5-Is administration (14-16,74). On the other hand, the maximal effect of chronic use of PDE5-Is might be achieved in patients with less favorable preoperative characteristics (14-16,74). Therefore, future studies should adopt less stringent criteria to evaluate the efficacy of PDE5-Is on EF recovery in these patients.

It should also be noted that a recent study demonstrated that patients receiving PDE5-Is after surgery might be more likely to experience biochemical recurrence compared to their counterparts left untreated (81). However, these data come from one single study and are not fully supported by preclinical evidences (81-85). Moreover, the lack of details on the type of PDE5-Is used, as well as dosing and duration of treatment strongly limits the applicability of these findings. Further well-designed studies are needed to better address this issue.

Intracavernosal injections

Montorsi *et al.* (35) in the year 1997 performed a pioneering study aimed at assessing the efficacy of intracavernosal injections of alprostadil for the recovery of spontaneous erections after nerve-sparing RP. Although the study was partially limited by the relatively small number of patients evaluated, early administration of alprostadil significantly increased the recovery rates of EF after surgery. From a biological standpoint, the administration of alprostadil might result into erections, which improve cavernosal oxygenation and penile stretch, finally resulting into a protective effect on erectile tissues (31). Of note, other non-randomized studies supported the efficacy of intracavernosal injections in the recovery of EF after surgery, even after initial administration of sildenafil (86-88). However, when considering this approach, high patient motivation and adherence to protocol are required to increase the compliance to this treatment modality and minimize the dropout rates (21,86). Concluding, intracavernosal injections might be effective in men who have tried oral agents and their condition has failed to respond. Despite this, evidences supporting the efficacy of intracavernosal injections in a rehabilitation setting are still scarce. Additionally, patient compliance is still sub-optimal. Taken together, these aspects prevent clinicians to routinely recommend the adoption of this treatment modality in penile rehabilitation after RP (49).

Vacuum devices

Vacuum devices create a vacuum around the penis. This results into a transient increase in arterial flow and oxygen supply to the erectile tissues (31,32,36,89). Preclinical studies in rats undergoing cavernous nerve injury demonstrated that VED therapy might facilitate EF recovery after surgery acting both on the preservation of smooth muscle and endothelial integrity via anti-hypoxia, anti-apoptosis, and antifibrotic mechanisms (90). These observations were only in part confirmed by randomized studies comparing EF recovery in patients receiving VED or placebo after nerve-sparing RP (91-93). In their pioneering study, Raina *et al.* (92)

228

Gandaglia et al. Penile rehabilitation after radical prostatectomy

evaluated 109 patients who developed ED after nerve-sparing surgery. The authors demonstrated that early use of VED facilitated early sexual intercourses, sexual satisfaction, and early return of natural erections sufficient for vaginal penetration. More recently, Basal *et al.* (93) randomized more than 200 patients treated with robotic-assisted RP to VED, PDE5-Is alone, VED and PDE5-Is, or placebo. Of note, the authors demonstrated that only PDE5-Is or the combination of PDE5-Is and VED had a beneficial effect on the recovery of EF after surgery. On the other hand, VED alone failed to show a beneficial effect with regards to postoperative EF recovery. These results were limited by the low number of patients and by the heterogeneity in preoperative characteristics, where a non-negligible proportion of these individuals had ED before surgery.

Concluding, VED alone or in association with PDE5-Is might represent a treatment option for penile rehabilitation in patients treated with nerve-sparing RP. However, evidences supporting the efficacy of this approach are scarce. Moreover, large well-designed and performed prospective randomized studies assessing the superiority of this approach compared to PDE5-Is and/or intracavernous injections are still lacking. Lastly, available studies do not support a long-term effect of this approach on postoperative EF recovery. As a consequence, VED is not recommended by clinical guidelines for the recovery of EF after surgery. Despite this, VED might represent a treatment option in selected patients.

Although we comprehensively reviewed the currently available literature regarding the role of penile rehabilitation after RP, our manuscript does not represent a systematic review and/or a meta-analysis. Therefore, it cannot provide the same level of evidence of these types of articles. Meta-analyses of currently available prospective randomized trials evaluating the role of PDE5-Is, intracavernosal injections, and vacuum devices are needed to definitively assess the role these therapies in the penile rehabilitation setting.

Conclusions

Currently available penile rehabilitation protocols are based on the administration of PDE5-Is, intracavernosal injections, and VED. Basic science evidences support the rationale of penile rehabilitation after nerve-sparing RP in patients with clinically localized PCa. However, clinical trials report conflicting results regarding the potential benefit of penile rehabilitation in terms of EF recovery and erectile tissue preservation after nerve-sparing RP. Although

rehabilitation, set as early as possible, seems to be better than leaving the erectile tissues unassisted, which is the optimal rehabilitation program still represents a matter of debate.

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Footnote

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Cytoreductive surgery in the era of targeted molecular therapy

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Abstract: Cytoreductive nephrectomy (CN) was regarded standard of care for patients with metastatic renal cell carcinoma (mRCC) in the immunotherapy era. With the advent of targeted molecular therapy (TMT) for the treatment of mRCC, the routine use of CN has been questioned. Up to date evidence continues to suggest that CN remains an integral part of treatment in appropriately selected patients. This review details the original context in which the efficacy of CN was established and rationale for the continued use of cytoreductive surgery in the era of TMT.

Keywords: Cytoreductive nephrectomy (CN); kidney cancer; targeted molecular therapy (TMT); tyrosine kinase inhibitors (TKI)

Introduction

Renal cell carcinoma (RCC) is a common malignancy that comprises approximately 3.9% of new cancers with up to 25% of RCC patients demonstrating evidence of systemic metastases at diagnosis (1). Historically, patients with metastatic RCC (mRCC) have poor prognosis with a 2-year survival of 10-20% (2). Over the last two decades, systemic management of metastatic RCC has significantly changed with increased understanding of the molecular biology of RCC. Agents such as sunitinib, sorafenib and temsirolimus, everolimus, and axitinib specifically target relevant biological pathways including vascular endothelial growth factor (VEGF) and mammalian target of rapamycin (mTOR), respectively and have revolutionized the treatment of advanced RCC and replacing immunotherapy as first line therapy (3).

Despite such advances in the medical treatment of mRCC, cytoreductive surgery continues to play a dominant role in managing patients with advanced disease. Evidence for surgery primarily originates from randomized control trials from the immunotherapy era. Similar prospective studies assessing the efficacy of surgery and newer targeted agents are still under accrual and are not yet available for scrutiny (4). This review examines the current evidence and controversies of surgical intervention in the new era of targeted therapy for mRCC.

The SWOG and EORTC trials—evidence for cytoreductive nephrectomy (CN)

Before the advent of targeted therapy, CN in conjunction with postsurgical immunotherapy for metastatic RCC was the standard of care. The use of immuno therapies such as interferon alpha (INF-α) or interleukin 2 (IL-2) were associated with substantial toxicity and questionable effectiveness (5). The rationale for using agents such as INF-α in advanced RCC was based on evidence from two prospective randomized trials, SWOG-8949 (Southwest Oncology Group) and EORTC-3047 (by the European Organization for Research and Treatment of Cancer). Both showed a significant survival advantage and delayed time to disease progression in patients who underwent CN followed by immunotherapy versus patients undergoing immunotherapy alone with INF-α (6,7). The SWOG study included 241 patients and showed a 3-month survival benefit in the nephrectomy group versus non-nephrectomy group (11.1 *vs.* 8.1 months, respectively). The difference in median survival between the two groups was independent of performance status, metastatic site, and the presence or

234

Thomas et al. Cytoreductive surgery in metastatic renal cell carcinoma
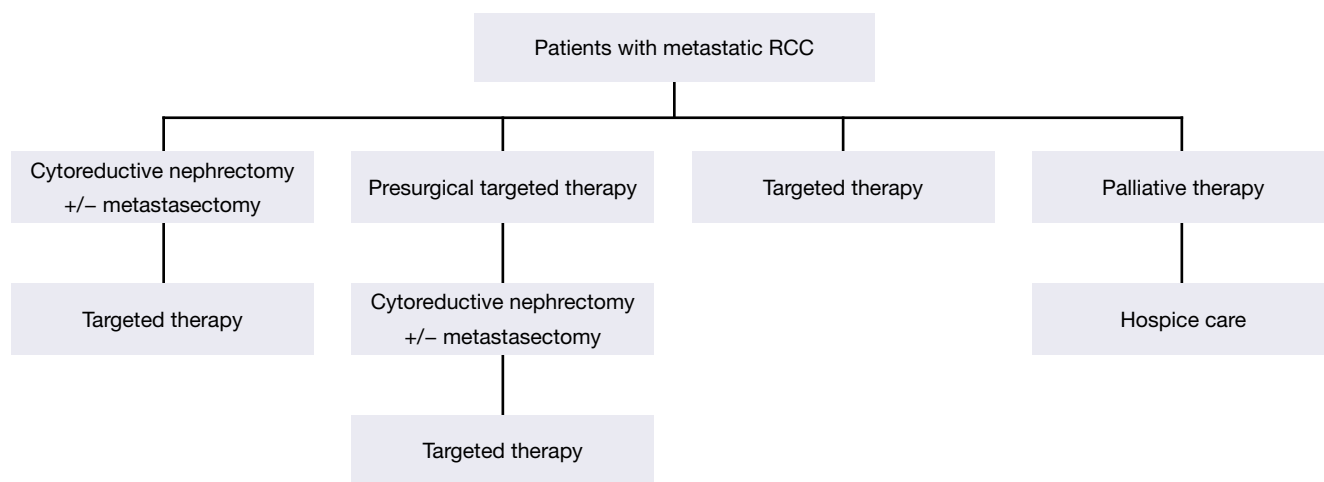
Figure 1 Treatment algorithm in patients with mRCC. mRCC, metastatic renal cell carcinoma.

absence of a measurable metastatic lesion (6).

Likewise the EORTC study showed an even more pronounced benefit in patients undergoing CN followed by INF-α (study group) *vs.* INF-α alone (control group). All patients had mRCC that had been histologically confirmed and was progressive at entry. Fifty-three percent of patients received at least 16 weeks of INF-α treatment, which was also the median duration of treatment. Time to progression (5 *vs.* 3 months), and median duration of survival (17 *vs.* 7 months) were significantly better in study patients than in controls, respectively. Toxicity resulting in dose modification was necessary in 32% of patients, most commonly because of non-haematological side-effects (7). However, both studies showed very low perioperative mortalities of less than 1%.

A combined analysis of the above SWOG and the EORTC trials by Flanigan *et al.* showed an overall survival of 13.6 months for nephrectomy plus INF-α *vs.* 7.8 months for INF-α alone (8). This 6-month survival advantage represented a 31% reduction in risk death in the CN group. A subsequent update of the SWOG data with 9 years of follow-up, continued to favor CN showing a 3-month survival benefit in the nephrectomy group or a 26% reduction in death (9). Multivariate analysis showed that performance status 1 *vs.* 0, high alkaline phosphatase and lung metastasis only were overall survival predictors. This analysis also highlighted that patients who progressed within 3 months after CN did not appear to benefit from surgery. Thus, CN prolongs overall survival, supporting its role as standard therapy in patients with advanced RCC in the immunotherapy era.

CN with post-surgical targeted therapy

The introduction of various tyrosine kinase inhibitors (TKI's) and other agents that target the VEGF and mTOR pathways have quickly replaced cytokines as the dominant systemic therapy in metastatic RCC. Several treatment strategies are now available for patients with metastatic RCC depending on both their performance and disease status (*Figure 1*). The benefits of targeted therapy over cytokine therapy include ease of administration, toxicity profile and superior efficacy in progression-free and overall survival (10). For example, targeted therapies used in patients who had not undergone CN still showed an improved treatment effect to standard immunotherapy. A randomized study of 626 patients by Hudes *et al.*, showed that patients who received temsirolimus alone had longer overall survival and progression-free survival than patients who received interferon alone (11).

Despite these advantages, the majority of evidence supporting the integration of surgery and systemic therapy from the cytokine era with newer targeted therapy has yet to be established. Furthermore, such advances in the treatment of metastatic RCC have led some investigators to question the benefit of CN. A study by Tsao *et al.* utilizing the SEER (Surveillance, Epidemiology and End Results)—Medicare dataset from 2001-2008 showed a decreasing trend in the utilization of CN in the targeted therapy era suggesting a potential uncertainty in survival benefit of CN with newer available targeted agents (12).

A recent Cochrane review highlights over 13 trials out of 28 that showed improved progression free survival with new

Table 1 Cytoreductive nephrectomy in molecular targeted therapy trials

References	Agent	Phase	Number of patients	Nephrectomy (%)
Motzer *et al.* 2006 (16)	Sunitinib	II	106	100
Motzer *et al.* 2007 (17)	Sunitinib	III	375	91
Escudier *et al.* 2007 (18)	Sorafenib	III	451	94
Hudes *et al.* 2007 (11)	Temsirolimus +/− IFN-α	III	419	67
Bukowski *et al.* 2007 (19)	Bevacizumab +/− Tarceva	II	104	100
Yang *et al.* 2003 (20)	Bevacizumab	II	76	90
Escudier *et al.* 2010 (21)	Bevacizumab +/− IFN-α	III	327	100
Sternberg *et al.* 2010 (22)	Pazopanib	III	258	89

targeted agents. Over all, nephrectomy status did not appear to be essential to benefit from targeted therapy, however it is important to note that patients who did not undergo nephrectomy were likely to have important different characteristics and comorbid status compared to the surgical group (5,13). Other trials have also questioned the benefit of CN in the context of adjuvant targeted systemic treatment. You *et al.* reported that CN provided no survival benefit in 78 patients with mRCC in receiving TKI therapy with or without nephrectomy despite the median OS in the CN group was twice that compared to the in the non-CN group (21.6 *vs.* 13.9 months) (14). Another retrospective review by Richey *et al.*, showed overall mean survival of 10.4 months in 188 patients with targeted therapy alone implying CN does not improve survival (15).

Even though these newer targeted treatments have revolutionized modern medical treatment of mRCC, these agents are not curative and complete responses are rare. In modern practice, despite the lack of level 1 evidence establishing the role of CN in patients receiving targeted therapies, CN continues to be an integral component of mRCC management. It is important to emphasize that the clinical trials that led to the approval of the seven current targeted agents available by the Food and Drug Administration (FDA), almost all patients had undergone prior nephrectomy, hence the benefits of such agents must be recognized within the context of a resected primary tumor (*Table 1*).

In contrast, a multi-institutional study by Choueiri *et al.* revealed a significant overall survival advantage in subjects undergoing CN with favorable and intermediate prognostic features described by Heng *et al.* (23) for targeted agents. These included performance score (PS) >80, age less than 75 years, more than one site of metastatic disease and absence of brain metastases (24). Only a marginal

benefit was observed in those patients with poor risk features, reinforcing the need for risk stratification and prognosticators to identify patients who will benefit from CN. Similarly, a further study by Shuch *et al.* reinforces the relationship between PS and improved survival after CN (25). In this study, the median disease-specific survival for patients post-CN with ECOG PS of 0, 1, and 2/3 was 27, 13.8, and 6.6 months, respectively suggesting that surgery in patients who have a poor performance may serve a palliative function, but should be performed with caution due to poor outcomes within this group.

The optimal answer to whether CN will be of benefit in the era of targeted therapy may be fulfilled by the ongoing CARMENA phase III trial. The trial hopes to recruit 700 patients with the primary tumor in place, randomizing patients to nephrectomy followed by sunitinib or sunitinib alone. Ongoing accrual difficulties and the fact that since its inception, there are an increasing number of available targeted agents will make it difficult to generalize its findings to newer agents. Regardless, evidence from CARMENA may help bridge the gap between the immuno- and targeted therapy eras providing level 1 evidence that CN continues to be beneficial to patients with mRCC in combination with these newer agents.

Patient selection/risk stratification for CN in metastatic RCC

Despite evidence that CN prolongs survival in patients with metastatic RCC prior to INF-α or targeted therapy, there are certain subgroups of patients that do not benefit from surgery. Prognostic variables that allow clinicians to discern if patients that will benefit from therapy are paramount in risk stratification and patient counseling prior to commencing medical and or surgical therapy.

236

Thomas et al. Cytoreductive surgery in metastatic renal cell carcinoma

Many publications have examined postsurgical outcome in the pre and post-targeted therapy era (2,24,26-29). One of the most widely used models in mRCC is the Memorial Sloan Kettering Cancer Center (MSKCC) model derived from 400 patients treated with INF-α. This model utilizes LDH, corrected calcium, serum hemoglobin, Karnovsky patient performance status and time from diagnosis to start of therapy to risk stratify patients for survival (30,31). However, the MSKCC risk factors were created during the immunotherapy era and it is uncertain whether these are still useful in the era of targeted therapy.

In 2009, Heng *et al.* retrospectively reviewed 645 patients with metastatic RCC treated with targeted therapy. They identified six predictors of survival similar to the MSKCC criteria including hemoglobin below lower limit of normal (LLN), calcium above upper limit of normal (ULN), Karnofsky score ≤80% and systemic disease within 1-year of diagnosis as independent predictors of decreased survival. Absolute neutrophil count greater than ULN and platelets greater than ULN were also independent adverse prognostic factors. Based on these six prognostic factors, patients were risk stratified to favorable (0 adverse factors: 75% 2-year survival), intermediate (1-2 adverse factors: 53% 2-year survival), or poor (3-6 adverse factors: 7% survival) (23). Both Heng and MSKCC models are useful in extrapolating which patients may most benefit from CN.

Clearly, appropriate patient selection is critical to the successful integration of surgery with systemic therapy. Within the literature there are also many published nomograms to facilitate better patient selection to identify those unlikely to benefit from CN (32-35). Such models may be helpful in selecting patients for CN, but all are inherently limited in their clinical use due to their retrospective nature. In a large retrospective analysis, Culp *et al.* compared 566 patients who underwent CN and 110 patients who received medical therapy alone (33). Surgical patients who died within 8.5 months of CN did not appear to benefit from surgery versus medical therapy alone. Within this group the authors identified seven significant pre-operative variables that were negative predictors of survival. These included serum albumin below the LLN, serum lactate dehydrogenase level above the ULN, a clinical tumor classification of T3 or T4, symptoms at presentation caused by metastatic disease, the presence of liver metastasis and radiological evidence (≥1 cm) of retroperitoneal or supra-diaphragmatic adenopathy at time of CN. Patients who had ≥4 risk factors did not benefit from CN versus medical therapy alone (33).

Even though this retrospective study did not standardize patients to any specific targeted regime, these pre-operative risk factors may be a useful aid in identify patients for CN. Another study by Margulis *et al.* developed a multivariable model examining cancer specific survival in patients following CN in 601 patients identifying both pre-operative and post-operative variables using previously identified negative risk factors for survival including LDH, albumin, pathological tumor and nodal stage (32). Other factors that likely impact outcome in CN and targeted therapy include presence of sarcomatoid differentiation and non-clear cell histology within the nephrectomy specimen, which have both been associated with worse survival (33,36-38).

Lastly percentage of tumor volume removed, defined as 'fractional percentage of tumor volume removed' (FPTV) may also impact outcome of CN. Fallick *et al.* showed that within the immunotherapy era reduction of >75% of overall tumor burden was required to be beneficial (39). More recently, studies suggest a much higher threshold (>90%) of tumor debulking is required to improve progression free survival and overall survival. Barbastefano and colleagues reported that FPTV remained an independent predictor of progression free survival in patients treated with a combination of targeted molecular therapy (TMT)'s where the median FPTV removed was 95% (40).

Timing of CN

The timing of CN, though controversial, is still most commonly performed prior to the commencement of systemic therapy. With higher response rates of targeted agents especially within the metastatic setting, there is an increasing interest in assessing neoadjuvant use of these agents in RCC supporting the treatment paradigm of initial systemic therapy followed by consolidative surgery.

The argument for initiating targeted therapy prior to CN includes timely delivery of systemic therapy to the patients with metastatic disease, and potentially down staging of the primary tumor to facilitate future surgical extirpation. Furthermore, excision of the primary tumor may remove a source of immunosuppressive cytokines or growth factors that stimulate the progression of metastatic sites (41). Another advantage of pre-surgical targeted therapy is that it may act as a litmus test allowing for better patient selection. Patients that respond to systemic therapy are most likely to benefit from CN where as those that rapidly progress could avoid potential surgical morbidity.

For example, a phase II study from the immunotherapy

era by Bex *et al.* (42) evaluated the response to immune therapy as a selection tool for subsequent CN in 16 patients newly diagnosed with metastatic RCC. Patients were treated with two cycles of low dose IL-2 and IFN-α prior to CN. Five patients (31%) had rapidly progressive disease and spared the morbidity of radical nephrectomy (RN) with a median survival of 3 months whereas 11 patients (69%) had tumor response or stability and underwent CN with a median survival of 11.5 months. In a follow-up study by Bex *et al.*, IFN-α was administered to intermediate risk patients with metastatic RCC (43). Similar to the previous study, patients who had tumor response or stability after receiving IFN-α underwent CN whereas 50% patients rapidly progressed and were spared surgery. Such approaches clearly highlight the feasibility of pre-surgical systemic therapy as a litmus test for patient selection.

Recently several groups have reported successful use of targeted agents in patients with the primary tumor *in situ* (38,44-46). For example, a study by Thomas *et al.*, daily sunitinib in 19 patients with locally advanced disease or metastatic RCC showed that after two cycles of therapy 16% of primary tumors demonstrated a partial response with an average shrinkage of 24%. Seven percent of patients had stable disease and 47% of tumors demonstrated progression (46). This same study highlighted four patients with locally advanced RCC were initially deemed unresectable due to the proximity to adjacent structures prior to medical therapy. After treatment with presurgical sunitinib, 3 out of the 4 patients were able to undergo nephrectomy with tumor shrinkage ranging between 11-24% (46).

In one of the largest retrospective series, Abel *et al.* reported 168 patients with metastatic RCC receiving targeted therapy with the primary tumor *in situ* resulting in a median tumor diameter shrinkage of 6.5 cm (7.1%) at 62 days. Most patients had a partial response or stable disease (59%) whereas 41% demonstrated disease progression (47). Other retrospective series also report similar tumor volume shrinkage between 24-31% with neoadjuvant targeted therapy such as sunitinib or sorafenib (45,48). Regardless, neoadjuvant therapy with any of the targeted agents have yet to be curative and the question arises whether the modest reductions in primary tumor burden is clinically meaningful. Furthermore, the definition of surgical resectability is poorly defined with subjective variability depending on surgeon, patient's clinico-pathological and radiological parameters. In the modern era, less than 1% of cases are deemed unresectable (49).

The safety of pre-surgical targeted therapy in patients is also important. A study by Chapin *et al.* compared complications between 70 patients receiving neoadjuvant targeted therapy prior to CN versus patients who had immediate CN. The use of pre-surgical therapy in patients with metastatic RCC did not result in increased overall complication rates or complications requiring intervention (Clavien >3) when compared to patients undergoing immediate CN. However, an increased risk of wound complications was noted. Patients were also more likely to have late complications or multiple events especially wound related events in the neoadjuvant therapy setting (50).

The disadvantage of performing CN first is that disease progression may occur during recovery after surgery and the window of benefit from systemic therapy is missed. Both the SWOG and EORTC trial in the cytokine era reported that 20-25% patients rapidly progressed and died within 4/12 after CN suggesting overtreatment (6,7). Despite these concerns, CN will continue to remain the standard of care for many patients with metastatic RCC until integrating CN and targeted therapy is shown to be inferior to targeted therapy alone (51). The value of pre-surgical targeted therapy may be further clarified from the ongoing SURTIME trial (randomized phase 3 trial) where 458 patients will be randomly assigned to either immediate CN followed by sunitinib or three cycles of pre-surgical sunitinib followed by deferred CN.

Presurgical targeted therapy downstaging of inferior vena cava (IVC) thrombus

The timing of targeted therapy in patients with IVC involvement of locally advanced RCC prior to surgery must also be reviewed. Venous tumor thrombi are present in approximately 10% of patients with RCC (52) and surgery for such thrombi is associated with increased morbidity and mortality (53). With the cytoreductive effects of TMT for RCC, there is hope that such therapy could also decrease the tumor thrombus burden, in turn potentially reduce the extent of morbidity and mortality of surgery. The use of targeted therapy in RCC to downsize caval thrombus has been documented in various case reports (38,54,55) and even though such cases are memorable, the current literature is extremely limited.

A study by Cost *et al.* examined the role of pre-surgical targeted therapy in patients with IVC thrombus in 25 patients (56). Before targeted therapy, thrombus level was II in 18 (72%) patients, III in 5 (20%) patients, and IV in 2 (8%) patients.

Following targeted therapy, 7 (28%) patients had a measurable increase in thrombus height, 7 (28%) had no change, and 11 (44%) had a decrease. One patient (4%) had an increase in thrombus-level classification, 21 (84%) had stable thrombi, and in 3 (12%) the thrombus level decreased. There was only 1 case (4%) where the surgical approach was potentially affected by tumor thrombus regression (level IV to III). No statistically significant predictors of tumor thrombus response to targeted therapy were found (56). This study implies that targeted therapy has minimal clinical effect on RCC tumor thrombi and CN and IVC thrombectomy should not be delayed in good surgical candidates.

Although the previous study is the largest reported experience within *in situ* caval tumor thrombi treated with TMT, most cases were treated with targeted therapy for reasons other than downsizing of the caval tumour thrombus and many of the patients were not even candidates for surgery. Furthermore, the current series lacks sufficient statistical power to adequately assess the usefulness of TMTs in tumor thrombus cytoreduction and further investigation is required (56).

Another retrospective study by Kwon *et al.*, reviewed 45 patients with synchronous mRCC with IVC thrombus. Twenty-eight patients underwent RN with IVC thrombectomy followed by targeted therapy and 17 received targeted therapy alone. Progression-free and overall survival were similar in both groups and surgical resection of the primary renal mass with IVC thrombus did not appear to affect the probability of progression or overall mortality suggesting a limited role for surgery in this patient population (57). In summary, the survival advantage of targeted therapy in the adjuvant setting after nephrectomy and IVC thrombectomy still remains to be further investigated with little in the literature to guide clinicians.

Cytoreductive surgery with metastasectomy

In selected patients with low volume metastatic RCC, surgical resection of metastatic foci can yield long-term disease-free survival, where metastasectomy may be performed concurrently with RN or shortly after. A study by Eggener and colleagues reported clinical benefit of metastasectomy in 44 patients across all three MSKCC risk categories in both the synchronous and metachronous metastatic settings. On multivariate analysis a better risk category and metastasectomy were each independently associated with more favorable survival (58).

Alt *et al.* described outcomes of complete metastasectomy in 125 patients (59). This study showed that complete metastasectomy was associated with a significant prolongation of median CSS (4.8 *vs.* 1.3 years). Patients who had lung-only metastases had a 5-year CSS rate of 73.6% with complete resection versus 19% without complete resection On multivariate analysis, the absence of complete metastasectomy was associated significantly with an increased risk of death from RCC (hazard ratio 2.91) (59). The authors conclude that complete resection of multiple RCC metastases may be associated with long-term survival and should be considered when technically feasible in appropriate surgical candidates.

Another study by Russo *et al.*, described 61 patients undergoing CN with complete metastasectomy during the immunotherapy era in patients with involvement of single and multiple organ sites (60). Median survival was 30 months in patients who underwent CN and complete metastasectomy compared to patients who underwent CN alone (median 12 months). More recently, Karam *et al.* reported on 22 patients who underwent consolidative metastasectomy after at least one cycle of targeted therapy. Fifty percent of patients remained disease free at a median of 10 months. Even though these patients were highly selected with limited disease burden, this study contributes further evidence of the feasibility of consolidative metastasectomy with acceptable morbidity in the TMT era (61). To date, even though evidence favors a survival advantage for metastasectomy in the TMT era in selected patients, the true benefit of adjuvant targeted therapy after metastasectomy still warrants further investigation.

Lastly, a recent sub-sectional analysis from the only systematic review within the literature, identified eight studies that assessed metastases from various organs examining complete metastasectomy versus no metastasectomy or both. The majority reported a significantly longer CSS and OS with complete metastasectomy compared with no metastasectomy or incomplete metastasectomy (median of medians 40.8 *vs.* 14.8 months, respectively). A summary of survival outcome using forest plot hazard ratios for CSS and OS regardless of organ site, unequivocally favored complete metastasectomy in all eight studies (62).

Conclusions

In conclusion, cytoreductive surgery continues to play an important role in the era of TMT. The largest survival benefit of CN in mRCC is seen in patients with favorable

risk categories according to the MSKCC/Heng criteria and especially in patients where a high percentage of tumor burden can be removed. Patient selection is paramount in the decision to perform CN judiciously, as some patient will not benefit due to rapidly progressive disease. Surgery should be based upon volume of resectable disease, performance status, and other prognostic features. Prognostic models developed based on patients treated with targeted agents may enhance our ability to select patients who will gain the most benefit from surgical debulking. It is likely that a subset of patients with poor risk disease treated with upfront systemic therapy will benefit from delayed CN. Currently ongoing clinical trials should help to further define the role of CN in the era of TMT.

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Footnote

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240

Thomas et al. Cytoreductive surgery in metastatic renal cell carcinoma

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Presentation, management, and outcomes of complications following prostate cancer therapy

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Background: To characterize complications of prostate cancer therapy and operative management in patients referred to our institution for surgical intervention.
Methods: Data was abstracted from a retrospectively collected single surgeon database at a large tertiary care referral-based medical center. Variables included age, prostate cancer therapy, complication(s) and their management, and number of operations. Descriptive statistics were used.
Results: From 2006-2010, 890 patients underwent genitourinary surgery, of which 139 were to treat complications arising from prostate cancer therapy. Complications stemmed from radical prostatectomy (RP) monotherapy, RP and external beam radiation therapy (EBRT) or brachytherapy (BT), EBRT only, BT only, and combination EBRT and BT. Complications included urinary incontinence (UI), urethral strictures, bladder neck contractures, and fistulas. UI and bladder neck contractures were more common in patients treated with RP or RP with EBRT or BT. Strictures and fistulas were common in patients treated with EBRT or BT. Interventions included direct vision internal urethrotomy, artificial urinary sphincter, urethral reconstruction, UroLume urethral stent, urethral sling, repair of fistulas, and balloon dilation. Forty eight percent of patients required multiple operations. The median number of interventions was two.
Conclusions: We operatively managed patients treated with non-surgical and surgical modalities for prostate cancer. Complications included UI, fistulas, strictures, and bladder neck contractures. These were managed with a variety of operative interventions. As more men undergo treatment for localized disease, more will inevitably have complications stemming from interventions.

Keywords: Urethral stricture; urethroplasty; prostate cancer; fistula

Introduction

Prostate cancer is the most common noncutaneous malignancy and second leading cause of cancer death in American men. Annually, about 240,000 new cases of prostate cancer are diagnosed in the United States and 30,000 men will die from the disease (1). Advancements and increased utilization of prostate specific antigen (PSA) have led to increased and earlier diagnosis of localized disease. Consequently, prostate cancer mortality is decreasing as more men undergo treatment, however treatment-related complications are increasing (2).

Treatment modalities for localized prostate cancer are variable, have various side effects, and depend on patient preference, disease extent, and patient co-morbidities. Treatment may involve active surveillance, radical prostatectomy (RP), or radiation therapies including external beam radiation therapy (EBRT) and brachytherapy (BT). Other interventions include cyroablation, high intensity focused ultrasound (HIFU), and particle beam therapy (3).

As more men are diagnosed with prostate cancer, more will inevitably undergo treatment and develop treatment-related complications. We report commonly observed complications from treatment of prostate cancer and

Table 1 Demographics of men with complications from treatment of prostate cancer

	Number of patients	%	Mean age	SD
Overall	139		72.4	8.3
RP	50	36.0	69.9	9.6
RP + EBRT or BT	31	22.3	73.8	5.9
EBRT	15	10.8	78.8	9.6
BT	11	7.9	70.5	7.3
EBRT + BT	23	16.6	74.0	5.2
Salvage RP	7	5.0	67.3	3.8
Other	2	1.4	72.5	9.2

RP, radical prostatectomy; EBRT, external beam radiation therapy; BT, brachytherapy.

management of these in a contemporary cohort of patients referred to our institution.

Methods

Patient population

After institutional review board approval, data was abstracted from a retrospectively collected single surgeon database from 2006-2010 at a large tertiary care referral-based medical center. Study inclusion criteria were any patient who underwent operative therapy at our institution for sequela or complications from treatment of prostate cancer, regardless of treatment modality. Patients were excluded if their operative intervention did not stem from complications related to prostate cancer treatment.

Data collection

Variables abstracted included age, type of prostate cancer therapy, complication(s) arising from therapy, number of interventions to manage these complications performed at our institution, and types of procedures performed. Complications stemming from initial management of prostate cancer were classified using the Clavien grading system, a validated instrument to characterize postoperative complications (4).

Statistical analysis

We used descriptive statistics to characterize the study population and outcomes. Data was accrued and analyzed

using Microsoft Excel.

Results

From June 2006 to June 2010, 890 patients underwent genitourinary reconstructive surgery at the University of California, San Francisco (UCSF) Medical Center by a single surgeon. Of these, 139 patients underwent surgeries to treat complications arising from prostate cancer therapy. The mean age of patients in our study was 72.4 years (range, 72.4±8.3 years). Fifty patients (36%) were referred with complications stemming from RP monotherapy. Thirty one (22%) underwent RP followed by EBRT or BT. In the RP group, 55 were radical retropubic prostatectomies, five radical perineal prostatectomies, five robotic assisted laparoscopic prostatectomies, three laparoscopic prostatectomies, and 18 had unspecified approaches. Fifteen (10%) underwent EBRT only, 11 (8%) BT only, and 23 (17%) underwent combination EBRT and BT. Seven (5%) underwent salvage RP, one (0.5%) underwent high intensity focused ultrasonography and cryoablation each (*Table 1*).

Complications

Complications managed were classified as Clavien IIIb, given that they required operative intervention with general anesthesia. We noted 59 cases of urinary incontinence (UI), 51 cases of urethral strictures or stenosis, 29 cases of bladder neck contractures, 25 cases of fistulas (vesico-rectal, rectourethral, ileoanal pouch-ureteral in a patient status post proctocolectomy with ileoanal pouch, and recto-prostatic fistula), and nine other complications (radiation cystitis, radiation proctitis, genital edema, bladder/urethral stones) (*Table 2*). With regards to UI, thirty (50%) occurred in the RP monotherapy group, 20 (34%) occurred in the RP followed by EBRT or BT group, 10 (12%) occurred in the radiation groups (EBRT, BT, or EBRT + BT), and two (3%) occurred in the salvage prostatectomy group. With regards to fistula formation, nine (36%) occurred in the RP monotherapy group, one (4%) in the RP followed by EBRT or BT group, ten (40%) in the radiation groups, and four (16%) in the salvage RP group. With regards to urethral stenosis, six (11%) occurred in the RP monotherapy group, eight (16%) in the RP followed by EBRT or BT group, 35 (69%) in the radiation groups, and one (2%) in the salvage RP cohort. With regards to bladder neck contracture, 14 (48%) occurred in the RP monotherapy group, 11 (38%) occurred in the RP followed by EBRT or BT group, two (7%) occurred in the radiation

Table 2 Complications stratified by prostate cancer treatment

	Urinary incontinence	Fistula	Urethral stenosis	Bladder neck contracture	Other
Overall	59 [%]	25 [%]	51 [%]	29 [%]	9
RP	30 [50.8]	9 [36]	6 [11.8]	14 [48.3]	1[a]
RP + EBRT or BT	20 [33.9]	1 [4]	8 [15.7]	11 [37.9]	3[b]
EBRT	3 [5.1]	1 [4]	9 [17.6]	1 [3.4]	0
BT	1 [1.7]	3 [12]	9 [17.6]	1 [3.4]	0
EBRT+BT	3 [5.1]	6 [24]	17 [33.3]	0 [0]	4[c]
Salvage RP	2 [3.4]	4 [16]	1 [1.9]	2 [6.9]	1[d]
Other	0 [0]	1 [4]	1 [1.9]	0 [0]	0

RP, radical prostatectomy; EBRT, external beam radiation therapy; BT, brachytherapy. [a], Post op bleeding with disruption of anastomosis; [b], radiation cysititis (3), genital lymph edema, bladder stones; [c], radiation proctitis (2), genital edema, bladder/prostatic stones; [d], bladder stone.

Table 3 Interventions stratified by prostate cancer treatment

Intervention	Overall [%]	RP	RP + EBRT or BT	EBRT	BT	EBRT + BT	Salvage RP	Other
Urethral sling	29	19 [65.5]	9 [31]	1 [3.4]	0 [0]	0 [0]	0 [0]	0 [0]
Urethral sling revision/explant/reimplant	9	8 [88.9]	1 [11.1]	0 [0]	0 [0]	0 [0]	0 [0]	0 [0]
AUS placement	36	13 [36.1]	11 [30.6]	2 [5.6]	3 [8.3]	4 [11.1]	3 [8.3]	0 [0]
AUS explant/reimplant/revision	12	6 [50]	4 [33.3]	1 [8.3]	0 [0]	1 [8.3]	0 [0]	0 [0]
Urethral reconstruction	34	7 [20.6]	6 [17.6]	9 [26.5]	5 [14.7]	4 [11.8]	3 [8.8]	0 [0]
UroLume placement	29	7 [24.1]	7 [24.1]	0 [0]	4 [13.8]	8 [27.6]	3 [10.3]	0 [0]
UroLume replacement/revision/explant	7	2 [28.6]	1 [14.3]	0 [0]	1 [14.3]	1 [14.3]	2 [28.6]	0 [0]
DVIU	46	13 [28.3]	11 [23.9]	3 [6.5]	6 [13]	9 [19.6]	3 [6.5]	1 [2.2]
Baloon dilation	10	3 [30]	4 [40]	1 [10]	1 [10]	1 [10]	0 [0]	0 [0]
Fistula repair	22	9 [40.9]	0 [0]	1 [4.5]	2 [9.1]	6 [27.2]	3 [13.6]	1 [4.5]
Multiple surgeries	67	23 [34.3]	16 [23.9]	5 [7.5]	8 [11.9]	10 [14.9]	5 [7.5]	0 [0]
Other	26	3[a]	7[b]	3[c]	2[d]	9[e]	2[f]	0

RP, radical prostatectomy; EBRT, external beam radiation therapy; BT, brachytherapy; AUS, artificial urinary sphincter; DVIU, direct vision internal urethrotomy. [a], cystolithalopaxy, cystectomy (bladder cancer), augmentation ileocystoplasty, bladder botox; [b], diverting urostomy (2), excision of edematous skin, stones (2), cystectomy (hematuria) (2); [c], subcapsular orchiectomy, TURP, urinary diversion; [d], wound debridement/VAC, TURP; [e], TURP, prostatic utricles, cystoscopy, stones (5), urethral tumor resection, drainge of abscess; [f], stones, wound debridement (VAC).

groups, and two (7%) in the salvage RP group (*Table 2*).

Interventions

Common interventions performed were direct vision internal urethrotomy (DVIU) in 46 cases, artificial urinary sphincter (AUS) placement in 36 cases, urethral reconstruction in 34 cases, UroLume urethral stent placement in 29 cases,

urethral slings in 29 cases, repair of fistulas in 22 cases, and balloon dilation in ten cases (*Table 3*).

Other surgical interventions included lithotripsy for bladder, urethral, and prostatic stones; transurethral resection of prostate in the non-operative groups for obstructive urinary symptoms; excision of edematous penile skin in patients with penile and scrotal edema following radiation therapy; wound debridement and incision and

Table 4 Median number of surgeries required

	Median	Range
Overall	2	1-6
RP	2	6
RP + EBRT or BT	2	6
EBRT	1	5
BT	2	3
EBRT + BT	1.5	5
Salvage RP	2	4
Other	1.5	1

RP, radical prostatectomy; EBRT, external beam radiation therapy; BT, brachytherapy.

drainage for abscesses following operative intervention; augmentation ileocystoplasty; and urinary diversion for concomitant bladder cancer, persistent hematuria in radiation therapy patient, severe urethral strictures following radiation therapy, failed repair of fistula, and severe UI not managed with sphincter.

Sixty seven (48%) patients required multiple operations at our institution. Of the 29 urethral slings placed, nine (31%) required revision or explantation. With regards to the 36 artificial urinary sphincters placed, 12 (33%) required revision or explantation. Reasons a urethral sling or AUS required revision included erosion, chronic pain associated with placement, non-functional prosthesis, or infection of prosthesis.

Fistulas included vesico-rectal fistulas, rectourethral, ileoanal pouch-ureteral in a patient status post proctocolectomy with ileoanal pouch, and recto-prostatic fistula. Repairs were via a transperineal approach with or without a bulbar corpora spongiosum interposition flap, transperineal approach with a dartos interposition flap, transperineal approach with gracilis muscle flap, and transrectal approach with an endorectal advancement flap. Fecal diversion was performed in all patients who had rectal involvement. One patient, who sustained a rectal injury during RRP and had had two prior attempts at repair of a rectourethral fistula with recurrence each time, underwent a urinary diversion via diverting ileostomy.

Number of interventions

The median number of interventions performed at UCSF was two and average was two. This does not include those performed at outside institutions prior to or after referral

to UCSF. In the RP monotherapy group, there were an average of 1.9 (±1.2) interventions with median of 2 and range from 1-7. The patient that required seven included a urethroplasty and bladder neck reconstruction, AUS placement then revision, augmentation ileocystoplasty, bulbar cuff sphincter prosthesis implant, radical cystectomy and ileal conduit urinary diversion, and finally an AUS explant. The RP followed by EBRT or BT group required an average of 2.1 (±1.5) interventions, median of 2, and range from 1-7. The patient requiring seven interventions included serial balloon dilations and DVIU. The EBRT group required an average of 1.7 (±1.5) interventions, median 1, ranging from 1-6. The BT group required 2.5 (±1) interventions, median 2, ranging from 1-4. The combined EBRT + BT group required 2.1 (±1.5), median 1.5, ranging from 1-6. The salvage therapy group required 2.4 (±1.51), median 2, ranging from 1-5 (*Table 4*).

Discussion

In this retrospective study of a single surgeon's experience in operatively managing complications stemming from treatment of prostate cancer referred to our practice, we were able to define complications by treatment modality and means of management. Complications we encountered included UI, fistula formation (vesico-rectal fistulas, rectourethral, ileoanal pouch-ureteral, and recto-prostatic), urethral strictures, and bladder neck contractures. UI and bladder neck contractures were more common in patients initially treated with RP. On the other hand, patients initially treated with radiotherapy developed fistulas and urethral strictures more commonly. These were managed with a variety of operative interventions, including urethral sling, artificial urinary sphincter, urethral reconstruction, balloon dilation, and fistula repair. On average patient's required two surgeries for management of their complication.

Complications following treatment for localized prostate cancer with monotherapy versus multimodal treatments have been well documented. UI is more common following RP than radiotherapy. With RP, 7.9-16% and 1.5-7% of patients will require at least a single pad per day at 12 and 24 months respectively (6,7). In radiotherapy groups, at 24 months, 0-5% of patients will require pads for leak, with a direct correlation to the dose of radiation utilized. Patients treated with multimodal radiotherapy have higher rates of UI (8). Urethral strictures are more common following radiotherapy than surgical therapy. These have been reported to be 1.7-1.8% with monotherapy radiotherapy

and 5.2-12% with BT and EBRT combined therapy (9). With high dose rate BT, the rate has been reported at 8% at 41 months, with 92% occurring in the bulbo-membranous urethra (10). However, rates of bladder neck contraction are much more common following RP, ranging from 2.7-25.7%. The highest rates of urethral strictures were seen in patients undergoing surgical and combination radiotherapy (9,11). Rectourethral fistula formation following RP ranges from 0.5-2% (12) versus 1-1.8% from radiotherapy (13). With regards to voiding and bowel symptoms, patients treated with radiotherapy have higher rates of irritative urinary and bowel symptoms versus those treated surgically (14-17).

Findings from our study are consistent with published data. Surgical groups had higher rates of UI and bladder neck contractures. Likewise urethral strictures and fistulas were more common in radiotherapy groups.

Limitations of our study include that it is a single surgeon's experience at a single large academic medical center. Consequently, patient population may not be representative of other practice centers and practitioners. Our data is limited to interventions performed at our institution. We did not have original operative reports, interventions performed at outside facilities, and dosing of radiation therapy. Likewise, follow up is limited to care at UCSF and interventions afterwards are not included in our analysis. With regards to study groups, there were more patients referred to our practice after operative intervention versus radiotherapy. Data regarding initial disease, PSA, and margins was not always clearly documented. We also did not evaluate erectile dysfunction following treatment of prostate cancer, although this is a major issue for patients following prostate cancer therapy. Evaluation of outcome was limited and ideally, quantifiable data such as imaging, urodynamics testing, or validated instruments would serve as a superior means to evaluate patient outcome.

As more men undergo treatment for localized disease, more will inevitably have complications stemming from interventions. Consequently treatment of these complications will become increasingly important in counseling patients. Our study evaluated 139 patients with complications from a variety of treatments for prostate cancer, how these were managed, number of treatments required and patient outcome. Given the multitude of choices patients have for localized prostate cancer, counseling with knowledge of potential complications of each is especially important. Additionally, should a patient develop one of these complications, knowing options for management and outcomes are equally important. Our

study sheds light on both of these issues. The unique challenges these patients present require innovation and determination.

Future directions of study include correlating patient specific factors including medical and surgical history with outcomes and complications. Additionally, better characterizing patient outcome is paramount as well. Also, as treatment of prostate cancer shifts from open to robotic or laparoscopic approaches, the evolution and frequency of complications would be interesting to evaluate, especially as more urologic surgeons are trained on the robot during residency and thus more proficient once in practice.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Risk based neoadjuvant chemotherapy in muscle invasive bladder cancer

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Abstract: Muscle invasive bladder cancer (MIBC) is an aggressive disease that frequently requires radical cystectomy (RC) to achieve durable cure rates. Surgery is most effective when performed in organ-confined disease, with the best outcomes for those patients with a pT0 result. The goals of neoadjuvant chemotherapy (NC) are to optimize surgical outcomes for a malignancy with limited adjuvant therapies and a lack of effective salvage treatments. Despite level 1 evidence demonstrating a survival benefit, the utilization of NC has been hampered by several issues, including, the inability to predict responders and the perception that NC may delay curative surgery. In this article, we review the current efforts to identify patients that are most likely to derive a benefit from NC, in order to create a risk-adapted paradigm that reserves NC for those who need it.

Keywords: Bladder cancer; neoadjuvant therapy; chemotherapy; risk assessment

Introduction

According to Surveillance, Epidemiology, and End Results Program (SEER) estimates, there were over 74,000 new cases of bladder cancer and greater than 15,000 associated deaths in 2014, which remains largely unchanged over the last 25 years (1). Of these patients, 30% have muscle invasive bladder cancer (MIBC) at presentation and another 10% will progress from non-muscle invasive tumors. Radical cystectomy (RC) is the established standard of care for organ-confined tumors and has proven efficacy with extended follow-up cohorts reporting 5-year disease free survival from 68-85% (2-4). However, patient survival diminishes with increasingly advanced primary tumors, with a steep drop off once the cancer becomes non-organ confined or metastatic. Since currently available salvage therapies have very low rates of durable responses, with the notable exception of the recently FDA approved MPDL3280A (5), efforts to increase the success of definitive treatment have led to the utilization of perioperative chemotherapy.

Neoadjuvant chemotherapy (NC) has emerged as the preferred modality of delivering systemic therapy to non-metastatic MIBC patients planning to undergo RC. Based on evidence that will be enumerated below, NC provides a statistically significant survival benefit to patients. The benefit, however, is modest and the toxicities are prevalent, which has resulted in infrequent use of NC because of the perceived high risk (HR) and low benefit of the therapy (6). In this environment, there is an increasing demand to develop strategies that inform medical decision making to ensure those who require more aggressive therapies receive them. This article will review current and ongoing research on risk-stratified methods of identifying ideal candidates for NC.

The Case for NC

The question of perioperative chemotherapy was first addressed in the adjuvant setting with patients with extravesical disease or lymph node metastasis. A prospective trial from Skinner *et al.*, demonstrated that adjuvant chemotherapy (AC) could improve relapse free survival in

cystectomy patients from 46% to 70% (7). While there were subsequent randomized trials that confirmed this finding (8,9), there were still others with negative findings (10-12). Ruggeri *et al.* performed a pooled analysis of published phase III trials (n=5) and found that both overall survival (OS) and disease-free survival (DFS) were improved by the use of AC [response rate (RR) 0.74 and 0.65, respectively, P≤0.001] (13). Vale *et al.* further expanded on these results by performing a meta-analysis of the individual patient data from these randomized controlled trials (n=6) and corroborated the previous findings, showing a 25% risk reduction for OS, albeit with limited power (14). The recently published results of EORTC 30994, which compared immediate to salvage chemotherapy, appear to challenge the previous conclusions, as they did not demonstrate an OS advantage (47% *vs.* 57% mortality, respectively, P=0.13), suggesting that timing is not important for survival (15). However, the authors note that despite limited power for OS outcomes, progression free survival (PFS) was significantly improved (OR 0.54 for immediate), and there may be subgroups that can benefit from immediate AC. These studies demonstrate the importance of multimodal therapy in improving survival outcomes for patients with a poor prognosis with surgery alone.

In nearly all of these trials, a significant portion of the study population did not receive the complete AC regimen, which contributed to the lack of survival advantage in some trials. The low AC completion rate is, in part, attributed to the well-known high morbidity of RC, with quoted complication rates as high as 64% (13% high grade complications) (16). Donat *et al.* further examined this concept in a comprehensive examination of complication profiles among RC patients, and concluded that up to 30% of patients would be unable to receive timely AC due to prolonged recuperation (17). Additionally, the toxicity of the AC regimen is known to be particularly severe (18,19), which is compounded with the recovering state of post-operative patients resulting in as few as 56% of patients getting complete therapy in contemporary series (20). These results suggest that administering chemotherapy prior to RC might be a more favorable strategy to ensure patients are able to receive a complete chemotherapy course within the perioperative period.

There are several potential advantages of using NC instead of AC. Whereas extended surgical recovery precludes many patients from receiving or completing AC, giving chemotherapy up front when patients are at their optimal performance status increases the chance they receive the full dose/course of NC. The possibility of a complete tumor response, with the associated dramatic increase in survival, is the most compelling argument for NC. Nodal downstaging is another desirable outcome since occult lymph node metastasis is seen in 30-40% of cases, most likely due to micrometastatic disease not visualized on routine radiologic imaging (21). Finally, the degree of tumor response to NC gives a measure of *in vivo* drug sensitivity, which may also provide information on prognosis and choice of adjuvant/salvage therapy.

Evidence for NC

Through numerous prospective clinical trials, it has been determined that the ideal regimen for bladder cancer includes cisplatinum, and that replacement with other platinum based agents was not sufficient (22,23). The combination of methotrexate, vinblastine, adriamycin and cisplatinum (MVAC), initially described by Sternberg *et al.*, has been shown to be the most effective regimen for bladder cancer, with overall RR of 65-72% in the metastatic setting (24,25).

Millikan *et al.* designed one of the early trials examining whether NC was a viable treatment alternative by comparing NC plus AC to AC alone, using the MVAC regimen (26). Although they did not demonstrate a survival benefit in this study, subgroup analysis showed that those rendered pT0 derived significant benefit. Based on these results, the utility for NC became more evident in this patient population. In the landmark SWOG-8710 study, Grossman *et al.* demonstrated that NC utilizing MVAC increased median survival from 46 to 77 months, and enhanced pathologic downstaging, with pT0 seen in 15% and 38% of RC and NC + RC patients, respectively (27). Griffiths *et al.* reported that cisplatin, methotrexate and vinblastine (CMV) could also produce a 16% reduction of the risk of death, with long term follow-up (median 8 years) (28). The analysis of the two Nordic trials by Sherif *et al.*, demonstrated that even when combined with preoperative radiotherapy, cisplatin based regimens yielded at 20% relative and an 8% absolute risk reduction in death (29). Schultz *et al.* defined the importance of pre and post NC tumor stage in predicting survival, and confirmed that NC improves outcomes in patients with tumor downstaging (30). In subsequent meta-analyses of NC trials, it was shown that an absolute 5-6.5% OS benefit is observed when using MVAC NC for MIBC (31,32). The efficacy of this regimen is, unfortunately, tempered by an unfavorable toxicity profile, with documented granulocytopenia (33% grade 4)

Table 1 Clinical trial summary

Study	Treatment type	Year	Sample size	Regimen	OS	DSS	RR
Skinner *et al.* (7)	Observation *vs.* AC	1991	91	Cisplatin, doxorubicin, cyclophosphamide	2.4 *vs.* 4.3 yrs	–	–
Martinez-Piñeiro *et al.* (22)	NC	1995	122	Cisplatin	36%	–	34%
von der Maase *et al.* (36)	Salvage chemotherapy	2000	405	MVAC *vs.* GC	38% *vs.* 37%	–	46% *vs.* 49%
Millikan *et al.* (26)	AC *vs.* NC + AC	2001	140	MVAC	4 yrs	–	40%
Grossman *et al.* (27)	RC *vs.* NC + RC	2003	307	MVAC	35% *vs.* 41%	–	38%
Sherif *et al.* (29)	XRT + RC *vs.* NC + XRT + RC	2004	620	Cisplatin, doxorubicin or cisplatin, methotrexate	48% *vs.* 56%	–	80%
Griffiths *et al.* (28)	RC/XRT *vs.* NC + RC/XRT	2011	976	CMV	30% *vs.* 36%	54% *vs.* 59.7%	–
Plimack *et al.* (34)	NC	2014	40	Dose dense MVAC	–	87.5%	53% [37-68]
Choueiri *et al.* (35)	NC	2014	39	Dose dense MVAC	–	79.5%	49% [38-61]
Sternberg *et al.* (15)	AC immediate *vs.* delayed	2015	284	GC, MVAC, or high dose MVAC	54% *vs.* 48%	61% *vs.* 57%	–

OS, overall survival; DSS, disease specific survival; RR, response rate; AC, adjuvant chemotherapy; NC, neoadjuvant chemotherapy; MVAC, methotrexate + vinblastine + driamycin + cisplatinum; GC, gemcitabine + cisplatin; XRT, radiation therapy; RC, radical cystectomy.

and gastrointestinal complications (17% grade 3-4) (27).

To mitigate these adverse effects, alternate regimens have been developed that retain the same efficacy of MVAC. Some centers have recently modified the standard 4 week cycle, to a 2-week cycle with granulocyte colony-stimulating factor (G-CSF) support, known as dose dense MVAC (ddMVAC) (33). Follow-up clinical trials in the neoadjuvant setting have demonstrated that ddMVAC results in effective RR (pT0 =26-38%), far less toxicity (0-10% grade 3-4) and more patients completing the full course (93-95% completion) (34,35). More popular is the combination of gemcitabine and cisplatinum (GC) which was shown by von der Maase *et al.* in a phase 3 study to have similar outcomes compared with MVAC, but with more tolerable toxicity (36). However, since the design of this trial was to establish superiority, not equivalence, the evidence does not strictly support the widespread use of GC as an alternative to MVAC. Zargar *et al.* recently published a multicenter retrospective study that compared GC to MVAC, with two important findings; GC was utilized in the majority of patients (64%) and no significant difference was seen in pathologic RR or OS (37). Regardless of the regimen used, NC has level 1 evidence to support its use (*Table 1*), which is

reflected in published guidelines that recommend offering NC to MIBC patients who will be treated with RC (38,39).

Are all MIBC patients equally responsive to NC?

There is a growing utilization of NC, with Zaid *et al.* reporting an increase from 7.6% to 20.9% over a 4-year period, but this still represents a minority of patients (40). The biggest factor behind the limited application of NC for RC candidates is the modest OS benefit observed in clinical trials, which is contrasted with notable toxicity and potential for delaying surgery in chemotherapy non-responders. Scrutinizing the data lends credence to this view and reveals that the survival advantage is largely seen in responders. Unfortunately, only 30% of patients achieve a complete response, and another 44% will have some degree of downstaging. This leaves the majority of patients over treated if NC was offered to all candidates. Additionally, when looking at the RC only arm, there is a 15% pT0 RR demonstrating that a complete TURBT may be sufficient to render these patients downstaged and likely accounts for half of the downstaging seen with NC (27). The survival outcomes of these patients are similar to those of NC pT0

patients, 82% and 85% 5-year OS, respectively (41,42). These results clearly point to the fact that only a certain subset of MIBC patients will respond favorably to cisplatin based NC.

To understand which patients most likely benefit from NC, Sonpavde *et al.* examined the patterns of survival and relapse from the SWOG-8710 study (43). Firstly, they observed that the RR was dependent on the baseline clinical stage, with cT2 patients downstaged 55% of the time, and cT3/4 patients 35% of the time. Similarly, the RR for pT0 was also stage dependent, 39% and 24% for cT2 and cT3/4, respectively. They also found that survival was dependent on the degree of response (median survival pT0 =13.6 years, pT1/a/is =10.6 years). Conversely, very poor outcomes were seen in patients who had no response or progression of disease (median survival of 3.7 years for pT2+). Paradoxically, when compared survival of non-responding cT2 and cT3/4 tumors (median survival of 1.8 and 5.1 years, respectively), the advanced clinical stage has a better prognosis. Overall, this report highlights that the baseline clinical stage is inversely related to the likelihood of response and that the final pathologic stage is prognostic of survival.

Risk factors

In order to selectively administer NC to the patients that will derive some benefit, researchers have tried to determine if there are preoperative factors that can be used to predict which patients will have the poorest outcomes. These can be roughly divided into those factors that represent locally advanced disease [palpable or fixed mass on examination under anesthesia (EUA)], cross-sectional imaging revealing signs of extravesical extension or local organ involvement, hydronephrosis) and those factors that predict regional/distant metastasis [lymphovascular invasion (LVI), and variant histology] (30,44-50).

Locally advanced disease is challenging to accurately diagnose, but has a significant impact on outcomes. The utility of a good physical exam can never be underestimated, and the presence of a 3-dimensional palpable mass on bimanual EUA is consistent with a cT3b stage, and if that mass is fixed, cT4b (51). Cross-sectional imaging is important for local and distant staging in any malignancy, however computed tomography (CT) imaging has a limited efficacy in bladder cancer, plagued by poor accuracy (49-55%) and high interobserver variability (κ=0.23-0.35) (52,53). Dynamic magnetic resonance imaging (MRI) has proven

itself to have moderate staging accuracy (62-63%), and good ability to distinguish organ confined from locally advanced disease (82-90%) with strong interobserver agreement (κ=0.80-0.89) (54,55). Hydronephrosis has been proven to be a surrogate for invasive disease for many years (46), with risk increasing from unilateral to bilateral involvement (90%$\geq$ pT3 with bilateral). Bartsch *et al.* showed in their study that hydronephrosis was an independent predictor of recurrence free survival (χ^2=10.1, P=0.0015) (56). In early trials with bladder sparing tri-modal therapy, it was quickly determined that patients with hydronephrosis had such an abysmal success rate, that it is now considered a standard exclusion criteria (57). Currently, the best predictive information on extravesical disease comes from a combination of physical exam and radiologic imaging.

Metastatic disease, either to the regional lymph nodes or to distant sites, portends the worst prognosis, and yet, in the absence of measurable disease, there are only a few options to help guide clinicians. LVI is the strongest pre-surgical predictor of poor outcomes, able to independently predict OS, disease specific survival (DSS), recurrence (local and distant) in pN0 patients (58,59). There is data that suggests that the presence of LVI may predict failure of MVAC AC to improve outcomes in organ confined, node negative patients (60). Variant histology in bladder cancer includes many subtypes, but the variants that are of interest regarding early metastasis are micropapillary, small cell/neuroendocrine and plasmacytoid. Micropapillary is likely underreported due to interobserver variability both in academic institutions and community practice (61,62), but it is universally accepted that invasive micropapillary disease is associated with a higher incidence of extravesical and metastatic disease, and poor OS (63,64). While there is some data suggesting that NC may have efficacy in this group, due to small sample sizes, no definitive recommendation can be made (65). Small cell or neuroendocrine histology is another urothelial variant that has a grim prognosis. The largest series is from MD Anderson, with 172 patients in the cohort, with 50% of RC candidates receiving NC. NC has been shown to have a dramatic effect in this disease, with 62% downstaged to $\leq$ pT1 and a median OS improvement from 18.3 to 159.5 months (66). Plasmacytoid is a very rare and extremely aggressive variant of bladder cancer that has a predilection for peritoneal metastasis (67,68). Dayyani *et al.* reported a series of 31 patients (median OS, 17.7 months) in whom 5 received NC, with 80% downstaging, but with early relapse and no demonstrable difference between upfront surgery (69). In total, these are the only significant

252

Jayaratna et al. Risk based NAC in bladder cancer

clues available that suggest early metastasis.

Recently, Culp *et al*. described the MD Anderson paradigm of classifying MIBC patients as low risk (LR) or HR based on these well-established factors, and correlated risk of upstaging and survival outcomes with RC alone (70). The classifiers of HR status are a palpable/fixed mass on EUA, radiologic evidence of cT3/4, the presence of hydronephrosis, LVI and variant histology. By analyzing their own series and using an external validation cohort, they found that LR patients had a 5-year OS and DSS of 64.8% and 82.7%, respectively, and 5-year OS and DSS for HR patients were dramatically worse at 47% and 68.2%. The surprising finding is that the risk of upstaging in the LR cohort was 49.2%, but the group overall had reasonable outcomes. Looking at the HR category, those patients that were downstaged to LR on final pathologic staging (26.5%), had a 5-year OS and DSS of 85.1% and 91%. These same findings were corroborated by the external validation cohort, which had a much larger sample size. This schema of risk assignment gives NC to those patients at the highest risk of poor outcomes, while allowing LR patients to be promptly treated with RC, and avoid NC toxicities.

Molecular classification of MIBC

There has long been an effort to characterize bladder cancer using molecular markers that represent the underlying biologic processes driving the disease course. The prototypical molecular target in bladder cancer was p53, which was identified in the early 1990's as being correlated with grade, stage and risk of tumor progression (71-73). A follow-up study was performed at Memorial Sloan Kettering using immunohistochemistry on patient samples from an NC MVAC trial, which revealed that nuclear accumulation of p53 was independently predictive of DSS, with a relative risk of 3.1 (74). Unfortunately, conflicting reports afterwards have led to an indefinite determination on whether p53 is truly a biomarker of survival (75). The robustness of the molecular findings was limited by the technology available at the time, and may account for the variable results generated by the different study groups.

With the advent of next generation sequencing (NGS) and high throughput microarrays coupled with bioinformatics techniques, genomics research has been able to make large leaps in the discovery and understanding of the mechanisms of oncogenesis. In early 2014, The Cancer Genome Atlas (TCGA) Research Network released the results of their whole exome sequencing and whole genome expression profiling analysis of MIBC (76). There were significant alterations in 32 somatic genes, including TP53, RB1, FGFR3, EGFR, PPARγ and many others. Unsupervised hierarchical clustering of the sequencing data yielded three intrinsic molecular subtypes; group A enriched with copy number alterations, group B mainly comprised of papillary histology and FGFR3 alterations, and group C enriched with TP53 and RB1 mutations. In addition to this the TCGA, in parallel with groups at MD Anderson Cancer Center and University of North Carolina, used similar clustering techniques to identify molecular subtypes based on with gene expression data (77,78). While there are important differences between the classification systems, in general, they were able to mirror the pattern seen in breast cancer, identifying basal and luminal subtypes that are enriched with gene sets that reflect the milieu of the different lineages. Damrauer *et al*. demonstrated that cluster K1 expressed high molecular weight keratins and CD44, which are seen in basal cells, and cluster K2 expresses low molecular weight keratins and uroplakins, both seen in urothelial umbrella cells. When correlating the subtypes with clinical outcomes, they observed that basal tumors had poorer survival compared with luminal tumors. Choi *et al*. identified a similar basal/luminal dichotomy, with basal enrichment of p63 and squamous differentiation and luminal tumors with PPARγ. Additionally, they identified a subset within the luminal subtype that was characterized as "p53-like" and displayed significant platinum chemoresistance, both in the clinical cohort and in subsequent cell line studies. In addition to setting a benchmark for comprehensive genomic analysis of bladder cancer, these groups have established a classification framework that researchers can continue to refine.

Using similar techniques, other groups have correlated genomic findings to clinical outcomes that may inform patient management. Turo *et al*. created a tissue microarray using samples from the primary tumor and metastatic lymph nodes in patients that were clinically node negative prior to RC (79). Examining FGFR3 specifically, the authors found that there was a high concordance between the specimens (OR 8.45), even when using multiple samples from each site to account for intratumoral heterogeneity. This suggests that FGFR3 protein expression in the primary tumor can be used to identify patients at a HR of occult lymph node metastasis, and candidates for NC. Groenendijk *et al*. used NGS methods to compare the mutational profile of complete responders and non-responders to NC (80). Their group found that ERBB2 activating mutations

were exclusively found in the responder cohort, with none present in the non-responders (P=0.003). ERCC2 mutations also appeared to be differentially expressed with 16% of responders and 6% of non-responders having the mutation, however this was not significant (P=0.27). Van Allen *et al.*, however, performed whole exome sequencing on patients receiving cisplatin-based NC and demonstrated that ERCC2 mutations were enriched in responders (81). Further *in vitro* work demonstrated that ERCC2 deficient cell lines increase cisplatin sensitivity, and this effect is rescued with wildtype ERCC2, but not the mutant form found in the patient cohort. Font *et al.* analyzed gene expression in NC patients and found that high BRCA1 expression in pre-treated tumors predicted lower NC response (22% *vs.* 66%, P=0.01) and lower OS (HR 2.73, P=0.02) (82). Using molecular characteristics to identify patients with HR disease or to predict patients likely to respond to NC, the major contribution of these efforts is that this information is correlated to a meaningful difference in clinical behavior, demonstrating the importance of translational research.

Conclusions

If we could perfectly identify responders to NC, or if the toxicities were minimal, utilization rates would be much higher than they are now. Unfortunately, neither of those conditions is currently true. We now know that there are factors that we can use to stratify patients into high and LR. Even with a high incidence of upstaging amongst LR patients, it has been shown that their outcomes with RC alone are similar to patients that had no stage change. But this binary system is still a relatively unsophisticated way of guiding decision making. Knowing that we can identify patients who do well with surgery alone, we now need to identify which HR patients will respond well to cisplatin based NC, and those who need alternate treatments based on novel targets.

New molecular classifiers are being created to characterize tumors based on the underlying cancer biology, with important implications concerning progression and chemoresistance. This is certainly the direction that bladder cancer research needs to follow, in order to refine decision making to attain the goal of personalized medicine. Already several genomic classifiers have been developed, which have been designed for predicting DSS after cystectomy. The most recent, developed by Mitra *et al.*, is a 15-gene classifier that predicts recurrence after RC without NC,

with superior performance compared with currently available clinical predictors. In a more prospective fashion, the recently activated SWOG-NCI sponsored COXEN clinical trial (S1314) plans to compare GC and MVAC NC, and simultaneously collect tissue, blood and urine samples to process through the COXEN algorithm (83). The COXEN algorithm has already been able to develop and validate a multivariate gene expression model for survival in NC treated bladder cancer, using a combination of publicly available human microarray data sets and *in vitro* drug sensitivity testing using the NCI-60 cell line panel (84). In the current prospective trial, gene sequencing and expression profiling will be performed to analyze oncogenomics, expression patterns of coding and non-coding RNA, and pharmacogenomics. Instead of simply comparing two different NC regimens, this trial is unique in the fact that it will allow investigators to discover patterns of sensitivity/resistance and develop molecular signatures to guide decision making in multimodality cancer treatment. With the initiation of more trials like this, we will be able to test new therapeutic agents, and ideally be able to predict the right drug for the right patient at the right time.

Acknowledgements

Footnote

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Beyond chemotherapy for advanced disease—the role of EGFR and PD-1 inhibitors

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Abstract: Penile squamous cell carcinoma (SCC) is a rare malignancy with limited treatment options when the tumor is unresectable and/or chemorefractory. Triplet systemic chemotherapy regimens including taxane and cisplatin are recommended, but the response duration can be short and the treatment-related toxicity high. Only a small proportion of patients survive 1 year or longer with the current standard treatment paradigm. Beyond chemotherapy, the use of novel targeted agents, either alone or in combination with traditional chemotherapeutic agents, has appeared to have promising efficacy in patients with platinum-refractory penile cancer. The frequent overexpression of PD-L1 in advanced penile SCC indicates the potential efficacy of PD-1 inhibitors. Upcoming clinical trials using the immune check-point inhibitors may provide exciting landscape and change the paradigm for patients in the future.

Keywords: Penile squamous cell carcinoma; immune checkpoint inhibitor; target therapy; EGFR; PD-1; PD-L1

Introduction

Penile cancer is a rare neoplasm in developed nations of Western Europe and the USA, where it represents approximately 0.4–0.6% of all malignancies in men (1). However, the prevalence of penile cancer in developing areas of Africa, Asia and South America is ranging from 6–20 per 100,000 men (2). General socioeconomic status, Human papillomavirus status (HPV), cigarette smoking, access to health care contribute to the discrepancies in this incidence (3). The dominant pathology is squamous cell carcinoma, accounting for 95% of cases. Other malignant tumor types described in published work include basaloid, warty, warty-basaloid, papillary, verrucous, sarcomatoid, adenosquamous and mixed (4).

Most early stage penile cancer will present with lesions affecting the glans and prepuce. Penile preserving

surgical techniques are now widely used and result in good functional and cosmetic results (5). However, up to 14% of cases may present as advanced penile cancer in association with extensive inguinal lymph node invasion and 2% patients present with metastasis due to aggressive histological subtype (6). With such advanced disease, surgical treatment may not be effective due to the presence of skin, subcutaneous tissue and vessels invasion by extra nodal disease. There may be little option but to commence palliative systemic treatment as the prognosis is generally very poor. Due to the rarity of penile carcinoma, the peer reviewed scientific literature on the value of systemic treatment is fragmented and the optimal therapy is yet to be determined as studies are generally limited to small single institution retrospective studies. Triplet chemotherapy regimens by adding taxane to cisplatin-based therapy have demonstrated better efficacy in patients with locally

advanced or metastatic penile cancer, as indicated by positive findings seen in patients with advanced head and neck squamous cell carcinoma (HNSCC). In a study of 26 patients with advanced penile SCC, the efficacy of a regimen consisting of docetaxel, cisplatin-5-fluorouracil (TPF) was studied. Ten of 26 cases (38.5%) had responded, and 2 patients with locally advanced disease exhibited complete remission. Similarly, another study of 30 cases with advanced penile SCC, patients received neoadjuvant treatment with 4 cycles of paclitaxel, ifosfamide, and cisplatin (TIP). Half of the patients (15/30,50%) had an objective response. Three patients (10%) had a complete pathologic response. The median time to progression (TTP) was 8.1 months, and the median overall survival was 17.1 months. However, the major limitation of this triplet regimen is the extremely high treatment-related toxicity with 65–70% patients experiencing grade 3 or 4 adverse events (7,8). With the development of new strategies including novel targeted therapy and immunotherapy, current treatment paradigms may shift to emphasize the implementation of epidermal growth receptor (EGFR) inhibitors and program death receptor 1 (PD-1) inhibitors in the treatment of advanced or metastatic penile SCC.

Novel targeted therapy: epidermal growth factor receptor inhibitors

The overexpression of EGFR is frequently observed in a variety of epithelial cancers, such as non-small cell lung cancer (NSCLC), HNSCC, colorectal cancer (CRC), and breast cancer (9-11). The expression of EGFR is often assessed by immunohistochemistry (IHC), and the overexpressing is prevalent in patients with penile SCC. In a study of 17 invasive cases, the overexpression of EGFR was examined in all samples, with most showing 3+ overexpression (12). Chaux and colleges assessed the expression of EGFR in 112 patients with the high expression rate was 44%, but the expression was not associated with grade, histologic subtype, or HPV status (13). In a study of 148 penile cancers, Stankiewicz and colleagues investigated the epidermal growth factor receptor (HER) family receptors and in HPV-positive and negative penile SCC and its impact on Akt activation. Differently from EGFR, they found the expression of phosphate-EGFR (p-EGFR) was present in only 25% of penile SCC, and the p-EGFR in tumor with HPV-negative significantly more expressed than HPV-positive cancers (14). The expression of EGFR appeared to be predictive of poor prognosis in a number

of malignancies, including non-small-cell lung cancer, oropharyngeal cancer as well as penile cancer. In a study of 30 cases with penile SCC, EGFR expressed was noted in all patients, and positivity for cytosolic p-EGFR were predictive for recurrence and poor survival (15). A recent study from Brazil found EGFR expression in half of the samples which correlated with recurrence. FISH analysis, as determined by signals of the EGFR gene and chromosome 7, revealed the alteration (polysomy and amplification) as an independent risk factor for poor survival (16). Thus, expression of EGFR detected by IHC has been frequently observed in penile SCC, but expression may not correlate well with response.

EGFR mutation is known as an actionable driver mutations in patients with NSCLC, and sensitizing the EGFR mutations play an important role, because of the high prevalence of approximately 10% in Caucasian patients and up to 50% in Asian patients (17-19). EGFR exons 18 to 21 encode a portion of the EGFR kinase domain. It is reported that up of 80% to 90% of patients with EGFR-mutated NSCLC will have either an exon 19 deletion or an L858R point mutation in exon 21 (20-22). It is noted that most mutations involving exons 18, 19, and 21 are considered predictive of sensitivity to EGFR tyrosine kinase inhibitors (TKIs), whereas mutations in exon 20 are typically resistant to these agents (23-25). In penile carcinoma, multiple studies have identified that overexpression of EGFR is not associated with gene amplification, or gene copy number gain. In a study of a series of 20 cases with penile SCC, targeted next-generation sequencing showed EGFR amplification was seen in about 4/20 (20%) patients (26). A recent study performed targeted next-generation sequencing to identify somatic genomic alterations in a cohort of 60 samples from 43 patients and the results showed EGFR expression by IHC does not appear to be correlated with EGFR copy number. The EGFR gains/amplifications accounts for approximately 10% of penile SCC cases, with significant heterogeneity between paired primary tumors and lymph node metastases (27). Moreover, we failed to detect any driver mutations in the tyrosine kinase domain of EGFR, which is known as a predictor of responsiveness in lung cancer (28).

The EGFR-RAS-RAF signaling pathway plays an important role in regulation of tumor cell survival and proliferation, especially in squamous cell carcinoma. The KRAS gene, as a member of the RAS proto-oncogene family, is an important component of the EGFR signaling

260

Gu et al. The role of EGFR and PD-1 inhibitors for penile cancer

pathway. KRAS mutations are mostly found in codons 12 and 13, which harbor in exon2. KRAS gene mutation has been recognized as a negative predictor for responsiveness of CRC to cetuximab. However, KRAS mutation has no effect on the overall survival of patients with CRC (29). KRAS mutations were reported to be rare in penile SCC. In a small sample size of 28 cases, Andersson and colleges found 1 mutations in KRAS gene (30). In an analysis of 107 samples from Brazil, only 1 sample presented a mutation in exon 12/13 of KRAS (16). Recently, Gou and colleges analyzed 94 tumor tissues of penile SCC, only 1 case of KRAS mutations at codon 12 was found. Moreover, the RAS-association domain family 1, acted as a tumor suppressor gene through RAS-mediated apoptosis, positively expressed in only 5/150 patients (3.33%) (31). Similarly, KRAS gene mutation was also rare in HNSCCs and was estimated to occur in <3 % (32). BRAF is another important component of the EGFR-RAS-RAF signal transduction pathway, which mediates cell growth, differentiation, apoptosis, and malignant transformation. Mutations of BRAF were found in several tumors, such as pilocytic astrocytoma, melanoma, colorectal, thyroid and ovarian cancers (33). The presence of BRAF mutations also very rare in penile SCC. In an analysis of 83 tumors, Gao and colleges found no BRAF V600E point mutation (31).

To date, several EGFR-targeted therapies have been developed and these drugs have been shown in efficacy in several solid tumors, including lung, head and neck and colon. The commercial EGFR-targeted therapies included monoclonal antibodies such as cetuximab, and EGFR tyrosine kinase inhibitors such as erlotinib, and gefitinib. These drugs have been reported to have promising efficacy in some small subset of patients with advanced or metastatic penile squamous cell carcinomas (*Table 1*).

A retrospective study by Carthon and colleges evaluated the safety and efficacy of EGFR-targeted agents in 24 patients with advanced penile SCC. Eight patients had received an EGFR-targeted drug alone, 13 had received cetuximab plus a platinum or carboplatin, and three patients had received TIP plus cetuximab. The patients with cetuximab with chemotherapy had overall response rate of 30%. Partial responses were seen in 1/5 patients (20%) who had received cetuximab alone, in 3/12 patients (25%) who had received cetuximab plus cisplatin, and in 2/3 patients (66%) who had received cetuximab and TIP. There were no objective responses to the small-molecule inhibitors gefitinib or erlotinib. The overall median TTP was

11.3 weeks, and the median overall survival was 29.6 weeks. The toxicity of EGFR-targeted therapy has been well tolerated, and only 4 cases had the grade 3 or 4 adverse events (34). Similarly, several case reports have also demonstrated the efficacy of other anti-EGFR drugs in addition of cetuximab in the treatment of advanced penile SCC. In a case report, a partial response was seen with the anti-EGFR monoclonal antibody, nimotuzumab, in combination of cisplatin-based chemotherapy (37). In a case report with 3 cases, Brown and colleges reported 2 of the 3 patients had clinical benefit who received cetuximab or panitumumab in the platinum-refractory settings (37). Necchi and colleges reported an experience of using single-agent panitumumab to treat a penile SCC with extensive cutaneous and subcutaneous metastatic nodules. Significant clinical response and rapid recovery of disease related symptoms were observed 2 weeks after the administration (39). Another study summarized cases retrieved from the published studies on using anti-EGFR monoclonal antibodies. Lorenzo and colleges presented a cohort of 28 advanced penile SCC who treated with cetuximab, panitumumab and nimotuzumab. About half patients received the EGFR agent as secondline therapy. Cetuximab was the most commonly used drug, which was administrated in 24/28 patients (85.7%). In the patients who received EGFR-targeted inhibitors plus chemotherapy, over a half of them showed a response to treatment, with a median TTP of 3.2 months. In contrast, patients who received EGFR-targeted inhibitors alone had a response rate of 28.6% and the median TTP of 2.1 months (40).

A number of commercial anti-EGFR agents have been used in patients with penile SCC outside the context of a clinical trial, and these agents seem to have promising efficacy as a salvage treatment after failure of first-line chemotherapy. The current data indicate that patients with penile SCC who received anti-EGFR monoclonal antibodies appear to have better response rate and longer TTP, whereas available anti-EGFR TKIs such erlotinib and gefitinib seem to have no activity, which is likely to be related to the lack of EGFR mutating activation.

Although the vascular endothelial growth factor (VEGF) receptor is overexpressed in approximately 50% of penile SCC cases (41), few studies use these agents in patients with advanced penile SCC. In penile cancers, Stankiewicz and a study of 6 cases, Zhu and colleagues described the experience with VEGF-TKIs after receiving at least 2 prior chemotherapy regimens. One partial response was observed,

Table 1 Published studies of EGFR-targeted therapy for penile squamous cell carcinoma

Authors	Treatment	N	Number of response
Carthon *et al.*, 2014 (34)	Cetuximab	5	1 (20%)
	Cetuximab + cisplatin/carboplatin	12	4 (33%)
	Cetuximab + TIP	3	3 (100%)
	Erlotinib	2	0
	Gefitinib	1	0
Rescigno *et al.*, 2012 (35)	Cetuximab + docetaxel	1	1
Pandey *et al.*, 2013 (36)	Nimotuzumab + paclitaxel	1	1
Men *et al.*, 2014 (37)	Nimotuzumab + paclitaxel + cisplatin	1	1
Necchi *et al.*, 2011 (38)	Panitumumab	1	1
Brown *et al.*, 2014 (39)	Cetuximab	1	0
	Panitumumab	1	1
	Panitumumab + cisplatin	1	1

TIP, paclitaxel, ifosfamide, and cisplatin.

and 4 patients showed stable disease. Three patients showed pain response and had an improvement in quality of life (42).

Immune checkpoint inhibitors: a new approach to trial design and potential treatment of penile squamous cell carcinoma

Immune checkpoint inhibition with PD-1 and PD-ligand 1 (PD-L1) emerged to play an important role in cancer immunotherapy for a number of cancer phenotypes. The success of immunotherapeutics was previously reported in squamous cell carcinomas of the lung, which led to significant interest in testing similar therapeutic strategies in penile squamous cell carcinoma (43,44).

Recent studies using immunochemical assay to exploit the PD-1/PD-L1 pathway in penile squamous cell carcinoma. The expression of PD-L1 in penile squamous cell carcinoma was reported in three studies (*Table 2*). The first study reported by Udager found 23 of 37 primary tumors (62.2%) were positive for the PD-L1 expression. Furthermore, the PD-L1 expression of primary tumors was strong positively correlation with usual type histology, regional lymph node metastasis and decreased cancer-specific survival (45). Another study evaluated PD-L1 expression in 200 tumor specimens from a European cohort. At a 1% cut-off level, PD-L1 expressed in 96 primary penile carcinomas (48%) and associated with

negative high-risk HPV status. Multivariable analysis revealed PD-L1 expression was independently associated with negative lymph node status and with poor survival. The results were more prominent in men with negative HPV status (46,48). A study access the PD-L1 expression in a North American cohort. Twenty-one (40%) of 53 penile squamous cell carcinomas had positive PD-L1 expression, which was expressed by a significant proportion of advanced penile cancer (47). Thus, 40–60% cases of primary penile SCC express PD-L1, which is associated with negative HPV status, high-risk clinicopathologic features, and poor clinical outcome. Although the sample size of these studies is relatively small, these findings provide a strong rational for the use of checkpoint inhibitors as therapeutic options in penile SCC.

The FDA approval of the immune checkpoint inhibitors, Ipilimumab, Pembrolizumab and Nivolumab, in metastatic melanoma has led to significant interest in rapid development of clinical trials in penile SCC. Ipilimumab, as a humanized IgG1 monoclonal antibody against cytotoxic T lymphocyte antigen (CTLA-4), has yielded a significant improvement in overall survival in metastatic melanoma. The other two immune checkpoint inhibitors, Pembrolizumab and Nivolumab, are humanized monoclonal antibodies against PD-1, and are approved for use in metastatic melanoma. Several clinical trials were designed to use these immune checkpoint inhibitors to treat penile

Table 2 Published data on PD-L1 expression in penile squamous cell carcinoma

Authors	N	PD-L1+ (%)	Antibody	Cut-off
Udager *et al.*, 2016 (45)	37	23 (62.2)	clone 5H1	≥5%
Ottenhof *et al.*, 2016 (46)	200	96 (48.0)	clone E1L3N (cell signaling)	≥1%
Cocks *et al.*, 2016 (47)	53	21 (39.6)	clone E1L3N (cell signaling)	any extent

SCC, and the results of these clinical trials would provide valuable insight to treatment of the aggressive disease. The ongoing clinical trials are phase 2 trial of Pembrolizumab for advanced penile SCC (NCT02837042), and the phase 2 trial for the evaluation of efficacy of Pembrolizumab in rare tumors (NCT02721732). The other two are going to open for recruiting patients, which are phase 2 trial for investigating the efficacy and safety of Nivolumab (NCT03012581) and phase 2 trials of evaluating efficacy of Ipilimuab and Nivolumab for selected rare cancer types (NCT02834013).

Summary

Penile SCC is a rare and lethal disease. In advanced disease, the results, even after aggressive surgical approaches in combination with conventional systemic chemotherapeutic agents, have been disappointing with high recurrence rates and poor survival. High expression of EGFR and the rarity of KRAS mutation make the rational in the use of anti-EGFR inhibitors in advanced penile SCC promising. Anti-EGFR monoclonal antibodies could be used in the neoadjuvant setting to increase radiological responses and in the adjuvant setting to decrease recurrence probability, as well as in the first-line setting in combination with chemotherapy or in more advanced lines of therapy as a single agent. Furthermore, immune checkpoint PD-1 inhibitors have changed the treatment paradigm in a variety of solid tumors. The overexpression of PD-L1 in advanced penile SCC lay biological rational in the potential efficacy of PD-1 inhibitors in this frequently chemo-refractory disease.

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Footnote

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Radiotherapy for prostate cancer and sexual health

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Abstract: Sexual dysfunction is very common after treatment of prostate cancer. Radiation therapy together with radical prostatectomy is the most effective treatment for localized disease. Percentages of erectile dysfunction (ED) reported in prospective studies after external-beam radiotherapy (RT) vary from 60-70%, and these are similar after brachytherapy. In randomized trials more realistic percentages of 30-40% are reported. Modern techniques do not seem to decrease post-radiation ED. No final conclusions can be drawn whether or not the radiation dose to the penile structures correlates with post-radiation ED in patients treated for prostate cancer. The etiology of ED after RT of prostate cancer is most probably multi-factorial. The phosphodiesterase type 5 inhibitors (PDE5-I) sildenafil and tadalafil have been shown to be effective to treat post-radiation ED in about half of the patients in randomized trials. Patients and their partners need to be adequately counselled on the effects of cancer treatment on their sexual life and relationship, and about the different treatment possibilities. Sexual counselling has not become yet a routine part of oncology care in most hospitals, but this should be provided. Due to the lack of robust data, prevention of post-radiation ED with PDE5-I cannot be recommended so far.

Keywords: Prostate cancer; radiotherapy (RT); erectile dysfunction (ED); sexual dysfunction; sexual counseling

Introduction

The number of cancer survivors continues to increase due to the aging and growth of the population, and improvement in early detection and treatment (1). Among men, the most common cancer affects the prostate. About 60% of prostate cancer survivors are aged 70 years or older, and many remain interested in sex (2). Therefore it is important to understand the medical and psychosocial needs of prostate cancer survivors and proactively address sexual health.

Primary treatments for prostate cancer are radical prostatectomy, radiotherapy (RT) (external-beam, brachytherapy or a combination), or observation. The choice of treatment depends on tumor staging, patient's age and comorbidity, urologist's and patient's preferences (3). More often, patient's quality of life, including sexual functioning, plays a significant role in the decision making on which treatment the patient prefers. The introduction of sildenafil (Viagra®) in the late 1990s, with media attention towards

erectile dysfunction (ED), has made sexual problems more normative and has increased acceptance of help-seeking (2,3). This paper is an extension on a previous published paper (3) and aims at specifically address sexual health after RT for prostate cancer.

Definition and evaluation of erectile (dys) function

The 3rd International Consultation on Sexual Medicine defined ED as the consistent or recurrent inability to attain and/or maintain a penile erection sufficient for sexual performance (4). Such a definition is strictly relevant in the presence of a willing partner, therefore the general term sexual activity (intercourse or masturbation), would be more appropriate (5). Rigidity of erections, presence of spontaneous morning and night erections should be addressed as well (5). Psychological factors may play a role in post-radiation ED. In most of the published studies,

authors referred to the general terms potency or impotence without giving a proper operational definition (5). The most practical way to evaluate erectile function is by using questionnaires, and different questionnaires have been used in the literature so far. In most of the cases questions on sexual functioning were limited to a few items, or were incorporated into a questionnaire on toxicity of radiation treatment, or on quality of life in general. With a few exceptions, the entire questionnaire was not included in the papers. Since the end of the 1990s, the International Index of Erectile Function (IIEF) has been introduced (6), followed later by the shortened IIEF-5 questionnaire (also known as the Sexual Health Inventory for Men or SHIM) (7). The IIEF and the SHIM have been translated and validated in many languages, though they have not been specifically developed for cancer patients. More recently a specific questionnaire on sexual functioning after treatment of cancer has been developed in the USA, but has not been validated yet in other countries (8).

Incidence of post-radiation sexual dysfunction

Only studies that prospectively evaluated erectile functioning, by using validated questionnaires and using a proper definition of potency are useful to draw conclusions on the incidence of post-radiation ED (5). In general, this reaches about 60-70% in prospective studies (5,9,10). Three recent prospective studies have shown an incidence of ED in 30-40% of the patients treated by external-beam RT. They showed an increase of post-radiation ED between one and two years, with no changes after three years (11-13). Brachytherapy was originally introduced not only to limit the detrimental effects of external-beam RT on bowel and urinary function, but also to help preserve sexual function. The introduction of sophisticated 3D-computer-assisted dosimetry, and the availability of intra-operative transrectal ultrasound in the late 1990s, led to more accurate and reproducible implants. In general, after permanent seed implantation, ED rates range from 5-51% (5,14).

A deterioration of sexual activity has also been associated with the severity of ejaculatory dysfunction, particularly with decreased volume or absence of semen (15). After RT for prostate cancer, ejaculatory disturbances vary from a reduction or absence of ejaculate volume (2-56%) to discomfort during ejaculation (3-26%) and hemospermia (5-15%) (5,14). Dissatisfaction with sex life has been reported in 25-60%, decreased libido in 8-53%, and decreased sexual desire in 12-58% of the patients (5). A decreased intensity of orgasm, decreased frequency and rigidity of erections, and decreased importance of sex have also been reported (5,9,10,14).

New radiation techniques and sexual (dys) function

In the last decade substantial improvements have been made in the irradiation techniques and doses, therefore post-radiation outcome and toxicity in prostate cancer patients have improved. Sophisticated planning systems have allowed the introduction of intensity-modulated RT (IMRT), which enables adequate dose delivery with better sparing of the normal tissues. IMRT has almost completely replaced the older conformal RT techniques (16,17). Image-guided RT techniques such as implanted fiducials and cone-beam computed tomography (CT) equipped linear accelerators have added even more precision and will hopefully allow for further decrease in toxicity. Several trials on hypofractionated RT for prostate cancer (higher dose per fraction) are on-going. These schedules could improve therapeutic gain, reduce toxicity as well offer economic and logistic advantages (18,19). Stereotactic body RT has been used for several years now in patients with prostate cancer, often using 2-5 fractions, with encouraging results and acceptable toxicity (18,19). Though the clinical advantages of all these new techniques have not been proven in randomized trials yet but only in comparative studies. These techniques, unfortunately, seem to cause post-treatment ED similarly to conventional RT (18,20) or even worse outcome (16,17). Proton therapy is a relatively new conformal technique to treat prostate cancer. This modality delivers less radiation dose to normal tissues compared to traditional RT. Few data are available on the effects of proton therapy on erectile function. Only one study has reported prospectively on the rates of potent patients before and after proton therapy using a validated questionnaire (21). It seems that this technique reduces the percentage of post-radiation ED: at 2 years 73% of patients reported no or mild ED compared to baseline levels. This study only included men of 60 years and younger, which may bias the results as seen in daily practice (21).

Mechanisms of post-radiation ED

Post-radiation ED in patients with prostate cancer has already been extensively and critically reviewed (5,9,10,14). Zelefsky and Eid concluded that the predominant etiology

of radiation-induced impotence was arteriogenic (22). Several studies investigated the relationship between the radiation dose to the neurovascular bundles, the penile bulb and the penile bodies and the incidence of post-radiation ED, presenting contradictory results (23). In a randomized dose-escalation trial comparing 68 and 78 Gy, the proximal corpora cavernosa (crura), the superiormost 1-cm segment of the crura, and the penile bulb were contoured on the planning CT-scan and dose-volume parameters were calculated in 96 patients (24). Two years after RT, 35 patients had developed ED. No statistically significant correlations between post-radiation ED and dose-volume parameters in the crura, the most superior 1-cm segment of the crura, or the penile bulb were found (24). Magnetic resonance imaging (MRI) appeared to be superior to CT for the imaging of erectile tissues, and showed that the dose to the penile bulb and corporal bodies is low, but the dose has not been correlated to post-radiation ED (25). Post-radiation ED has more likely a multi-factorial etiology, and is not only based on the radiation dose to one single anatomical structure. It is very likely that the structure responsible for ED has not been investigated yet (24). Carrier *et al.* conducted an experiment in which 47 male rats were treated at the prostatic area with a single fraction (10 or 20 Gy) and found a decrease in nitric oxide synthase (NOS)-containing nerve fibers in the proximal shaft of the penis (26). They concluded that there were defects in the vascular supply of the erectile tissue and a decrease in cavernous smooth muscle (26). The first animal experimental study demonstrating fibrotic changes in the arteries of the rat penis after fractionated irradiation of the prostatic area was conducted by van der Wielen and colleagues (27). The prostate of twelve rats was irradiated in 5 daily fractions of 7.4 Gy. Three control rats were sham irradiated. Prostatic and penile tissue was evaluated for general histology and the penile tissue was further evaluated after combined staining for collagen and a-smooth muscle actin (SMA) (27). The prostate showed adequate irradiation with fibrosis occurring at 9 weeks after irradiation. The corpora cavernosa showed arteries that had developed loss of smooth muscle cells expressing SMA, thickening of the intima, and occlusions (27). All the control rats maintained normal anatomy. These data suggest that post-radiation ED might be caused by radiation damage to the arterial supply of the corpora cavernosa (26,27). Further experimental studies are needed to support these data.

To date, no final conclusions can be drawn whether or not the radiation dose to the penile structures correlates with post-radiation ED in patients treated for prostate cancer.

Therapy of post-radiation ED

Prior to the introduction of sildenafil in 1998, only very limited data were available on treatment of post-radiation ED. Sildenafil citrate is a selective inhibitor of cyclic guanosine monophosphate (cGMP) specific phosphodiesterase type 5 inhibitors (PDE5-I), and hence inhibits the degradation of cGMP in the cavernosal smooth-muscle cells, restoring erectile response to sexual stimulation in patients with ED of different etiologies. Sildenafil has been reported to be effective in the treatment of post-radiation ED in up to 90% of the patients in open-label studies (28-32), but less effective in double-blind studies (33-35). Incrocci and colleagues performed a randomized, double-blind, placebo-controlled, cross-over trial in 60 patients, who complained of post-radiation ED (33). Sildenafil 100 mg improved erections significantly as compared to placebo; 55% of the patients had successful intercourse with sildenafil. In a follow-up study of patients who had participated in the sildenafil double-blind study only 24% were still using the drug two years after (34). Reasons were lack of efficacy (60%), costs (24%) and side effects (16%). Almost half of the patients were dissatisfied with their sexual life. This indicates that patients with a history of prostate cancer treatment and subsequent ED should be informed on treatment modalities but also followed-up, and adequately counseled to improve their sexual life (34). Similar results on the efficacy of oral drugs have been reported in randomized, double-blind trials using tadalafil (36-38). Tadalafil once-daily showed similar efficacy, and even better compliance, than on-demand in a recently published randomized trial (38). In patients treated with both RT and androgen deprivation therapy sildenafil seems to be less effective (35).

Prevention of post-radiation erectile ED

If one accepts the hypothesis that radiation induces vascular damage, then decreasing the dose to pelvic vascular structures could decrease ED incidence (24). Even modern techniques do not appear to spare the neuro-vascular bundles as these are always entirely in the high-dose prostate field (10). So far, no conclusive data are available that show a correlation of the radiation doses in these structure with ED (23). It has been written a lot in the literature about

the role of PDE5-I in the penile rehabilitation process for patients after radical surgery for prostate cancer (39). This is not the case for patients undergoing radiation therapy (40). As PDE5-I have been found effective in the treatment of post-radiation ED in randomized trials of prostate cancer (33,35,36,38), one may speculate that these drugs might be useful in the rehabilitation process as well (40). Schiff and colleagues reported in a non-randomized, non-blinded study, that the early use of PDE5-I after brachytherapy was associated with a significant improvement in and maintenance of erectile function compared with late use (41). Recently two large trials have been published on the use of sildenafil (42,43) and tadalafil (44) for the prevention of post-RT ED. An Australian trial randomized 27 men undergoing RT for localized prostate cancer to receive daily sildenafil 50 mg or placebo starting one month after completion of radiation therapy for 6 months (42). Primary end point was erectile function measured by the IIEF at 2 years. The results showed no difference in erectile function between the two groups (42). Zelefsky and coauthors randomized 279 patients to double-blinded daily sildenafil 50 mg or placebo (43). Medication was started 3 days before RT (external-beam, brachytherapy or both) through 2 weeks after, and used daily for 6 months. The IIEF questionnaire was administered at 3, 6, 9, 12, 18 and 24 months (43). At 12 months erectile function scores were statistically significantly better for the active drug; 73% of the patients taking sildenafil reported no or mild ED compared to 50% of those taking a placebo. Erectile function and IIEF scores were not better anymore for sildenafil at 24 months (43). Sexual desire and overall satisfaction scores though were still better for those patients who received sildenafil. Patients on hormonal manipulation experienced worse erectile function than those who were not, regardless of treatment arm (43). No difference in erectile function was found in patients treated with external-beam RT, brachytherapy or a combination of these. The authors concluded that a longer course of treatment with sildenafil might be required to provide better functional outcomes beyond 12 months after therapy (43). Pisansky and coauthors performed a placebo-controlled, multicentre, double-blinded, randomized trial (n=242) to assess the efficacy of tadalafil once-daily in maintaining erectile function in patients undergoing radiation therapy for localized prostate cancer (44). Almost two thirds of the patients received external-beam RT (almost all them IMRT), one third received brachytherapy. Patients received tadalafil 5 mg or placebo for 24 weeks. Two-hundred-

twenty-one patients were evaluable. Primary outcome was spontaneous erectile function at 28-30 weeks after RT was started (i.e., 4-6 weeks after tadalafil was stopped). The patient was considered to maintain erectile function without the study drug at week 28-30 if he answered "about half of the time" or more (score 3-5) to question 1 of the IIEF: how often were you able to get an erection during sexual activity? Seventy-nine per cent and 74% of the participants assigned to the tadalafil group or to placebo maintained spontaneous erections, respectively, showing a difference of 5% (P=0.49), at primary end-point (44). Although patients younger than 65 years seemed to maintain erectile function more frequently than older patients, the difference did not reach a statistically significant difference. At one year similar results were found: 72% of the patients who received tadalafil versus 71% who received placebo maintained erectile function (P=0.93) (44).

The strengths of the trial are the multicentre distribution, covering different types of medical practices, the use of standardized, modern radiation techniques and doses, the use of validated questionnaires, and the assessment of other aspects of sexual functioning than erectile function only (45). Though it might be doubted whether question 1 of the IIEF is the right choice to evaluate erectile function, getting an erection does not mean that this is rigid enough for penetration (question 2) and whether this is maintained during sexual performance (question 4) (45). Another possible limitation might be the relatively short administration of the study drug; 24 weeks might be too short to prevent penile fibrosis as a consequence of radiation therapy (45). We can speculate that a PDE5-I, by increasing nightly, spontaneous, and voluntary erections, might improve oxygenation of the corporal bodies and therefore preserve endothelial and cavernosal function. This could prevent fibrosis occurring in the first 6-12 months after RT by restoration and preservation of nitric oxide-mediated vasodilation in the irradiated corporal bodies and maintain erectile function of patients undergoing radiation therapy (45). The previously mentioned animal studies (26,27) may also help to explain the beneficial effect of PDE5-I in patients complaining about ED after radiation therapy and their possible role in preventing post-radiation ED. Because of the extended period of effectiveness, tadalafil, which lasts up to 36 hours after intake, might have advantages above other PDE5-I because of its prolonged and continuous enhancement of vascular responsiveness. Unfortunately the results of the prevention trials, similar to the one with sildenafil, do not allow us yet to advice patients to take

PDE5-I to prevent ED when undergoing RT for localized prostate cancer (45).

Sexual counseling

Quality of life in general and sexual functioning in particular has become very important in cancer patients. Patients need to be correctly informed on the pelvic anatomy and on the possible sequelae of radiation on their sexual life and functioning (40). Sexual desire, satisfaction with sexual life, libido and frequency of intercourse has to be discussed as well. Patients should be offered sexual counseling and informed about the availability of effective treatments for sexual dysfunction. Being treated for prostate cancer is detrimental to patient's frequency of sexual activity. Sexual activity dropped from 2 times weekly to once a month in one study (46). The stability of sexual function in husbands and wives of cancer patients suggest that the problem developing after cancer treatment are caused by the emotional and medical impact of illness rather than by stress in the couple's relationship (46). In busy oncology clinics where outpatient visits must include educating patients about their disease, prognosis and treatment, physicians and nurses often do not have the time of assessing quality of life issues (47). Sexuality in general, and in relation to cancer in particular, should be an integral part of training at the undergraduate and postgraduate level (48). This does not happen in most medical schools and training programs in most countries around the world. The great majority of oncology professionals are scared to address sexuality and the great majority of sexological professionals are scared by cancer (48). It is time that cancer specialists and sexologists better understand each other. The challenge for any health care professional is to address both components with compassion. The recommendations of the Committee on chronic disease and cancer of the 3rd International Consultation on Sexual Medicine (4) are very useful to help developing research and educational programs in oncology and sexual medicine (40).

Conclusions

Although vascular damage to the pelvic organs seems to play a role in post-radiation ED, no reliable data are available to correlate the radiation dose received by the penile bodies and the penile bulb and ED. Furthermore, nerve injury cannot be excluded. A multi-factorial etiology has to be considered, taking into account age, comorbidity, previous pelvic surgery, drugs, pre-treatment erectile function and hormonal manipulation. The time elapsed between RT and ED evaluation is important as one should wait at least 18-24 months when ED occurrence reaches a maximum, and remains stable further on. It is important to standardize procedures to assess quality of life and sexual functioning in cancer patients, using validated questionnaires and using the definition of (im)potence advocated by the 3rd International Consultation on Sexual Medicine (4,40). Patients need to be correctly informed on the pelvic anatomy, on the possible sequelae of radiation on their sexual life and functioning, and about the availability of treatments for sexual dysfunction. Cancer clinics should offer a specific consultation for sexual function and dysfunction. Cancer affects quantity and quality of life. Any health care professional should address both components with compassion.

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Footnote

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Stereotactic body radiotherapy for organ-confined prostate cancer

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Abstract: Stereotactic body radiotherapy (SBRT) is the precise external delivery of very high-dose radiotherapy to targets in the body, with treatment completed in one to five fractions. SBRT should be an ideal approach for organ-confined prostate cancer because (I) dose escalation should yield improved rates of cancer control; (II) the unique radiobiology of prostate cancer favors hypofractionation and (III) the conformal nature of SBRT minimizes high-dose radiation delivery to immediately adjacent organs, potentially reducing complications. This approach is also more convenient for patients, and is cheaper than intensity modulated radiotherapy (IMRT). Several external beam platforms are capable of delivering SBRT for early-stage prostate cancer, although most of the mature reported series have employed a robotic non-coplanar platform (i.e., CyberKnife). Several large studies report 5-year biochemical relapse rates which compare favorably to IMRT. Rates of late GU toxicity are similar to those seen with IMRT, and rates of late rectal toxicity may be less than with IMRT and low dose rate (LDR) brachytherapy. Patient-reported quality of life (QOL) outcomes appear similar to IMRT in the urinary domain. Bowel QOL may be less adversely affected by SBRT than with other radiation modalities. After five years of follow-up, SBRT delivered on a robotic platform is yielding outcomes at least as favorable as IMRT, and may be considered appropriate therapy for stage I-II prostate cancer.

Keywords: Prostate cancer; stereotactic body radiotherapy (SBRT); hypofractionation

Background

Prostate cancer is the most common malignancy in men. An estimated 233,000 cases will be diagnosed in the United States in 2014 (1). PSA screening has led to earlier stage diagnoses; in 1998, 92% of prostate cancers were diagnosed with clinically organ-confined disease (2). The 7th edition of the AJCC Staging Manual (3), adopted in 2010, added Gleason score and PSA to the TNM staging system. Nearly 50% of patients (4) diagnosed with prostate cancer fall in prognostic Group 1, which includes patients with a clinical stage of T1-T2a, PSA <10, and Gleason 6. Active surveillance has become a suitable alternative for AJCC stage I, also referred to as "low-risk", patients (5). The PIVOT trial randomized PSA-era diagnosed patients between radical prostatectomy and observation; in the low risk group, treatment afforded no cancer-specific or overall survival

benefit, bolstering the argument against definitive treatment in this subgroup. In intermediate- and high-risk patients, the PIVOT trial showed surgery afforded, respectively, 50% and 60% reductions in prostate cancer deaths. This clear benefit justifies treatment in these subgroups.

According to the NCI Consensus Conference (6) and the Prostate Cancer Panel of the American Urological Association in 1995 (6), treatment options that should be discussed include radical prostatectomy, external beam radiation therapy (RT), interstitial brachytherapy and watchful waiting.

Historical evolution of radiotherapy for prostate cancer

Radiotherapy was first used to treat prostate cancer in the

Table 1 bDFS outcomes for low-risk prostate cancer

Rx	Institution/Author	Details	# pts	Median f/u yrs	5-yr bDFS & definition (%)			Ave[‡]
					Nadir +2	ASTRO	PSA ≥0.2	
HDR + EBRT	Seattle, Kiel, Beaumont (12)	45-50 Gy +2-4 fx boost	46	5		96		92
	CA Endocurietherapy (13)	36 Gy +5.5-6 Gy ×4 boost	70	7.25	93	90		
HDR alone	CA Endocurietherapy (14)	6-7.25 Gy ×6	117[#]	8		96		97
	Beaumont (15)	9.5 Gy ×4	95[†]	4.2		98		
LDR	RTOG 9805 (16)	145 Gy I125 alone	95	5.3	99	93		88
	11 inst meta-analysis (17)	I125 & Pd103 alone	1,444	5.25	86	88		
Exter Beam	Clev Clin (18) hypofract	IMRT: 70 Gy, 2.5 Gy/fx	36	5.5	97	97		88
	MSKCC (19)	IMRT: 81 Gy, 1.8 Gy/fx	203	7	93	85		
	9 instit meta-analysis (20)	3dRT/IMRT: >72 Gy	70	5.7		79		
	Thames meta-analysis (21)	3dRT/IMRT: 70-76 Gy	231	6.3	95			
	MDA rand dose-esc (22)	3dConformal: 78 Gy	32	>5	93	92		
	MGH Loma Linda: PROG (23)	Proton boost to 79.2 Gy	116	5.5		95		
Radic prost	Baylor: Hull (24)		299	3.9			92.5*	94
	Clev Clin, MSK: Kupelian (25)		524	5.5			92	
	Univ Penn: D'Amico (26)		322	5		88		
	Johns Hopkins: Han (27)		899	5.9			98	

bDFS estimated based on proportions within each risk group. [#], 75% low risk, 25% intermediate; [†], Included T2b in low-risk group; [‡], weighted average of ASTRO bDFS or of stated bDFS definition in prostatectomy series; *, PSA ≥0.4; bDFS, biochemical disease-free survival; EBRT, external beam radiotherapy; IMRT, intensity modulated radiotherapy; LDR, low dose rate brachytherapy; HDR, High-dose rate brachytherapy.

first half of the 20th century; the application of radium or kilovoltage therapy yielded disappointing results (7,8). The development of megavoltage external beam platforms in the 1950's (9-11) allowed higher doses to be delivered, with encouraging outcomes. The next important development was CT imaging and computerized treatment planning, which facilitated 3-dimension conformal external beam planning and intensity modulated radiotherapy (IMRT). These more sophisticated treatment plans yielded better dose conformity to the target, allowing further dose escalation. Conformal, dose-escalated techniques have yielded varying disease-free outcomes, approximately similar to those seen with radical prostatectomy (see *Table 1*), although not without toxicity.

Several randomized trials (28-30) have confirmed that dose escalation yields improved relapse-free survival rates. Fowler's dose-response analysis in intermediate-risk patients (31) (see *Figure 1*) indicate doses exceeding 90 Gy are necessary to minimize recurrence rates. A meta-analysis of seven randomized dose-escalation trials yielded the same

conclusion (32). A variety of strategies have been employed to further escalate dose and/or reduce toxicity to surround normal tissues.

Modern radiotherapy plans still had to account for variations in patient positioning, inaccuracies in treatment delivery, and internal organ motion. Radiation oncologists account for these uncertainties by adding a radial margin around the intended target, creating a "planning target volume (PTV)". This expanded target extends the high-dose treatment region into the surrounding normal structures. A PTV expansion of about 1 cm in required when skin marks are used for positioning. Set-up uncertainty can be reduced by placing gold fiducials in the prostate and imaging prior to treatment delivery. This does not account for movement within a given treatment session, or "intrafractional" motion. Kupelian (33) demonstrated that in 15% of treatment sessions, the prostate moved more than 5 mm. A study from the Mayo Clinic (34) recommended a 5-mm margin to account for intrafractional motion. The expanded PTV required in IMRT employing pre-treatment image
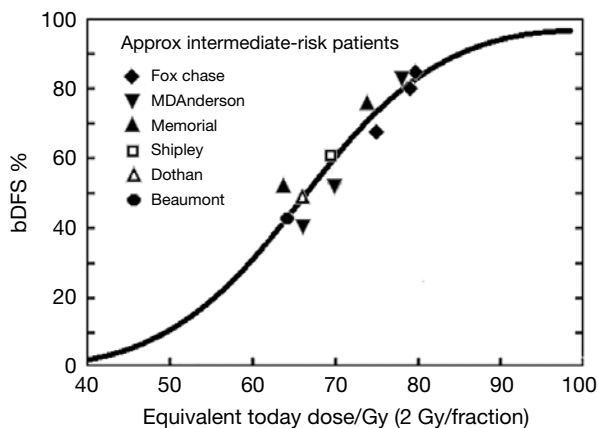
Figure 1 Relationship between dose and 5-year freedom from PSA failure for intermediate-risk patients treated with EBRT. [Adapted from Fowler (31)].

guidance has limited the maximum safe dose around 82 Gy, if delivered at 2-Gy per fraction.

Proton therapy offers the prospect of prostate dose escalation while limiting exposure to normal tissues. Proton beams deposit radiation until after passing beyond the target, where the dose then falls off rapidly. This reduces the radiation dose to normal tissues, potentially yielding fewer side effects. However, like IMRT, proton beam plans must account for prostate motion, thus the same large PTVs must be targeted. Also, since most proton beam plans employ only two beams, conformal dose sculpting around the prostate is not possible. While proton therapy reduces the volume of normal tissues receiving low dose radiation, large volumes of the rectum still receive high-dose radiation. In one study (35), protons yielded a 50% greater incidence of rectal toxicity compared to IMRT. The American College of Radiology Study 03-12 demonstrated (36) significant (8%) late grade 3+ rectal toxicity when proton dose was escalated to 82 Gy. Proton dose escalation beyond 82 Gy is thus not possible with current technology, and long-term toxicity GI toxicity appears to be no better, and perhaps inferior to IMRT.

Transperineal ultrasound-guided brachytherapy allows the delivery of conformal, high-dose radiotherapy to the prostate, with a rapid dose fall-off outside of the implanted region. In low dose rate (LDR) implants, 70-100 iodine-125 (I-125) or palladium-103 (Pd-103) sources are permanently placed within the prostate; these "seeds" slowly deliver radiation over the ensuing 2-6 months. For patients with low-risk prostate cancer, a single

LDR implant (monotherapy) yields favorable long-term outcomes (37-39). Patients with intermediate- or high-risk disease usually require a five-week course of external beam radiotherapy plus the LDR implant (40,41). When post-implant dosimetry demonstrates the prostate received a biologically equivalent dose (BED) of around 200 Gy, LDR brachytherapy yields exceptionally high relapse-free survival rates (42). This is equivalent to about 110 Gy at 2 Gy/fx, assuming $\alpha/\beta=1.5$. Unfortunately toxicity following LDR brachytherapy appears to be greater than IMRT. Fox-Chase (43) reported 3-year grade 2+ GI and GU toxicities rates were three- and five-fold greater following seed implants. Sanda's patient-reported quality of life (QOL) study (44) did not directly compare treatments, however greater declines in urinary and bowel scores were observed following brachytherapy than after external beam radiotherapy.

Hypofractionation

High-dose rate (HDR) brachytherapy has been used in the treatment of prostate cancer since the 1980's (45-52). Catheters are placed temporarily in the prostate, and then loaded with a high-dose Iridium-192 source, delivering a few fractions of very high-dose RT. Initial protocols employing HDR combined conventionally fractionated external beam RT with an HDR boost. More recent reports have employed HDR as monotherapy (14,15,53-56). Adjusting for pre-treatment risk factors, these studies yield biochemical disease-free survival (bDFS) outcomes at least as favorable to those seen with LDR brachytherapy or conformal dose-escalated RT or IMRT (see *Table 1*). A prospective study from William Beaumont Hospital (15) comparing HDR monotherapy versus LDR brachytherapy (Pd-103) showed a superior 5-year event-free survival (98% *vs.* 85%, P=0.01) and a trend towards improved freedom from cancer failure (98% *vs.* 92%, P=0.1) in the HDR cohort. The same group showed toxicity and QOL following HDR brachytherapy was more favorable than either LDR brachytherapy or conformal external beam RT (54,57). These results suggest prostate cancer favorably responds to hypofractionated regimens.

Radiation oncologists fractionate RT dose to reduce toxicity to surrounding normal tissues. For most cancers, by delivering dose over several weeks, equivalent cancer-killing effect is achieved with reduced long-term toxicity. The effect of dose fractionation on both cancer and normal tissues can be estimated using the "linear-quadratic model". In this model, the alpha-beta ratio reflects the response

of normal tissues or cancers to changes in RT dose per fraction. Most cancers respond to RT as do rapidly-dividing normal tissues (e.g., skin or mucous membranes), and thus have high α/β ratios, in the 8-12 Gy range (58). Tissues with lower α/β ratios are more sensitive to large dose per fraction (also known as hypofractionated) RT.

The results of HDR and other hypofractionated regimens led radiobiologists to reconsider α/β ratio of prostate carcinoma. Numerous studies have concluded that prostate cancer has an unusually low/ratio of about 1.5 Gy (31,59-62). A pooled analysis (63) of 5,093 patients yielded a α/β ratio of 1.55 Gy. A low α/β ratio is consistent with other biologic properties of prostate cancer: an unusually long tumor doubling times (64), and a very low proportion of proliferating cells (65). If the α/β ratio for prostate cancer is smaller than the α/β ratios for late effects in the surrounding normal tissues (3-5 Gy), then a therapeutic gain could be achieved by hypofractionation. In this setting, larger doses per fraction should result in equivalent or improved cancer control with reduced toxicity (66-68).

Several prospective clinical trials have evaluated the efficacy of hypofractionated radiotherapy in organ-confined prostate cancer. A large prospective study from the Cleveland Clinic (69) demonstrated favorable relapse-free survival and low toxicity with 70 Gy given in 2.5 Gy fractions. A trial from Royal Adelaide Hospital in Australia (70) randomized 217 patients between 64 Gy in 2 Gy/fx versus 55 Gy in 2.75 Gy/fx; these schedules are isoeffective if prostate $\alpha/\beta=2.5$. The hypofractionated arm showed a significantly better bDFS (53% *vs.* 43%), with equal toxicity in the two arms. In an Italian trial (71), 168 high-risk patients were randomized between 62 Gy in 3.1 Gy/fx versus 80 Gy in 2 Gy/fx (isoeffective if prostate $\alpha/\beta=1.8$; both arms received 9 months of androgen ablation). Toxicities were equal. Overall relapse rates were equivalent, although improved cancer control was suggested if presenting PSA was 20 or less. Thus the radiobiologic assertion that the α/β ratio for prostate cancer is low (1.5-1.8) has been confirmed by class 1 evidence.

Stereotactic body radiotherapy (SBRT) is the precise external delivery of very high-dose radiotherapy to targets in the body, with treatment completed in one to five fractions. Dose conformality is achieved using cross-firing ionizing radiation beams and image-guidance. By concentrating dose in the targeted cancer, SBRT maximizes cell-killing. Rapid dose fall-off minimizes radiation-related injury to adjacent normal tissues. Organ-confined prostate cancer should be ideally suited for SBRT as (I) dose escalation should yield

better outcomes; (II) the toxicity from treatment is due to high-dose radiation exposure to the organs immediately adjacent to the prostate; and (III) the unique radiobiology of prostate cancer favors hypofractionation.

SBRT platforms

Several external beam platforms can theoretically deliver stereotactic radiotherapy for prostate cancer. *Table 2* summaries the capability of these devices. At a minimum, target localization prior to daily treatments is required. This can be performed using x-ray imaging of implanted fiducials, or on-board CT imaging. If intra-fractional image guidance is not employed, then at least 5 mm PTV expansions are required to account for target motion. If the target can be localized during treatment, then smaller PTV expansions can be employed, potentially reducing dose to surrounding organs. The accuracy of different real-time localization systems can vary considerably. For example, with the Novalis or Varian TrueBeam systems, the operator may opt to perform intrafractional localization and correction multiple times during treatment, or only once prior to treatment. With the Calypso system, the operator sets a threshold (typically 3-5 mm) beyond which treatment is interrupted and the patient positioning corrected. With the CyberKnife, continuous image acquisition and target correction occurs routinely; the Stanford group showed that when intrafractional correction is done every 40 seconds, this device achieves sub-millimeter accuracy (72).

Correction for target motion must account for translational (i.e., anterior/posterior, right/left, and superior/inferior) motion. Since rotational motion, particularly pitch, can be substantial, correction for rotations may be beneficial, although this potential benefit has not been quantified. The use of multiple non-coplanar beams should yield better dose conformality than single-plane treatments. While non-coplanar delivery is possible for any platform, in practice centers employing gantry-based linacs treat in a coplanar fashion, as non-coplanar delivery adds complexity and time. The intrinsically non-coplanar CyberKnife platform is reported (73) to yield more conformal treatment plans than IMRT.

Clinical SBRT outcomes

The first report (74) of hypofractionated stereotactic radiotherapy treated 40 low-risk patients using a conventional linear accelerator with daily localization of

Table 2 SBRT platforms

Platform	Description	Target localization method	Real-time correction	Rotational correction
CyberKnife	Linac on robotic arm, non-coplanar delivery, variable aperture or multileaf	Orthogonal X-rays image implanted fiducials	Continuous, automated sub-mm correction	Yes, continuous automatic
Varian (Trilogy, TrueBeam etc) w/ Novalis, BrainLab	Linac on gantry. Multileaf collimator. Volumetric arc therapy available	Cone-beam CT; orthogonal X-rays image implanted fiducials	Intermittant; tx interruption & manual correction	6D couch available
Electa (Synergy, VersaHDetc)	Linac on gantry. Multileaf collimator. Volumetric arc therapy available	Cone-beam CT	No	6D couch available
Calypso	Used with gantry-based linacs	Implanted beacons provide real-time localization	Continuous; tx interruption & manual correction	No
Tomotherapy	Linac, helical delivery, multileaf collimator	Megavoltage CT	No	No

implanted fiducials. 33.5 Gy was delivered in 5 fractions to the prostate plus a 4-5 mm margin. Toxicities were acceptable. Four-year nadir +2 bDFS was 90%, suggesting further dose escalation would be beneficial.

The feasibility of SBRT employing further dose escalation was first reported by King at Stanford University (75) using the CyberKnife platform. 36.25 Gy in 5 fractions of 7.25 Gy was delivered to the prostate plus a 3-5 mm margin. In the most recent update (76) of long-term outcomes in 67 patients, there were no grade 4+ toxicities. Two patients had a grade 3 urinary toxicity, and there were no grade 3 GI toxicities. Toxicities compared favorably to other radiation modalities. Five-year Kaplan-Meier PSA relapse-free survival was 94%. The majority of subsequent reports of prostate SBRT have employed the same platform. In a series of 304 patients treated with CyberKnife at Winthrop hospital, five-year bDFS was 97%, 90.7%, and 74.1% in low-, intermediate- and high-risk groups, respectively. Five grade 3 complications were reported, all GU, for an incidence rate of 2%. In a pooled analysis of eight institutions (77), 1,100 patients were treated with CyberKnife SBRT and followed a median of 36 months. Five-year bDFS rates were 95%, 84%, and 81% in low-, intermediate- and high-risk groups, respectively. In a multi-center study (78) Cyberknife treated 129 intermediate-risk prostate cancers 40 Gy in 5 fractions of 8 Gy each, with only one grade 3 toxicity reported (GU: bladder injury). More recent reports (79,80) have shown similar favorable outcomes with gantry-based platforms.

The mature series evaluating dose-escalated SBRT are summarized in *Table 3*. In low-risk patients treated to 35-36.25 Gy in 5 fractions, 5-year bDFS ranges from 94-97%. In the low-risk patients treated in the 8-institution pooled analysis (77) and in Katz' series (84), no difference in 5-year bDFS was seen when dose was escalated from 35 to 40 Gy. Sunnybrook (79) demonstrated 97% 5-year bDFS in 84 low-risk patients treated to 35 Gy in 5 fractions with a gantry-based system. In a series (80) of 98 low-risk patients treated to 40 Gy in 5 fractions with real-time tracking on a gantry-based linac, only one biochemical failure was reported at 5 years. Current data shows no evidence of a dose response beyond 7 Gy ×5 in low risk patients. These SBRT outcomes compare favorably to the 92-94% 5-year bDFS typically reported with conventionally fractionated external beam radiotherapy (see *Table 1*).

In intermediate-risk patients treated with SBRT, bDFS outcomes vary. In a multi-center study (85) of 137 intermediate-risk patients given 8 Gy ×5 fractions on the CyberKnife platform, 5-year bDFS was 97%. In a pooled analysis of eight institutions (77), 5-year bDFS in intermediate-risk patients was only 84%. However, those patients that received biologically higher doses (38 Gy in 4 fractions or 40 Gy in 5 fractions) had 5-yr bDFS of 96.7%. The apparent improvement in bDFS in the higher-dose cohort was not statistically significant. Longer follow-up and comparisons of larger populations will be necessary to confirm trends suggesting dose escalation beyond 7.25 Gy ×5 yields better relapse-free survival in intermediate risk

Table 3 Prostate SBRT series with mature follow-up

Institution	Platform	Details	Median F/U yrs	Risk group	# pts	5-yr bDFS[†] (%)
Virginia Mason (74)	Gantry-based linac	6.7 Gy ×5	3.4	Low	40	90*
Stanford (76)	CyberKnife	7.25 Gy ×5	2.7	Low & low-interm	67	94
Stanford, Naples (81)	CyberKnife	7-7.25 Gy ×5	5	Low & low-interm	41	93
Winthrop Hospital (82)	CyberKnife	7-7.25 Gy ×5	5	Low	211	97
				Intermed	81	91
				High	12	74
San BortoloHosp (83)	CyberKnife	7 Gy ×5	3	Low, interm & high	100	94
Pooled 8 institutions (77)	CyberKnife	36-40 Gy in 4-5 fxs	3	Low	641	95
				Interm	334	84
				High	125	81
Katz & Kang (84)	CyberKnife	7-7.25 Gy ×5	5	High	97	68
Multi-institution (85)	CyberKnife	8 Gy ×5	3	Interm	137	97
Sunnybrook (79)	Gantry-based linac	7 Gy ×5	4.75	Low	84	97
21st Century Onc (80)	Gantry-based linac	8 Gy ×5	5	Low	98	99

[†], Nadir+2 definitions; *, 4-year bDFS reported; bDFS, biochemical disease-free survival; SBRT, stereotactic body radiotherapy.

patients. These 5-year relapse-free survival rates compare favorably to fractionated EBRT (23,86) outcomes, which are typically around 85%.

Mature data evaluating SBRT in high-risk prostate cancer are limited. The largest series is a pooled analysis of 8 institutions (77), in which 125 high-risk patients received Cyberknife with or without androgen deprivation therapy (ADT). 5-year bDFS was favorable at 81%. Katz (84) reported on a series of 97 high risk patient treated with either 5 fractions CyberKnife (35-36.25 Gy) or CyberKnife boost (19-21 Gy in 3 fractions following 45 Gy pelvic RT). 46 of the 97 patients received ADT. 5-year bDFS was 68%. The addition of pelvic radiotherapy or ADT had no impact on relapse free survival, although pelvic RT was associated with greater GI toxicity.

SBRT toxicity

Rates of late physician-reported GI and GU toxicities from mature SBRT series and from 3D conformal, IMRT, proton and LDR brachytherapy series are summarized in *Table 4*. Since median follow-up on the SBRT series is the 3-5 year range, these rates may underestimate the true rates of toxicities, as more toxicities may develop with longer follow-up. Nevertheless, *Figure 2A*, which illustrates the rates of grade 2+ toxicities for various modalities, suggests SBRT late urinary toxicity rates compare favorably to

external beam. Late rectal toxicity rates appear to be consistently less than those seen with external beam radiotherapy (*Figure 2B*). These series employed a robotic non-coplanar delivery platform which corrected for target motion in real-time (Cyberknife), although recent reports of SBRT employing conventional gantry-based platforms (79,80) also suggest favorable toxicity. A recent study (88) comparing Medicare claims found SBRT was associated with 38% more diagnoses of urethritis, incontinence and obstruction, compared to IMRT. This study did not evaluate patients treated with G0039 and G0040 codes (used with CyberKnife delivery) so the increased toxicity may be related to the differences in treatment technique and/or platforms. Finally, most SBRT series limited PTV doses to 35-40 Gy in 5 fractions. In a multi-center dose-escalation SBRT study (89), 5 of 91 patients treated to 50 Gy in 5 fractions required colostomy for rectal injury. This emphasizes the need to respect dose constraints for critical structures surrounding the prostate.

Patient-reported toxicity

Following definitive therapy for prostate cancer, prospective patient-completed QOL questionnaires more accurately estimate treatment-related toxicity, compared to physician reports (90,91). In Katz' report of 304 patients treated with CyberKnife SBRT, urinary and

Table 4 Toxicity rates for SBRT *vs.* EBRT, protons, brachytherapy

Technique	Institution	Details	Median F/U yrs	# pts	Late GU toxicity (%)		Late GI toxicity (%)	
					Gr2	Gr3	Gr2	Gr3
SBRT CyberKnife	Stanford (76)	7.25 Gy ×5	2.7	67	5.3	3.5	2	0
	Winthrop Hosp (82)	7-7.25 Gy ×5	5	304	8.2	1.6	4.6	0
	San BortoloHosp (83)	7 Gy ×5	3	100	3	1	1	0
	Multi-institutional (78)	8 Gy × 5	3	129	11	0.8	1	0
3D-ConfRT	Dutch Random Trial (28)	78 Gy	4.2	333	26	13	27	5
	MDA Random Trial (87)	78 Gy	8.7	151	7.3	3.3	19	6.6
IMRT	Memorial SKCC (86)	86.4 Gy	4.4	478	13	2.5	3.3	0.4
Protons	MGH PROG (30)	79.2 Gy	8.9	196	21	1.5	24	1
LDR brachy	RTOG 9805 (16)	145 Gy	8.1	94	20	3.1	5	0

SBRT, stereotactic body radiotherapy; EBRT, external beam radiotherapy; IMRT, intensity modulated radiotherapy; LDR, low dose rate; RT, radiation therapy.
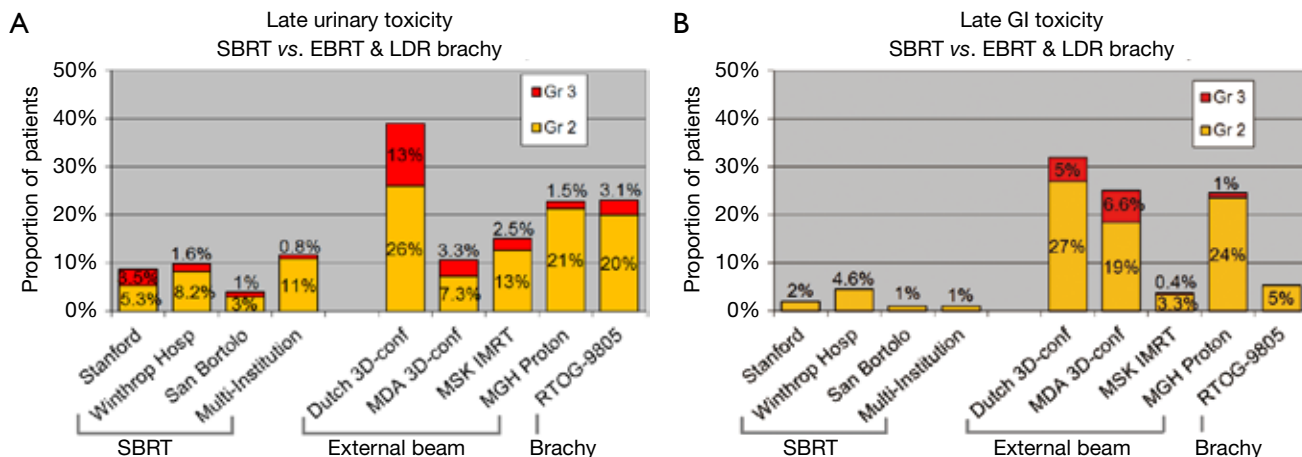
Figure 2 Late urinary (A) and GI (B) toxicity rates following SBRT, external beam radiotherapy, and brachytherapy. SBRT, stereotactic body radiotherapy; LDR, low dose rate.

bowel QOL decreased immediately following treatment, and then returned to baseline. Patient-reported QOL outcomes from a prospective multi-institutional study (85) of 309 patients treated with Cyberknife are illustrated in *Figures 3-6* below. QOL outcomes of various organ domains from the validated EPIC instrument are superimposed on the benchmark external beam and brachytherapy outcomes reported in Sanda's (92) study. Long-term changes in urinary incontinence scores following SBRT were similar to those observed in external beam and in brachytherapy (*Figure 3*). Urinary irritation/obstruction scores following SBRT appeared to be less adversely affected than after brachytherapy

(*Figure 4*). While there were small changes in bowel QOL following SBRT (*Figure 5*), these declines appeared less prominent than following EBRT and brachytherapy. EPIC sexual score declined progressively during the four years after treatment (*Figure 6*). Because this methodology does not account for potential differences between SBRT and EBRT/LDR patient populations, no firm conclusions can be drawn. Nonetheless, these patient-reported SBRT QOL outcomes are encouraging.

Cost effectiveness

Although delivery of SBRT is technically more involved that
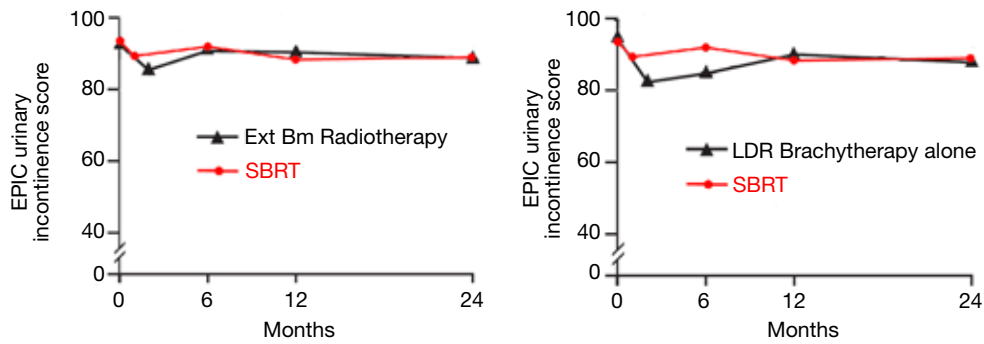
Figure 3 EPIC urinary incontince scores at baseline and at various intervals following treatment (months) from Sanda (92) (black: left graph is for external beam RT and right is for brachytherapy) and SBRT (red). SBRT, stereotactic body radiotherapy; RT, radiation therapy; LDR, low dose rate.
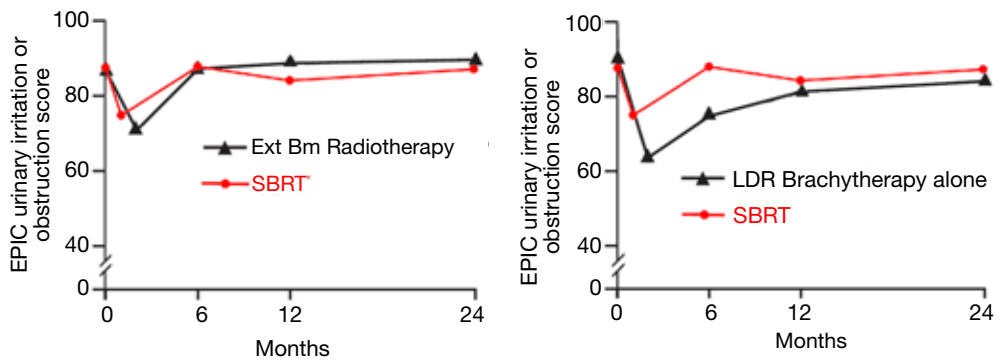
Figure 4 EPIC urinary irritation/obstruction scores at baseline and at various intervals following treatment (months) from Sanda (92) (black: left graph is external beam RT and right is brachytherapy) and SBRT (red). SBRT, stereotactic body radiotherapy; RT, radiation therapy; LDR, low dose rate.
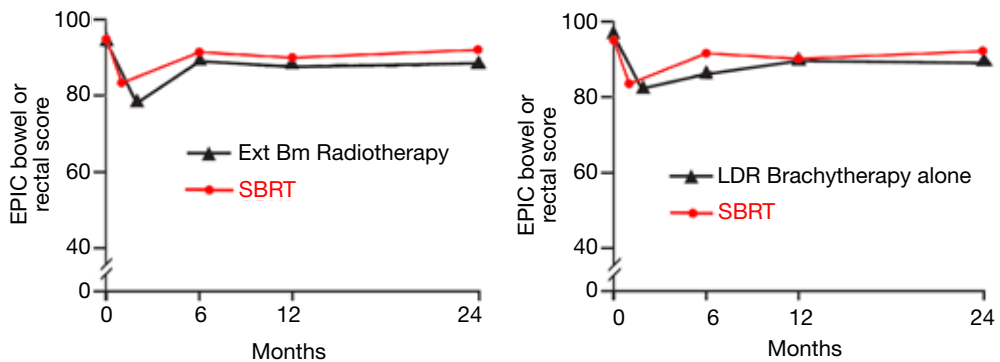
Figure 5 EPIC bowel scores at baseline and at various intervals following treatment (months) from Sanda (92) (black: left graph is external beam RT and right is brachytherapy) and SBRT (red). SBRT, stereotactic body radiotherapy; RT, radiation therapy; LDR, low dose rate.
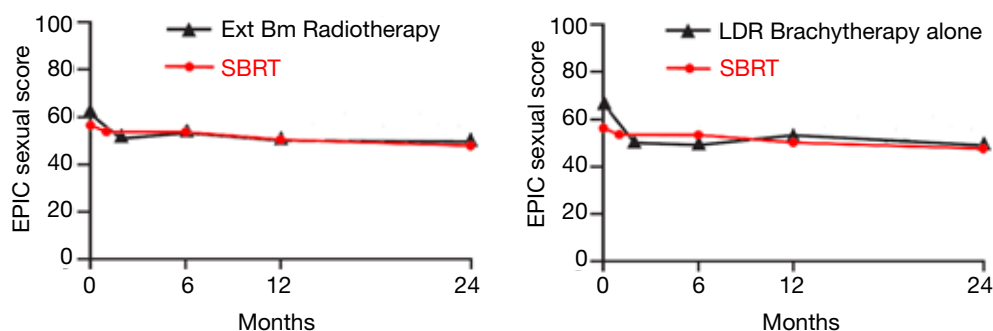
Figure 6 EPIC sexual scores at baseline and at various intervals following treatment (months) from Sanda (92) (black: left graph is external beam RT and right is brachytherapy) and SBRT (red). SBRT, stereotactic body radiotherapy; RT, radiation therapy; LDR, low dose rate.

IMRT, treatment is completed in only 5 fractions, rather than the 39-48 fractions required for IMRT. A Markov decision analysis model (93) showed the mean cost of $22,152 for SBRT versus $35,431 for IMRT. Another study of Medicare claims (88) reported mean costs of $13,645 and $21,023 for SBRT and IMRT, respectively. Furthermore, the substantial time-cost to patients (94) for conventional prostate treatment can be mitigated with SBRT.

Conclusions

SBRT offers a cost-effective alternative to external beam radiotherapy which is much more convenient for the patient. The radiobiology of prostate cancer would predict that this approach should yield superior outcomes compared to conventional protracted courses. For low- and intermediate-risk prostate cancer patients treated on a robotic, non-coplanar RT platform, five-year relapse-free survival rates are at least equivalent, or possibly superior to conventionally fractionated RT. Physician-reported late urinary toxicity appears to be similar to external beam RT, and late GI toxicity appears to be less than with external beam and LDR brachytherapy. Patient-reported QOL outcomes show urinary and bowel function return to near baseline levels two years following robotic SBRT. Long-term changes in rectal QOL appear to be superior to those reported after IMRT or LDR brachytherapy. For high-risk prostate cancer, initial CyberKnife series suggest favorable outcomes. Emerging outcomes in low-risk patients treated on gantry-based platforms are similarly encouraging. A prospective randomized trial would be required to confirm these favorable SBRT outcomes relative to other modalities. But given these excellent cancer control rates and toxicity profiles, SBRT delivered on platforms which have real-time

image guidance appears to be an acceptable approach for stage I-II prostate cancer. Further studies are also required to determine if similar favorable outcomes are possible with SBRT delivered on other platforms, and in patients with high-risk disease.

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Footnote

Conflicts of Interest: The author has no conflicts of interest to declare.

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Proton beam radiation therapy of prostate cancer - history, results, and future directions

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Abstract: Proton beam radiation therapy of organ-confined prostate cancer now constitutes one of the most commonly treated malignancies with this modality. This paper will provide a concise review of the history of proton beam therapy of prostate cancer, discuss the most recently published clinical data, examine the future uses of proton beam radiotherapy in prostate cancer treatment, and seek to place proton beam therapy in the context of other technological evolutions in radiation oncology.

Keywords: Protons; prostate cancer; dose-escalation

Introduction

Prostate cancer continues to present a major oncologic dilemma for the developed world. In the United States there were an estimated 240,000 new cases diagnosed in 2011, with approximately 33,000 deaths from this disease (1) . Prostate cancer is the second leading cause of cancer deaths among American men and accounts for approximately 10% of all cancer related deaths in men. A similar incidence and death rate is seen in Western Europe, with the lowest reported incidence being in Eastern/Southern Asia. Beginning in the early 1990's the discovery and use of Prostate Specific Antigen (PSA) as a screening tool has led to both an increase in the number of cases being diagnosed and a decrease in the proportion of men being diagnosed with advanced disease. This encouraging trend towards diagnosis with organ-confined disease has prompted the development and refinement of treatment methods directed at the prostate in the entirely reasonable hope of providing long-term disease free survival and cure.

From the standpoint of radiotherapy virtually all technical advances in prostate cancer treatment have been implemented to reduce normal tissue toxicity by limiting the volume of adjacent bladder and rectum which receive moderate to high doses of radiation. A direct consequence of this improvement in dose conformity has been dose escalation (2), a concept which has been tested and confirmed in one proton beam-based prospective randomized trial.

The unique physical properties inherent in proton beams makes them particularly attractive to the radiation oncologist, for they permit a reduction in "integral dose" (defined as the total radiation dose given to the patient) over and above anything which can be achieved with photon-based external beam treatment systems (3-5).

Initial proton beam treatment results

The ability to use proton therapy to treat deep organs was, and remains, greatly dependent on the concurrent development and refinement of cross-sectional imaging technology [CT, MRI] and modern computers, hence it is not surprising that proton beam therapy of prostate cancer did not commence until the late 1970's. Beginning in 1977, Shipley and associates at the Massachusetts General Hospital [MGH] initiated a Phase I trial in which proton beam radiotherapy was used to give a boost dose to patients with locally advanced disease that were also receiving photon radiotherapy. At that time, this boost dose was felt to be over and above what could be safely given with existing 2-dimensional photon technology. Seventeen

Table 1 Patient characteristics in initial LLU trial (adapted from Slater *et al*. 1998)

	Patients
T-stage	
1A/1B	28
1C	91
2A	157
2B	173
2C	157
3	37
Gleason score	
2-5	232
6-7	324
8-10	54
Initial PSA	
≤4.0	53
4.1-10.0	280
10.1-20.0	175
>20	85

patients with stage T2-T4 disease received a perineally-directed proton beam boost of 20-26 GyE (given at a rate of 1.8-2 GyE/day) following treatment to the prostate and pelvis to a dose of 50.4 Gy with 10 MV photons given via a four-field box approach. A perineal approach was chosen because this was the only anatomical pathway that allowed the 160 MeV proton beam generated by the Harvard Cyclotron to reliably encompass the entire prostate gland. Acutely, the treatment was well tolerated and after a follow up period ranging from 12-27 months no severe late rectal reactions were noted (6).

These favorable toxicity results led directly to the initiation of a prospective randomized trial designed to test the benefits of proton beam dose escalation in patients with locally advanced disease. Patients with stage T3-T4 tumors were chosen as it was felt that this group stood to gain the most benefit from high doses. All patients received 50.4 Gy to the prostate and pelvis with megavoltage photons, administered via a four-field box-technique. They were then randomly assigned to receive either an additional 16.8 Gy of photons (for a total prostate dose of 67.2 Gy) or 25.2 GyE of protons for a total prostate dose of 75.6 Gy. Adjuvant hormonal therapy was not permitted. The limited availability of the Harvard Cyclotron affected patient accrual; nonetheless, two hundred and two patients were eventually enrolled,

with one hundred and three being treated in the high dose arm and ninety nine in the standard dose arm.

With a median follow up of 61 months there were no differences seen in overall survival, disease-specific survival, total relapse-free survival, or local control between the arms. Patients with high-grade tumors who were treated on the high dose arm did experience a trend towards improvement in local control at five and eight years (92% and 77% *vs*. 80% and 60%, P=0.089). Patients whose digital rectal exams normalized following treatment and who underwent subsequent prostate biopsy revealed a lower positive biopsy rate in the high dose arm (28% *vs*. 45%) and, perhaps most surprisingly, the local control rates for patients with Gleason grade 4-5 tumors (57 patients total) were significantly better at five and eight years in the high dose patients (94% & 84% *vs*. 68% & 19%, P=0.001). High dose treatment was associated with an increase in late grade 1-2 rectal bleeding (32% *vs*. 12%, P=0.02) (7).

These results have been erroneously cited by some as evidence that proton-beam dose escalation is of doubtful utility (8). However, it must be noted that the patients treated in this trial were at a high risk of not only local failure but of distant failure and therefore it is not surprising that overall survival was unaffected. In addition, patients with these adverse characteristics would not, if diagnosed today, receive radiotherapy as monotherapy and instead would be treated with a multi-modality approach (9-12). What the trial did demonstrate is that (I) high dose radiotherapy did decrease local failure, and this decrease was most profound in those patients with the most aggressive tumors and (II) Dose-escalation by means of a perineal proton beam (an approach which has largely been abandoned today as higher energy proton beams have become available) can be performed safely with acceptable toxicity.

The completion in 1990 of the world's first hospital-based proton treatment center at Loma Linda University Medical Center [LLUMC] marked the beginning of a transition in proton beam therapy from the research laboratory setting to clinical radiation oncology (13). Beginning in late 1991 prostate patients at LLUMC were treated on a clinical trial who's goal was to confirm the efficacy and toxicity data generated at MGH. Between December 1991 and December 1995 643 patients were treated to total prostate radiation doses of 74-75 GyE. Patients who were deemed to be at a low risk for occult nodal metastasis were treated with lateral proton beams alone while those who were felt to benefit from elective nodal radiation received 45 Gy to the pelvis with 18-23 MV photons delivered via a multifield

Table 2 Predictors of biochemical failure-from Slater *et al.* 1998

		% Disease-free survival @ 5 years	Univariate P	Multivariate P
Initial PSA	≤4.0	100		
	4.1-10.0	88	<0.001	0.001
	10.1-20.0	68		
	>20.0	48		
Gleason	2-5	82		
	6-7	76	<0.001	0.007
	8-10	48		
T stage	1A/1B	79		
	1C	94		
	2A	87	<0.001	0.003
	2B	73		
	2C	59		
	3	59		

3-D conformal technique. Patient characteristics are shown in *Table 1*.

With a median follow up of 43 months, the overall biochemical disease-free survival [bNED] rate was 79% as per the American Society for Therapeutic Radiology and Oncology [ASTRO] definition of three successively rising PSA values above a nadir equating to biochemical failure. The risk of biochemical failure was strongly dependent on the pre-treatment PSA with five-year bNED survival rates varying from 53% in patients with pre-treatment PSA's of 20-50 to 100% with PSA's of <4.1. BNED survival was also significantly influenced by post-treatment PSA nadir. A multi-variant analysis of failure predictors demonstrated that initial stage, PSA, and Gleason Score were all strong predictors of biochemical failure at five years (*Table 2*). Acute toxicity was minimal and all patients completed the prescribed course of radiotherapy. Proctitis remained the most common late toxicity with Grade 2 proctitis occurring in 21% of patients at three years; for the majority of patients this represented a single episode of rectal bleeding. No > Grade 3 GI toxicity was seen. Grade 2 GU toxicity (primarily gross hematuria) was seen in 5.4% of patients at three years, with two patients developing Grade 3 bladder toxicity. No significant difference in late toxicity was seen between those patients treated with protons alone and those receiving pelvic x-ray therapy (14).

An update of the initial LLUMC experience was published in 2004. This study encompassed 1,255 patients with stage T1-T3 disease who were treated with proton beam radiotherapy alone (i.e., no prior or concurrent hormonal therapy) to a dose of 74-75 GyE. As was seen in the earlier trial initial PSA, Gleason Grade, and PSA nadir were all strong predictors of bNED survival. Treatment continued to be well tolerated with rates of RTOG Grade >3 GI/GU late morbidity of <1% (15).

PROG/ACR95-09 randomized dose-escalation trial

Beginning in 1996, LLUMC and MGH embarked on the Proton Radiation Oncology Group/American College of Radiology [PROG/ACR] 95-09 trial, a prospective, randomized dose-escalation study for patients with organ-confined prostate cancer. This study was designed to test the hypothesis that a dose escalation from 70.2 to 79.2 GyE would result in a statistically significant decrease in local failure, biochemical failure, and overall survival. Eligibility criteria included stage T1b-T2b disease (as per the 1992 American Joint Committee on Cancer staging system), a PSA of <15 ng/mL, and no evidence of metastatic disease on imaging studies (bone scan, abdominal-pelvic CT scan). All Gleason scores were allowed, but no prior or concurrent androgen-deprivation therapy was permitted. Pre-treatment patient characteristics are shown in *Table 3*.

Patients were randomly assigned to receive a total prostate dose of 70.2 or 79.2 GyE. Radiotherapy was administered sequentially in two phases. In Phase I, conformal proton beams were used to treat the prostate alone. Depending on randomization either 19.8 or 28.8 GyE in 11 or 16 fractions was delivered. The clinical target volume [CTV] was the

Table 3 PROG/ACR 9509 patient characteristics-from *Zietman et al.* 2005

Characteristic	#Patients [% of group]	
	70.2 GyE [n=197]	79.2 GyE [n=195]
Age, years		
50-59	43 [21.8]	37 [19.0]
60-69	92 [46.7]	103 [52.8]
70-79	61 [31.0]	55 [28.2]
≥80	1 [0.5]	0
Race		
White	176 [89.3]	178 [91.3]
Hispanic	4 [2.0]	7 [3.6]
Black	12 [6.1]	5 [2.6]
Other	5 [2.5]	5 [2.6]
PSA level, ng/mL		
<4.0	24 [12.2]	21 [10.8]
4-10.0	145 [73.6]	145 [74.3]
10-15	28 [14.2]	29 [14.9]
Median [range]	6.3 [1.2-14.7]	6.2 [0.67-14.3]
Combined gleason score		
2-6	148 [75.1]	147 [75.4]
7	30 [15.2]	30 [15.4]
8-10	18 [9.1]	15 [7.7]
Unknown	1 [0.5]	3 [1.5]
Tumor stage		
T1b	1 [0.5]	0
T1c	120 [60.9]	120 [61.5]
T2a	43 [21.8]	50 [25.6]
T2b	33 [16.8]	25 [12.8]
Node stage		
N0	0	2 [1.0]
NX	197 [100]	193 [99.0]
Risk group		
Low	111 [56.4]	116 [59.5]
Intermediate	68 [34.5]	61 [31.3]
High	18 [9.1]	15 [7.7]
Not classified	0	3 [1.5]

prostate plus a 5 mm margin. Beam arrangement was facility dependent with patients at LLUMC being treated with lateral proton beams of 225-250 MeV energy, while at MGH a perineal 160 MeV proton beam was employed. Before each proton beam treatment session a water balloon was inserted into the rectum and inflated with 100 mL of saline; this served the dual purpose of distending the rectum lumen to decrease the volume of rectum receiving any radiation and minimizing prostate motion.

In the second phase of treatment all patients received 50.4 Gy of photons given in twenty-eight 1.8 Gy fractions. The CTV was the prostate and seminal vesicles. No effort was made to include the pelvic lymphatics. Three-dimensional planning was used on all patients and photon energies of 10-23 MV were employed. The use of photons for a portion of the treatment was done solely to allow both institutions to participate in this trial, for at the time the trial commenced MGH patients were still restricted to treatment at the Harvard Cyclotron Laboratory and the limited throughput of that facility meant that the most efficient use of protons was as a boost and not as monotherapy. A total of 393 patients were randomized between January 1996 and December 1999.

The results of the trial were initially published in 2005 (16), with an update in 2010. At a median follow-up of 8.9 years there is a persistent and statistically significant increase in biochemical freedom from relapse amongst patients randomized to the high dose arm (*Figure 1*). This difference was seen when using both the ASTRO and the more recent Phoenix definition (17) (in which biochemical failure = a PSA elevation of >2 ng/mL above a nadir). Subgroup analysis showed a particularly strong benefit in 10-year bNED survival amongst the "low risk" patients (defined as PSA <10 ng/mL, and Gleason score <7 and stage < T2b), with 92.2% of high dose patients being disease free *vs.* 78.8% for standard dose (P=0.0001). A strong trend towards a similar finding was seen in the intermediate risk patients but this has not reached statistical significance (*Figure 2*). In addition, patients in the standard dose arm are twice as likely to have been started on androgen deprivation therapy as high dose patients (22 *vs.* 11, P=0.47) with such treatment usually being initiated due to a rising PSA. To date, there is no difference in overall survival between the arms (18).

As was seen in the previously reported proton trials treatment was well tolerated. Only 2% of patients in both arms have experienced late GU toxicities of Grade >3 and 1% have experienced late GI toxicity of Grade >3. Interestingly, as opposed to what has been reported in some photon-based randomized dose escalation trials high dose radiotherapy delivered via a conformal proton beam boost did not result in an increase in late Grade >3 GI morbidity amongst the high dose patients (*Table 4*). This encouraging finding has been confirmed by a patient-reported sensitive Quality of Life instrument which did not report any greater
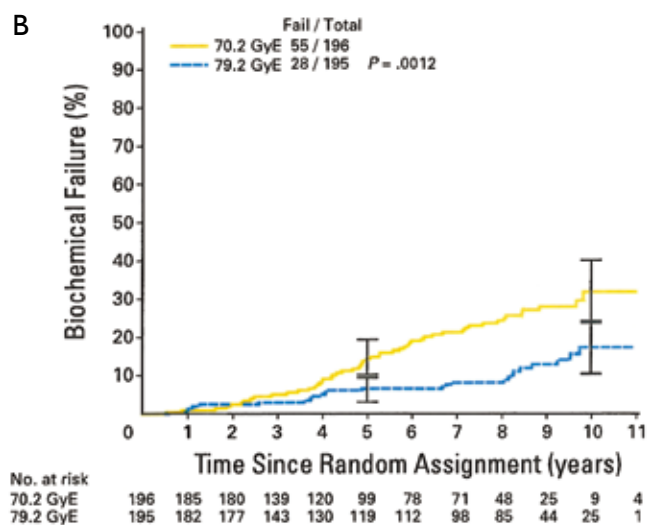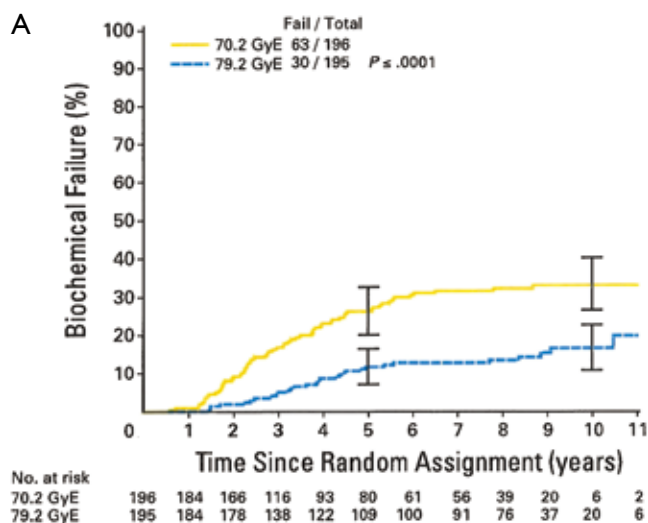
Figure 1 Biochemical failure for all patients. A. represents failure as per the American Society for Therapeutic Radiology and Oncology Definition; B. represents failure as per the Phoenix Definition. Adapted from Zietman, 2010

Figure 2 Biochemical failure for low-risk patients. A. as per ASTRO definition. B. as per Phoenix definition. Adapted from Zietman, 2010

morbidity than the physician-reported scores, and which revealed equal and high satisfaction with quality of life between both arms (19).

Thus, the PROG/ACR 9509 trial provides "Level One" evidence verifying the importance of radiation dose-escalation in organ confined prostate cancer and while this study was not designed to directly compare the efficacy of conformal proton beam radiotherapy against other conformal techniques or modalities it does demonstrate that conformal proton beam radiotherapy is an effective treatment for this disease, with minimal risk of experiencing severe treatment-induced toxicity.

University of Florida experience

The University of Florida Proton Therapy Institute opened in the summer of 2006 with prostate cancer treatment commencing at that time. From August, 2006 to October 2007 patients were treated on one of three prospective trials: 78 GyE/39 fractions for low-risk disease, dose escalation from 78-82 GyE for intermediate-risk disease, and 78 GyE with concomitant taxotere, followed by androgen-deprivation therapy, for high-risk disease. Preliminary GI and GU toxicity data was reported in 2010 with a minimum of two year follow up. Forty-two percent of the patients experienced Grade 2+ GU symptomatology requiring management after treatment, including four transient Grade 3 symptoms (all of which occurred in patients who required medical or surgical management of GU symptoms prior to radiotherapy). The overwhelming majority of Grade 2 symptoms (98%) were retentive symptoms requiring treatment with alpha-blockers. Multivariate analysis suggested that Grade 2+ GU toxicities were correlated with pre-treatment prostatitis, pre-treatment International Prostate Symptom Score [IPSS] score and, as time progressed, with patient age and pre-treatment GU symptom management. This strongly suggests that the predominant predictors of early GU toxicity were pre-treatment clinical factors.

Table 4 Acute and late GU and GI toxicity. From Zietman *et al*. 2010

Toxicity	Assigned dose																P
	70.2 GyE (n=196)								79.2 GyE (n=195)								
	Grade 1		Grade 2		Grade 3		Grade 4		Grade 1		Grade 2		Grade 3		Grade 4		
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	
Acute																	
GU	72	37	100	51	5	3	0	0	56	29	117	60	4	2	1	1	0.0745
GI	76	39	87	44	2	1	0	0	50	26	123	63	2	1	0	0	0.0006
Late																	
GU	82	42	44	22	4	2	0	0	88	45	52	27	3	2	0	0	0.7934
GI	68	35	25	13	0	0	0	0	79	41	46	24	2	1	0	0	0.0895

GI toxicities were considerably less common, 10% of the patients experiencing a cumulative incidence of Grade 2+ GI toxicities over the first two years post-treatment, including a single Grade 3 toxicity. Univariate analysis revealed a significant correlation between Grade 2 or higher GI toxicity and the percentage of rectal wall receiving radiation doses from 40-80 GyE, and the percentage of rectum receiving 10-80 GyE, while multivariate analysis revealed the rectal wall V70 correlated with the cumulative incidence of Grade 2+ rectal bleeding and/or proctitis at 24 months. The authors concluded that treatment was well tolerated with minimal and acceptable GI/GU toxicity, again mirroring the results from other proton centers (20).

ACR 0312 trial

Following the completion of patient accrual to the PROG/ACR9509 randomized trial, LLUMC and MGH opened a Phase II dose-escalation study designed to determine the toxicity and efficacy of proton-beam based dose escalation in patients with organ-confined disease. The ACR 0312 trial delivered a total dose of 82 GyE/41 fractions to the prostate, with the initial 50 GyE also including the caudal 2 cm of the seminal vesicles. PTV volumes were identical to those used in the PROG 9509 patients. The trial enrolled eighty-five patients who were treated between May 2003 and March 2006. The rate of acute GI/GU > Grade 3 complications were 1%. With a median follow up of 31.6 months six patients have developed a late Grade 3 GI/GU toxicity with one additional patient developing Grade 4 toxicity. The median time to Grade 3+ toxicity was 9.5 months with an estimated rate of Grade 3+ toxicity at eighteen months of 6%. Dose-Volume Histogram [DVH] analysis of the radiation dose to the anterior rectal wall failed to reveal a demonstrable association between dose to various volumes of the anterior wall and the risk of subsequently developing a Grade 2+ late rectal toxicity. The authors noted that the observed late morbidities compare favorably with that reported in IMRT dose-escalation studies, but that the dose of 82 GyE/41 fractions may represent the safe limit of what can be delivered with passive-scattered proton beams. They speculated that further dose-escalation should be possible with the forthcoming implementation of intensity modulated proton beams and real-time image-guided proton treatment delivery (21).

Japan

The Hyogo Ion Beam Medical Center began treating prostate patients with proton radiation in April 2001. Between 2001-2002 a series of Phase I-II protocols were performed to verify treatment techniques and assess toxicity. Once these revealed minimal toxicity proton beam therapy passed into general clinical use (22). In 2003-2004, 287 patients with stage T1-T4 N0 M0 prostate cancer were treated with lateral proton beams to a dose of 74 GyE in 37 fractions. Planning margins were similar to those used at the US proton centers, although a rectal balloon was not used. Patient characteristics are shown in *Table 5* (23). Seventy-one percent of the patients also received androgen-deprivation therapy.

The observed morbidities are shown in *Table 6*. Mirroring the US experience, Grade 3 GU toxicities were extremely rare, and no Grade 4 events occurred. On Univariate analysis CTV size and patient age were significantly associated with a greater incidence of Grade >2 GU morbidity. Multivariate analysis confirmed that large CTV's [P=0.001] and the use of androgen suppression therapy [P=0.017] independently

Table 5 Patient characteristics. From Mayahara *et al.* 2007

Characteristic	Patient [% of group]
Age [y]	
<70	146 [51]
≥70	141 [49]
T stage	
T1c	107 [37]
T2a	81 [28]
T2b	39 [14]
T3	59 [21]
T4	1 [0.3]
Gleason score	
2-6	91 [32]
7	161 [56]
8-10	26 [9]
Unknown	9 [3]
Initial PSA ng/mL	
<10	135 [47]
10.0-19.9	79 [28]
20-49.9	53 [18]
≥50	20 [7]
Risk group [MSKCC]	
Favorable	62 [22]
Intermediate	100 [35]
Unfavorable	125 [43]
Use of AAT	
No	83 [29]
Yes	204 [71]

Abbreviations: MSKCC = Memorial Sloan-Kettering Cancer Center; AAT = Androgen Ablation Therapy

predicted acute GU Grade 2-3 morbidity. These acute toxicities were comparable to those seen in published IMRT, 3-D conformal, and Brachytherapy series.

Protons *vs.* IMRT

In a widely quoted 2012 study, Sheets and colleagues at the University of North Carolina performed a comparison of prostate cancer patients treated with IMRT to those receiving 3-D conformal radiation therapy or proton beam treatment. The study reviewed patients from the SEER and Medicare databases who were treated between 2000 and 2007. Disease-free status was assessed by the need for additional cancer therapy and late morbidity was assessed by the need for additional diagnostic and/or therapeutic procedures to address radiation-induced problems.

The authors concluded that while IMRT was superior to 3-D conformal radiation therapy in terms of disease-free status and late morbidity, proton beam therapy carried with it (as compared to IM RT) an increased risk of late gastrointestinal morbidity for no therapeutic gain (24).

I believe that there are substantial methodological flaws in the study, which could easily explain the observed results:

(I) The authors made no attempt to account for likely 10-15% difference in radiation dose between the proton and IMRT patients. During the time period encompassed by this study, the "typical" IMRT radiation dose was between 70-74 Gray, the largest series of randomized data favoring dose escalation in prostate cancer was not published until 2005 and even after this paper was published it still took several years for the radiation oncology community to accept the increased external beam radiation dose of 79-81 Gray as "standard". In contrast, all the proton patients analyzed in this trial were treated at a single SEER institution, and all received a minimum radiation dose of 79.2 Gray, with many receiving 80-81 Gray. As has been previously published late gastrointestinal morbidity is highly dependent upon both total radiation dose and normal-organ delineation (13,25), so the difference in late gastrointestinal morbidity between the proton beam and IMRT patients can be easily explained simply by the higher radiation dose routinely given to the proton beam patients.

(II) In contrast to the situation prevalent in the community, all of the proton beam patients were treated on protocols that called for close and regular follow-up with particular attention being paid to gastrointestinal issues, and which mandated gastrointestinal evaluation for any late gastrointestinal complaints. Since this study did not analyze severity of gastrointestinal issues but only the need for additional diagnostic and/or therapeutic procedures, this inherent bias in the proton patients towards protocol-mandated gastrointestinal referral can explain the greater number of gastrointestinal event seen in the proton beam patients.

(III) No attempt was made by the authors to analyze any potential differences in prostate gland and rectal wall coverage between the IMRT and Proton patients via a dose-volume-histogram analysis. Indeed, the authors fail to comment on any of the technical aspects of the two different types of radiotherapy analyzed. Were identical treatment margins used on all patients? How was the dose proscribed?

Table 6 Acute GU and GI morbidities as per NCI-CTC. From Mayahara *et al*. 2007. Patient # [% of group]

	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Toxicity					
Dysuria	52 [18]	134 [47]	101 [35]	0	0
Frequency	69 [24]	179 [62]	36 [13]	3 [1]	0
Retention	204 [71]	73 [25]	9 [3]	1 [0.3]	0
Hematuria	231 [81]	50 [17]	5 [2]	1 [0.3]	0
GU overall	18 [6]	154 [54]	111[39]	4 [1]	0
Proctitis	282 [98]	5 [2]	0	0	0
Bleeding	0	0	0	0	0
GI overall	282 [98]	5 [2]	0	0	0

What immobilization, if any was used? Was image-guidance employed and if so what type? When one considers the heterogeneous nature of the IMRT patients who were treated at multiple facilities versus the homogeneous nature of the proton patients, all of whom were treated at a single center with well-defined and adhered to protocols for dose prescription, patient immobilization, and daily positioning, these technical factors become even more important as they could easily in and of themselves result in the difference in morbidity noted between the two groups.

All this serves to illustrate the risks and potential inaccuracies inherent in attempting to use large patient registries to perform a detailed data analysis. Unfortunately, papers such as the Sheets paper, once published, are often quoted as having "proved" a particular point when in fact they have done nothing substantive to settle the issue. The definitive way to answer the protons *vs*. IMRT question would be to perform a prospective randomized trial but this is no more likely to occur than were randomized 3-D conformal X-ray *vs*. IMRT trials when the latter technology was first being introduced, and for the same reason-randomizing patients to potentially receive more of a toxic substance (radiation) whose toxicity is beyond questioning and which is of no benefit whatsoever to the patient is ethically suspect and in all likelihood such a trial would, if attempted, fail to reach its accrual goal (26).

Hypofractionation

Modern radiobiologic theory predicts that prostate cancer has a low "alpha/beta ratio". This is a numeric description of the sensitivity of a particular tissue to radiation fraction size. For example, tissues with a low alpha/beta ratio are more sensitive to changes in fraction size than those with a high

alpha-beta ratio, with most estimates for prostate cancer cells being in the range of 1.5-2.0 (27). This is substantially lower than the alpha/beta ratio of 3-4 that has been assumed for late bladder/rectal toxicity. This difference in alpha/beta ratios implies that prostate cancer cells are more sensitive to changes in radiation fraction size than those of the bladder or rectum, meaning that by increasing the daily fraction size and reducing the total radiation dose one can potentially shorten the overall treatment time without compromising tumor control and without increasing the risk of incurring a late GI/GU injury.

Hypofractionation has a long-established history in proton beam therapy, and is now routinely used in proton beam treatment of ocular melanomas, intracranial metastasis, arterial-venous malformations (28), lung cancer (29), and breast cancer (30). It also is being actively investigated in prostate cancer, although to date this investigation has employed primarily IMRT-based approaches (31-34). There is an emerging body of data supporting its safety and efficacy in this setting to the point that at least one prominent radiation biologist has declared that hypofractionation should be considered the treatment of choice for prostate cancer (35).

At the time of this writing there are at least four hypofractionated conformal proton beam treatment protocols actively accruing patients in the USA. At LLUMC, a Phase I-II trial of 60 GyE/20 fractions (which is designed to be isoeffective with 81 GyE/ 45 fractions, if one assumes an alpha/beta ratio of 1.5 for prostate cancer) began accruing patients in 2009. Eligibility is limited to "low risk" patients (PSA <10 ng/mL, Gleason <7, and Stage < T2b). Preliminary analysis indicates that treatment is well tolerated with no patient (n=50) experiencing a Grade >3 acute GI/GU complication. Post-treatment PSA decreases

are consistent with expectations. At the University of Florida hypofractionation is being investigated in a similar protocol in which patients with low to intermediate-risk prostate cancer are treated on a 5-week hypofractionated schedule to a total dose of 70 GyE/28 fractions for low-risk patients, and 72.5 GyE/29 fractions for intermediate risk patients. The Proton Collaborative Group is performing a Phase III randomized trial of standard *vs.* hypofractionated proton radiation in low-risk patients, while the University of Pennsylvania is performing a feasibility trial of "mildly hypofractionated" proton radiation therapy or IMRT in intermediate-risk patients.

Proton treatment-summary

The published peer-reviewed data conclusively demonstrates that conformal proton beam radiotherapy is extremely well tolerated and can produce bNED survival rates equivalent to other modern radiotherapy modalities, and to radical prostatectomy. Conformal proton beam dose-escalation has been tested in a prospective randomized trial and has been shown to improve bNED survival without [as opposed to what has been seen in some x-ray based trials (36)] concurrently increasing the risk of late Grade >3 GI/GU morbidity. However, attempts to escalate dose to 82 GyE have been met with a substantial increase in late GI morbidity; this may reflect the "limit" beyond which treatment with passive-scattered beams and their attendant substantial penumbra may not be safely possible, although it is likely that the pending introduction of intensity-modulated proton therapy [IMPT] via active beam scanning and the implementation of novel image-guided techniques will permit further increases in dose. Hypofractionation is currently being tested in protocols at several proton centers and preliminary data on the safety and efficacy of this technique will be available within the next 12-18 months.

Future directions

Prostate cancer is an excellent site in which to test and perfect the implementation of new treatment techniques and dose-fractionation schedules. Ongoing technical advances in proton beam therapy will lead to further dose-specificity within the target organ and a further reduction in normal tissue radiation dose. Development of these techniques, including IMPT and real-time particle beam IGRT, will require their testing in a large number of patients who have similar disease characteristics and

anatomic constraints. Prostate cancer represents an excellent "test bed" for these important developments. It is an extremely common disease so large numbers of potential patients exist and, as opposed to some other common tumors (most notably lung cancer) it is typically diagnosed while confined to its organ of origin so that treated patients are likely to live for the many years post treatment required to perform a comprehensive analysis of late effects. Organ motion is minimal, which aids in the development of beam-scanning techniques that are inherently more sensitive to target motion than passive-scattered arrangements. That fact that tumor response can be assessed biochemically as opposed to clinically or radiologically means that the effects of alterations in treatment techniques on tumor can be analyzed (and potentially adjusted or even abandoned) far more rapidly than when less exacting measures are available. Lastly, in contrast to other sites like the base of skull, the prostate is adjacent to only two critical organs about which a good deal is already known concerning dose-volume effects and their impact on acute and late morbidity, thereby providing for a more accurate extrapolation of the effects of any potential treatment alterations than would be true of other, less frequently treated sites.

One of the often-voiced complaints about proton beam treatment is the cost of providing this therapy. This concern is commonly raised whenever any new treatment technology or, for that matter, any new technology, is introduced into society. In the health care arena, new technology is increasingly being met with the demand that the new method be subjected to randomized trials *vs.* existing treatment methods before the medical community and health care payers accept the new method.

This clamor for randomized data is not new, nor is it confined to the introduction of proton beam treatment. It is imperative to remember that virtually all other advancements in radiotherapy treatment technology, including the widespread embracement of IMRT, have not occurred only after this technology was first tested in prospective trials but solely because this technology promised a higher degree of dose conformality than its contemporaries. When considered from this perspective, proton beam therapy is best viewed as simply a further large step along the same road of technological advancement that has been followed diligently by radiation oncologists for the last century. A randomized proton/IMRT trial would expose (literally) one group of patients to an integral dose 3-4 times greater than the other, with no expected gain in terms of disease control. Attempts to convince educated patients to

294

Rossi. Proton beam radiation therapy of prostate cancer

participate in such a study in meaningful numbers will be difficult at best may well prove to be impossible.

It is also quite likely that the cost of proton beam radiation therapy (again, mirroring the cost of any new technology, with computers being a prime example) will inevitably decline as demand for this technology fosters the continuing development of newer, less expensive treatment units. Once the cost of proton beam treatment approximates that of IMRT arguments over relative efficacy will in all likelihood come to an abrupt end. In order for proton beam treatment to achieve this goal it has to be used for treatment of common cancers like prostate cancer. Again, this pathway is not new, and it simply follows the trail already blazed by other technologies, including IMRT.

The prostate represents perhaps the ideal proving ground for proton beam treatment. Rather than discourage its use on prostate cancer I believe that its use should be encouraged. The techniques perfected and lessons learned will serve to benefit all patients, including those treated with other radiotherapy modalities, and will add invaluable data to the widespread clinical implementation of proton beam radiotherapy.

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Footnote

Conflicts of Interest: The author has no conflicts of interest to declare.

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Impact of comorbidity in elderly prostate cancer patients treated with brachytherapy

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Objective: To analyze the correlations among comorbidity and overall survival (OS), biochemical progression-free survival (b-PFS) and toxicity in elderly patients with localized prostate cancer treated with ^{125}I brachytherapy.

Methods: Elderly men, aged ≥65 years, with low-intermediate risk prostate cancer, were treated with permanent ^{125}I brachytherapy as monotherapy. Comorbidity data were obtained from medical reports using age-adjusted Charlson comorbidity index (a-CCI). The patients were categorized into two age groups (<75 and ≥75 years old), and two comorbidity score groups (a-CCI ≤3 and >3). Toxicity was scored with Radiation Therapy Oncology Group (RTOG) scale.

Results: From June 2003 to October 2009, a total of 92 elderly patients underwent prostate brachytherapy, including 57 men (62%) with low-risk prostate cancer, and 35 men (38%) with intermediate-risk prostate cancer. The median age of patients was 75 years (range, 65-87 years). Forty-seven patients (51%) had a-CCI ≤3 and 45 patients (49%) a-CCI >3. With a median follow-up period of 56 months (range, 24-103 months), the 5-year actuarial OS and b-PFS were 91.3% and 92.4% respectively, without statistical significance between two Charlson score groups. Toxicity was mild. None of the patients experienced gastrointestinal (GI) toxicity, and only 4 patiens (4%) experienced late genitourinary (GU) grade-3 (G3) toxicity. No correlation between acute GU and GI toxicity and comorbidity was showed (P=0.50 and P=0.70, respectively).

Conclusions: Our data suggest that elderly men with low-intermediate risk prostate cancer and comorbidity can be considered for a radical treatment as ^{125}I low-dose rate brachytherapy.

Key Words: Prostate cancer; brachytherapy; elderly; comorbidity; toxicity; overall survival; biochemical control

Introduction

Comorbidity is an important consideration in oncology practice, particularly among older patients. One of the most extensively studied instruments for measuring comorbidity was Charlson comorbidity index (CCI) (1).

In a variety of common cancers, including prostate cancer, increasing comorbidity was associated with a lower likelihood of receiving potentially curative therapy, a shorter overall survival (OS), and a greater likelihood of dying from other causes (2-6). However, elderly patients are an extremely heterogeneous population as regards comorbidity: subjects can vary from very fit to not being able to live independently due to comorbidities. Thus, unlike advanced age, clinicians may be offering a radical tratment, as brachytherapy, in a selected series of patients with low-intemediate risk disease. Dispite the lack of randomized studies and only elderly population-based studies, some institutional case series showed brachytherapy to produce excellent biochemical control (b-DFS) in elderly patients with localized prostate cancer compared with alternative curative treatments (7-10). Similar survival outcomes were showed comparing patients aged >60 years

than younger with clinically localized prostate cancer and treated with brachytherapy (11,12).

The aim of our study was to evaluate the impact of comorbidity on toxicity profiles and outcomes in a series of elderly patients who underwent low-dose rate brachytherapy (LDR-BT) with ^{125}I seeds implant.

Patients and methods

Patients characteristics

Patients aged more than 65 years and treated with LDR-BT as monotherapy, were selected for this analysis. LDR-BT was offered to patients with clinically localized prostate cancer: low-risk [T1-T2a; Gleason score (GS) ≤6; prostate specific antigen (PSA) <10 ng/mL] and intermediate-risk (T2b-T2c or GS=7; PSA=10-20 ng/mL). Risk groups were defined according to National Comprehensive Cancer Network (NCCN). Clinical stage was based on the 2002 International Union Against Cancer (UICC) (13,14).

A PSA relapse was defined according to the Phoenix definition (PSA nadir+2 ng/mL) (15). Karnofsky performance status (KPS) ≥70 and life expectancy longer than 5 years were eligible criteria for our study.

All patients underwent blood tests, including PSA level, digital rectal examination, computed tomography (CT) scan of the pelvis, bone scan, transrectal ultrasonography with multiple (≥12) needle biopsy cores of the prostate to stage disease.

Follow-up was performed every 3 months in the first year, and every 6 months in the following years. At each follow-up, PSA level was assesed and acute and late toxicity was scored using Radiotherapy Oncology Group (RTOG) toxicity scale (16).

Brachytherapy tecnique

The brachytherapy procedure was performed using a transrectal ultrasound-guided (TRUS) approach, with planned total dose of 145 Gy, according to the protocol of the American Association of Physicist in Medicine (APPM-TG 43) (17).

For each patients, a transrectal ultrasound was performed 2 weeks before the implantation date to estimate the number of radioactive sources to order and implant into prostate.

After spinal anaesthesia, the seeds implant was run with intraoperative transrectal guidance (images at 5-mm were acquired). The treatment planning was performed using the planning system (TPS) Vari Seed 8.0 (Varian Medical System, Palo Alto, CA, USA). The prostate and the organs at risk were contoured according to ESTRO guidelines (urethra, rectum and penile bulb); the dose constraints used for treatment plan evaluation were 217 Gy to 0.1 cc of the urethra and 145 Gy to 0.3 cc of the anterior rectal wall (18). A mean of 78 seeds (range, 46-135 seeds) were implanted, with the activity of 0.400 mCi (19). Seven weeks after the implantation, a CT scan was performed for each patient to compare the planned dose distribution and the effective dose received by prostate and other organs at risk.

Charlson comorbidity index (CCI)

Comorbidity data were obtained from medical reports using age-adjusted Charlson comorbidity index (a-CCI). The Charlson score takes into account the presence of 19 diseases weighted on the basis of their association with mortality. A Charlson sum is calculated according to the number of morbidities affecting an individual. For each morbidity, a number of points are allocated and the sum of these points gives an overall score. This sum can be used in conjunction with the patient's age as the Charlson score to calculate a probability of survival.

A malignant solid tumor is one of clinical condition associated to CCI score, in example for patient without other comorbidities, malignancy was scored with a point of 2 or 6 for metastatic desease, thus we arbitrarily decided to not considered prosate cancer as a morbidity in a-CCI calculating, firstly because all patients of our series were affected by prostate cancer and second to obtain a more homogeneous evaluation of other comorbidities in the fianl calculation of overall score.

For each patients, CCI-aged adjusted score was computed, defining two comorbidity levels: ≤3 (low-moderate) and >3 (high).

Statistical analysis

The follow-up period was calculated from the end of brachytherapy. Analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Survival curves were obtained with Kaplan-Meier method. Log-rank test was used to evaluate influence of comorbidity on OS. We used a cut-off point of 75 years, whereby the patients were subdivided into age ≤75 years and age ≥75 years. P<0.05 was considered statistically significant. Fisher exact test was used to determine association between age-adjusted comorbidity and acute toxicity.

298

Chiumento et al. Comorbidity in elderly prostate cancer patients

Table 1 Clinical characteristics of all patients (N=92)

Characteristics	No. of patients	%
Age (year)		
≤75	51	55
>75	41	45
AJCC tumor classification		
T1a	4	4
T1b	3	3
T1c	24	26
T2a	31	35
T2b	17	18
T2c	13	14
Gleason score		
≤6	85	92
7	7	8
Pretreatment PSA (ng/mL)		
≤10	84	91
>10	8	9
NCCN risk group		
Low risk	57	62
Intermediate risk	35	38

Table 2 Age and comorbidity (N=92)

Variables	No. of patients	%
Age (years)		
65-75	43	47
75-85	42	46
85-95	7	7
Co-morbidity		
None	50	54
MI	14	15
DM (without end-organ damage)	11	12
COPD	4	4
Ulcers	3	3
Cerebrovascular disease	6	6
Mild liver disease	6	6
CRF	3	3
Malignance solid tumour	3	3
Lymphoma	2	2
a-CCI score		
2	10	11
3	37	40
4	22	25
5	10	11
6	9	8
7	3	3
8	2	2

MI, myocardial infarction; COPD, chronic obstructive pulmonary disease; DM, diabetes mellitus; CRF, chronic renal failure

Results

Patients

From June 2003 to October 2009, a total of 92 elderly patients with localized prostate underwent low-dose rate ^{125}I brachytherapy implant at the Department of Radiation Oncology, IRCCS-CROB. The median age of patients was 75 years (range, 65-87 years). Low-risk disease occurred in 57 patients (62%) and intermediate-risk disease in 35 patients (38%). The clinical characteristics of patients are reported in *Table 1*. The median follow-up time was 56 months (range, 24-103 months).

At the time of statistical analysis, 80 patients (87%) were alive without disease, and only 4 patients (4%) were alive with disease (half of these patients had an age of greater than 75 years). There were 8 deaths (9%) over the period of follow-up, including 5 deaths from other causes and only 1 patient (age: 85 years) died for prostate cancer 72 months after brachytherapy implantation. Among this group, 6 patients were ≥75 years old and 2 patients were <75 years old.

a-CCI

The a-CCI score was calculate to be ≤3 in 47 patients (51%) and >3 in 45 patients (49%). In our series, all died patients had a-CCI ≥3. Descriptive characteristics of a-CCI score are shown in *Table 2*.

OS and biochemical disease-free survival

Biochemical recurrence occurred in 7 patients (8%) while 85 patients (92%) were free from biochemical failure. The median time to PSA failure was 27 months (range, 8-40 months). The actuarial 5-year b-DFS and OS were 92.4% and 91.3% respectively (*Figure 1A,B*).

Survival analyses of non-prostate cancer mortality across Charlson groups revealed no statistical significance between
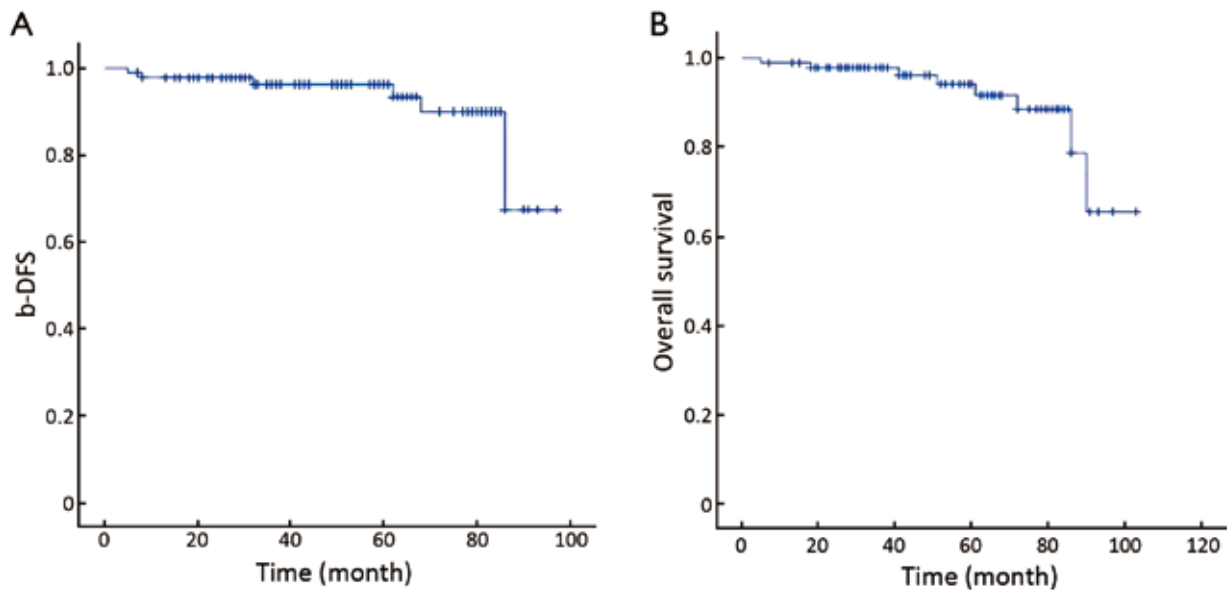
Figure 1 Survival curves. A. b-DFS; B. OS
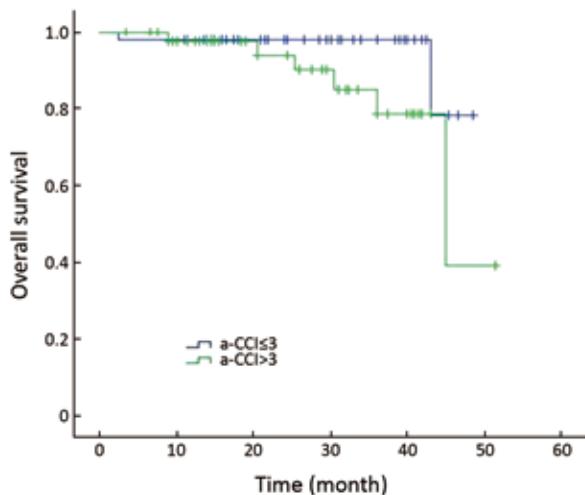
Figure 2 Overall survival and a-CCI

two groups of patients, showing an actuarial 5-year OS of 95.7% for patients with lower-moderate comorbidity (a-Charlson score ≤3) and 5-year OS of 86.7% for those with most significant comorbidity (a-Charlson score >3) (P=0.08) (*Figure 2*).

Toxicity

About toxicity profile: 24 patients (26%) experienced grade-2 (G2) acute genitourinary (GU) toxicity consisting in a frequency of urination less than one hour and dysuria requiring local anesthetic drugs. Only 4 patients (4%) experienced late grade-3 (G3) GU toxicity, because of obstructive symptoms requiring transurethral resection of prostate (TURP) in 3 patients (3%) and catheterization for longer than 2 weeks in 1 patient (1%). There was no acute G3 gastrointestinal (GI) toxicity.

A pure nonparametric correlation analysis between acute toxicity and a-CCI was also performed using Fisher exact test, which was tailed on two groups of patients adjusted for a-CCI (≤3; >3). About acute toxicity (GU and GI), the difference between two groups was not statistically significant (P=0.50 and P=0.70 respectively) (*Table 3*).

Discussion

The optimal treatment choice for clinically localized prostate cancer is controversial particularly in elderly men with presumed multiple concomitant medical morbidities. In a treatment decision making, age at diagnosis is an important determinant of therapy as remaining life expectancy, tumor grade, and comorbidity. Generally active surveillance is appropriate for men with very low risk prostate cancer when life expectancy <20 years or men with low risk disease with life expectancy <10 years (20). Literature data showed equivalent outcome, comparing

300

Chiumento et al. Comorbidity in elderly prostate cancer patients

Table 3 Acute toxicity and comorbidity

	No. of patients (%)		P
	Age <75 years (N=42)	Age ≥75 years (N=50)	
GU toxicity			
G0	4 (4%)	5 (5%)	
G1	28 (30%)	22 (24%)	0.50
G2	18 (20%)	15 (17%)	
GI toxicity			
G0	28 (30%)	26 (28%)	
G1	16 (17%)	10 (11%)	0.70
G2	5 (5%)	7 (9%)	

radical prostatectomy, external beam radiotherapy and brachytherapy as monotherapy in clinically localized prostate cancer patients (8-21,22). However, excellent outcomes were reported in some institutional case series about elderly patients with localized prostate cancer treated with LDR-BT (23,24). In literature, there were limited data on prognostic value of comorbidity. The impact of comorbidity on survival outcomes (using Charlson score) was reported both with conservative management and with active treatment for patients with prostate cancer (25,26).

Several studies reported a clear association between comorbidity and mortality in men with prostate cancer, but others didn't find any association (27,28). In a retrospective analysis on 107 patients aged ≥75 years, received radical external beam radiotherapy for prostate cancer, Fiorica *et al.* reported an acceptable rate of toxicity and a better survival for patients with mild comorbidities or good performance status (29). Neide*r et al.* in a largely unselected cancer prostate population, including also high-risk patients, treated with radical prostatectomy or external beam radiotherapy or with endocrine treatment alone, showed a statistically significant correlation between high comorbidity and early death (30). The aim of our study was to evaluate the impact of comorbidity on survival outcomes and toxicity profiles in a series of elderly patients who underwent LDR-BT with ^{125}I seeds implant.

However, it's difficult to compare existing studies due to: different study design (samples, treatments), the lack of wide use of a standard comorbidity assessment and various comorbidity tools used. It's not clear whether comorbidities can influence the acute and late toxicities due to an active treatment.

We found only one study reported urinary, bowel and erectile morbidity in unselected population-based sample of older men affected by prostate cancer who underwent brachytherapy alone, with none survival outcomes evaluation. With respect to explanatory variables (demografic variables, treatment variables, tumor related variables, risk factor for complications), age and higher CCI were associated to major urinary and bowel complications (31).

We examined 92 patients to evaluate the role of comorbidity in treatment outcomes: survival and toxicity. None correlation between toxicity and comorbidity was found in our study, perhaps because our simple was "fit" to treatment with a KPS ≥70 and not had major comorbidities; furthermore in the a-CCI score calculation, each decade of age over 40, contributes 1 point to the risk index score, which is added to the score from CCI. Thus, since over half of our patients (54%) had an age ≥75 years, probably, age had more impact on the a-CCI score than the medical comorbidity conditions.

About OS, we found no significant difference between two comorbidity groups, although the 5-year OS was shorter in patients with a-CCI >3 with respect to those with a-CCI ≤3. These data, probably, depend on the small simple size and the follow-up period.

Many different tools are available for the assessment of comorbidity but only few studies examined the performance of different comorbidity measures in prostate cancer setting, thus the optimal comorbidity index for clinical use remains unclear (32,33).

A radical approach is a safe and effective strategy, in elderly cancer patients (34,35). Our data suggest that comorbidity does not affect compliance to treatment and even results in terms of OS and b-DFS. As reported in a previous work, we believe that LDR-BRT could have a great role in elderly men treatment choice because it is a safety and efficacy treatment (36).

Although several studies have investigated the value of CCI to predict outcomes after radical prostatectomy, further studies are needed to investigate about ideal comorbidity assesment tool in elderly prostate cancer patients treated with brachytherapy (37-39).

Acknowledgements

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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302

Chiumento et al. Comorbidity in elderly prostate cancer patients

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Current clinical challenges in prostate cancer

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Abstract: Prostate cancer is the most common malignancy and the second leading cause of cancer death in men in the United States. Close to $12 billion are spent annually on the treatment of prostate cancer in the US alone. Yet still there remain tremendous controversies and challenges that exist in all facets of the disease. This review and discussion will focus on issues and challenges for clinicians and patients diagnosed with the disease. Appropriate risk stratification for men with newly diagnosed prostate cancer is an appropriate first step for all patients. Once risk-stratified, for those with low-risk of death, it is increasingly recognized that overtreatment creates an unnecessary burden for many patients. This is particularly evident when put in the context of competing comorbidities in an elderly population. For those with advanced or high-risk localized disease, under-treatment remains too common. For those with a high-risk of recurrence or failure following primary treatment, adjuvant or salvage therapies are an option, but how and when to best deploy these treatments are controversial. Recently, tremendous progress has been made for those with advanced disease, in particular those with metastatic castrate-resistant prostate cancer (mCRPC). Within the last 4 years, five novel FDA approved agents, acting through distinct mechanisms have been FDA approved for mCRPC. With the introduction of these new agents a host of new challenges have arisen. Timing, sequencing and combinations of these novel agents are welcomed challenges when compared with the lack of available therapies just a few years ago. In this summary of current clinical challenges in prostate cancer we review critical recent studies that have created or shifted the current paradigms of treatment for prostate cancer. We will also highlight ongoing issues that continue to challenge our field.

Keywords: Prostate neoplasms; active surveillance (AS); chemotherapy; castrate-resistance; hormone therapy; survival

Introduction

Prostate cancer is a highly heterogeneous disease, often with a long natural history. Nearly 240,000 men in the United States are newly diagnosed with prostate cancer annually, and more than 90% of these patients have local disease at diagnosis (1). Though statistics are variable, some autopsy reports indicate that the majority of men over age 50 harbor detectable prostate cancer after careful microscopic examination of the prostate (2). Although this data would suggest that prostate cancer follows an indolent course, it results in the death of nearly 30,000 American annually

and approximately 2.7% of men in the United States are estimated to die from prostate cancer in their lifetime (3). The incidence/mortality ratio for prostate cancer is approximately 8, making it distinct from any other major cancer (*Table 1*) (1). This perplexing series of dichotomous facts were eloquently summarized by the late the late Dr. Whitmore, "when a cure is possible is it necessary? And when it's necessary is it possible?" Reconciling this data involves stratifying patients by their risk of progression and offering appropriate therapy (or non-therapy) based on the risk of disease, comorbidities and life expectancy. After cancer progresses, additional challenges are encountered.

Only radiation and surgery have been shown to reliably cure patients and when these modalities fail, additional management problems ensue within each disease state that follows. Much progress has been made in metastatic castrate-resistant disease of late and this progress is highlighted herein. This summary is an introduction to many of the pertinent clinical challenges that face clinicians in treating and managing this complex and multi-faceted disease.

Risk-classification and disease categorization

It is now customary to divide localize prostate cancer into low-, intermediate-, and high-risk categories (*Table 2*). These categories were initially proposed by D'Amico and colleagues and are now endorsed by the National Comprehensive Cancer Network (NCCN) and the American Urologic Association (AUA). Disease classification is based on the clinical stage, PSA, and digital rectal examination results. Despite the relatively simplistic nature of these categories, they have stood the test of time and continue to be relevant in therapeutic discussions. Low-risk prostate has a Gleason score of 6 on prostate biopsy, clinical stage of T1a, T1c, or T2a and a PSA <10 ng/mL. Intermediate risk prostate cancer can have a Gleason of 7, or a PSA of 10-20 ng/mL, or a clinical stage of T2b or T2c. High-risk localized cancer has a Gleason score between 8 and 10, or a PSA of >20 ng/mL, or a clinical stage of T3a. Patients with T3b or T4 disease are classified as locally advanced.

Table 1 Incidence: mortality ratio for various cancers in 2013 (1)

Cancer	Incidence:mortality ratio
Prostate	8.04:1
Female Breast	5.86:1
Colon	2.02:1
Lung	1.43:1
Pancreas	1.18:1

The D'Amico/NCCN risk (4) classification for categorical distinctions in risk stratification in those initially diagnosed with prostate cancer is one of many that now have been published. More sophisticated models evaluating similar variables in a continuous model such as the UCSF-CAPRA (5) score or Kattan nomograms (6) allow better discrimination of individual risk of progression but are more complex.

The clinical challenges in prostate cancer are many and depend on the disease category at presentation as well as a number of other factors including previously administered treatments. In order to best understand prostate cancer it can be viewed from a disease state model which was originally put forth by Scher and colleagues and subsequently modified many times (*Figure 1*) (7). It is helpful to view prostate cancer in a series of distinct clinical categories as these categories will define not only the appropriate treatments, but also the current clinical challenges.

PIVOT: critical review of treatment versus no treatment

There has only been one trial of PSA detected localized prostate cancer that has looked at a cohort of prostate cancer men that were treated with radical prostatectomy, or not treated, and followed for a minimum of 10 years. This trial termed PIVOT deserves special comment (8). The PIVOT trial was performed primarily in Veterans Administration centers in the United States along with some academic centers. Inclusion criteria required age less than 75 with a PSA ≤50 and the trial was initiated in 1994. Any Gleason score was allowed and a total of 731 patients were randomized with a mean age of 67. About 75% of men presented with a PSA elevation or rise as the primary indication for biopsy, making it distinct from other studies (i.e., Scandinavian Prostate Cancer Group Study-4) where PSA detection drove diagnosis only in a small minority.

In PIVOT, 40% of the men had low-risk, 34%

Table 2 Risk assessment per NCCN guidelines (NCCN.org)

	Low risk	Intermediate	High risk	Locally advanced
Gleason	6	7	8-10 or	Any
Clinical stage	T1c/T2a	T2b or T2c	T3a or	T3b or T4
PSA	<10 ng/mL	10-20 ng/mL	>20 ng/mL	Any

Note: low risk patients must have Gleason 6 and PSA <10 and clinical stage T1c or T2a. For intermediate and high risk categories the Gleason scores or PSAs or clinical stages can result in categorization.

Figure 1 Clinical disease states of prostate cancer [adapted from Scher (7)].

intermediate-risk, and 21% high-risk prostate cancer (about 5% were missing data). After 10 years median follow-up, 77% of the men randomized to surgery underwent surgery and 20% of the men randomized to observation had definitive treatments with curative attempt. Over the course of the study 48.4% of the men died but only 7% died from prostate cancer. Given that it is generally accepted that men need to survive at least 10 years to benefit from surgery, this clearly indicates that the population was not ideal for this type of study.

There were no differences in prostate cancer specific mortality noted between the surgery and observation groups and a number of subsets were underpowered. Within the low-risk prostate cancer group, 62 deaths out of 148 were noted in the surgery arm and 54 out 148 men died in the observation arm. The hazard ratio (HR) for overall survival (OS) for low-risk disease was 1.15 (95% CI: 0.80-1.66). The intermittent- and high-risk diseases had favorable HRs for surgery with the HR for OS at 0.69 (95% CI: 0.49-0.98) and 0.74 (95% CI: 0.49-1.13), respectively, despite being underpowered with regard to subset analysis. Those with a PSA of >10 ng/mL also had as HR of 0.67 (95% CI: 0.48-0.94) favoring surgery. Thus some subsets favored surgery and some did not in the OS analysis.

In PIVOT, approximately 40% of the men had died

by 10 years of followup indicating that either the age or comorbidity was suboptimal in this trial which has been characterized as being a trial of surgery in men appropriate for watchful waiting (instead of a trial of observation in men appropriate for surgery). It is clear that OS was suboptimal for a surgical-treated population and there was inadequate power to accurately assess various subsets. Regardless, the data indicate that patients with low-risk disease had no benefit from treatment. Of men in the low-risk category treated by surgery (N=148), 6 men died from prostate cancer, whereas in the observation group (N=148), 4 men died from prostate cancer. Taken together there was a strong trend toward benefit in men treated with surgery for those with intermediate and high risk disease but no trend toward benefit in low risk disease at 10 years of followup in a population which included many men who died less than 10 years after randomization. This trial points to the importance of risk stratification in decision making but also demonstrates that our current stratification schemes are imperfect. Better risk stratification is one of the key challenges for prostate cancer research going forward.

Life expectancy in prostate cancer management

The ability to predict an individual patient's life expectancy

Table 3 Treatment of prostate cancer by risk category: adapted from Cooperberg *et al.* (16)

Risk category	Watchful waiting (%)	Radical surgery (%)	Brachytherapy (%)	External beam (%)	Cryotherapy (%)	Androgen deprivation (%)
Low	9.2	56.8	16.0	7.3	3.1	7.6
Intermediate	4.8	52.9	13.5	12.3	4.5	11.9
High	3.2	32.2	7.5	18.1	6.1	32.8

Note: watchful waiting and active surveillance or not distinguished herein. Radical surgery is radical prostatectomy.

is critical for screening, diagnosis, and/or treatment of localized prostate cancer (9). This is particularly important and difficult for prostate cancer patients due to the cancer's variable and generally long natural history coupled with its prevalence in older men with competing comorbidities. Physicians are poor at predicting overall life expectancy. Several tools are available to assist in predicting life expectancy (10). The first are actuarial life tables, which represent an average number of remaining life years based on the age/sex of a group of individuals. While actuarial tables are easily accessible (11) and rapidly provide a reasonable estimation, they fail to account for individual medical comorbidities. The second tool available for life-expectancy calculations are comorbidity indices, perhaps the best known is the Charlson comorbidity index (12). This index assigns weights to 19 medical conditions and adjusts life expectancy based on those weights. The tool is limited in that patient's comorbidities are dichotomized rather than considered in a continuous fashion and it may over emphasize the importance of some medical conditions. Nomograms for life expectancy that incorporate multiple variables are also available. Such nomograms, predict 10 years life expectancy following treatment for localized prostate cancer with a predictive accuracy in the range of 69-84% (13-15). Nomograms to predict life expectancy in patients electing active surveillance (AS) are currently under development.

Low-risk localized prostate cancer: concepts and challenges

Unfortunately there has largely been a failure of clinicians to meet the challenges of low risk prostate cancer with the great majority of patients receiving aggressive therapy (see *Table 3*) regardless of age or disease risk (16,17). Patients with low risk disease have a much greater probability of dying from causes other than prostate cancer, even 20 years after diagnosis (18). Clearly many patients with low-risk prostate cancer will not benefit from active treatment.

While there may be multiple reasons for the over treatment of low-risk disease, perhaps the most difficult to overcome is the fear, on the part of both the clinician and the patient, of missing the opportunity for high probability of cure with therapeutic intervention. Watchful waiting (WW) refers to conservative management of prostate cancer until the development of local or systemic progression at which point palliative measures are employed. A recognized alternative to WW or active treatment is AS; a therapeutic strategy that involves actively monitoring the patient's disease with the expectation to intervene with intent to cure if the cancer progresses. AS is a recognized strategy that has emerged in the past decade and is endorsed by the NCCN, the American Urological Association (AUA), and the European Association of Urology (EAU) for select patients.

Although multiple ongoing clinical studies are evaluating the effectiveness of AS, existing data is largely from non-randomized, immature single institution with follow-up of less than 10 years. All agree that followup is suboptimal. Inclusion criteria are typically based on predictors of progression of disease and vary somewhat from study to study. Inclusion criteria include pathologic assessment of prostate biopsy with a particular emphasis of Gleason grading, clinical staging via digital rectal exam of the prostate, various measures of volume of cancer within the prostate (based on the number biopsy cores with cancer and the length of cancer in those cores), total PSA, and (to some extent) PSA adjusted for the size of the prostate (PSA density). More recently studies have assessed use of novel bio- and genetic-markers as part of AS cohorts, however determining which markers to use and how to best incorporate them is currently is investigational (19). Unfortunately all of these predictors of progression have significant limitations and better characterization of the extent and aggressiveness of disease at the time of diagnosis remains a challenge. Clinical staging with DRE is subjective and lacks precision. PSA or PSA density reflect not only the burden of cancer but the volume of benign prostatic hyperplasia and/or the presence of

inflammation. PSA levels may fluctuate and a single test may be unreliable (20). Gleason score is subjective and dependent on the interpretation of individual pathologists. Biopsies fail to sample the entire gland and changes in Gleason grading from biopsy to radical prostatectomy have been demonstrated to be 36% at tertiary care centers with expert dedicated genitourinary pathologists examining both specimens (21). Perhaps most controversial of all is determining volume of disease; the number of biopsy cores containing cancer may depend in part on the total number of cores taken, and the length of the core containing cancer, but biopsy techniques are not standardized among urologists and methods of measurement not standardized amongst pathologists.

Several studies, despite nuanced differences in inclusion criteria, and intensity of follow up, have confirmed that, in well-selected patients with low-risk prostate cancer undergoing AS there is a low rate of cancer-specific death, but longer follow-up is needed before definitive conclusions can be reached (22-30). The randomized PIVOT trial is consistent with these observations as well as the SPCG-4 study (31). Both studies emphasized that long term followup is key to understanding cancer-specific survival (8,31). What is novel is that patients in these AS studies have undergone repeat evaluation including prostate biopsy and were offered curative treatment upon evidence of progression.

Two important and largely unresolved clinical challenges emerge from the AS studies; first is how do we define progression? Defining progression is challenging because the prostate is incompletely sampled on biopsy and it is unclear if increases in grade or volume on subsequent biopsies is a result of de-differentiation of the original tumor(s) or merely a result of more/better sampling (30). Most progression of tumors usually comes in the form of upgrading and occur in first two years of enrollment in AS, supporting the theory of better sampling. One study demonstrated that immediate repeat biopsy prior to enrollment in AS resulted in upstaging or upgrading in 27% of patients (28). More follow will be needed to determine if rates of progression begin to rise as the cohorts are followed for longer periods. Better biopsy schemes (MRI-guided) have been proposed and this may help to answer some of the questions related to under-grading of biopsies (32). It is clear that conventional prostate biopsies are "blind" and that imaging plays little role in current standard of care.

The second major challenge with AS is to determine whether intervention for patients who experience progression (however it is defined) have outcomes that approximate their initial projected outcome? If patients who experience progression on AS protocols have worse prognosis, earlier intervention may be of benefit. Two randomized studies aimed to address these issues by randomly assigning men with low risk prostate cancer to AS or radical intervention; the ProtecT (Prostate testing for cancer and Treatment) has completed accrual at nine centers in the United Kingdom and the Surveillance Therapy Against Radical Treatment (START) which has has recently been terminated due to poor accrual. Results for both are many years away.

High risk localized prostate cancer

High-risk clinical localized prostate cancer shares many of the same challenges with low risk prostate cancer; appropriate risk stratification based on an imprecise physical exam, limited random sampling of the prostate, and a variation in PSA. However, that is where the similarities end. While the primary challenge associated with low-risk prostate cancer is often an over treatment of disease, the primary challenge of high-risk prostate cancer is often under treatment. Many patients with high-risk disease who are likely to benefit from aggressive local therapy with curative intent only receive palliative treatment with androgen deprivation therapy (ADT). The CaPSURE database, a provider-based registry from a number of community based urology practices has demonstrated that 41% of high-risk patients receive ADT alone, compared with 24% and 28% that undergo RP and RT respectively (17,33).

The cause for this under treatment is not completely clear, but is likely based on the erroneous belief that treatment offers little benefit as these patients are likely to fail and die of disease. However, depending on the definition of high-risk disease, local treatment with either RP or RT results in progression free probability (PFP) of 49-80% (34,35). Perhaps even more convincing are several randomized trials which have demonstrated improved survival in men with high-risk prostate cancers who have received active treatment compared with observation or ADT alone. In PIVOT, men with intermediate- and high-risk prostate cancer had a strong trend toward improved OS with RP compared with observation (8). Similar findings were reported in the SPCG-4 study randomizing men with non-PSA detected prostate cancer to RP or observation (31). Finally, the randomized trial SPCG-7 for high-risk prostate cancer, demonstrated improvement in OS with external beam radiation plus ADT compared with ADT alone (36).

Although no adequately powered randomized trial has determined the best active treatment for high-risk localized prostate cancer, monotherapy with ADT has the potential for significant harms, reduces QoL, and is not indicated for patients with asymptomatic localized prostate cancer. Its persistent use as monotherapy represents a challenge for the field (37).

Death from prostate cancer post-radical prostatectomy

A large data-base study consisting of over 11,000 patients (and confirmed in a data set of over 12,000 patients) with projected 15 years of followup from a series of excellent cancer centers around the country indicated that Gleason 8 or higher, seminal vesicle invasion, and lymph node positivity were particularly associated with a higher risk of prostate cancer death, regardless of the age group examined (37). The 15-year prostate cancer specific mortality risk was estimated as being 0.8% to 1.5%, 2.9% to 10%, 15% to 27% and 22% to 30% for organ confined cancer, extra-prostatic extension, seminal vesicle invasion, and lymph node metastasis, respectively. Nomograms have been developed to assess prostate cancer-specific mortality risks with long term follow-up (38). This study emphasizes the very low risk of death from prostate cancer in patients with low-risk disease, while demonstrating the potentially aggressive nature of other tumors in a manner that can be quantitated over time.

Adjuvant radiation therapy post-radical prostatectomy

With regards to the utilization of adjuvant radiation therapy, there is a randomized prospective Southwestern Oncology Group (SWOG) trial which supports the concept of OS benefit for adjuvant radiation therapy in individuals with pathologic T3a and T3b post-surgery (39). The data however are somewhat controversial in that there is a substantial proportion of these patients who will never recur post-operatively and the use of adjuvant radiation therapy may clearly be associated with over treatment. An important European trial (EORTC 22911) looked at adjuvant radiation therapy and demonstrated no survival benefit despite a PSA recurrence benefit (40). The clearest conclusions to be reached are that the PSA benefit was not translatable into a life expectancy benefit

because so many of the patients who have a PSA recurrence post-prostatectomy are not destined to die from their disease. This emphasizes that PSA recurrence does not equate to death, a finding clearly demonstrated in careful analyses of the Johns Hopkins database (41).

Salvage radiation post-radical prostatectomy

One problematic area that has been not carefully examined in the context of the current clinical prostate cancer debate is the issue of salvage radiation and whether or not hormones may provide an additional positive benefit to external beam radiation (42). Although hormonal therapy in the context of radiation for localized intermediate or high-risk disease is certainly standard of care (43), the utility of hormonal therapy in combination with salvage radiation in the post-prostatectomy setting is not clear. The RTOG trial 0534 is addressing this issue in a prospective randomized manner with an accrual goal of nearly 1,700 patients (42). To date well over 1,000 patients have been accrued and this trial should be definitive in terms of answering the question of whether or not ADT adds value to salvage radiation for those with a PSA rise post-radical prostatectomy.

Timing of hormonal therapy

Another controversy in prostate cancer management is the timing of hormonal therapy for people who have failed primary treatment with curative intent and who have a rising PSA. To date there have been no trials that clearly indicate that earlier therapy is better for this particular patient population.

The data demonstrating that early ADT in combination with external beam radiation is superior to radiation alone, is plentiful and the original studies performed by the EORTC lead by Bolla and colleagues have stood the test of time (43). The use of hormonal therapy in the absence of radiation, as compared to hormonal therapy plus radiation, clearly leads to an inferior outcome (36).

In one trial, important though very small, patients with lymph node metastases detected at the time of radical prostatectomy were randomized to receive ADT for life or observation. In this context the hormonal therapy was found to be better with regards to OS as well as other intermediate endpoints (44). Unfortunately, the small size of this trial, and the lack of additional prospective randomized trials supportive of these conclusions, are problematic.

In a prospective study that utilized hormonal therapy early or later for those deemed to be unsuitable for definitive local therapy (EORTC 30891), there was slight improvement in OS for immediate androgen deprivation but quite oddly the prostate cancer specific mortality was not improved (45).

A retrospective study performed in hospitals associated with the US Department of Defense, found overall that there was no difference in bone-scan radiographic progression-free survival for early as compared to later ADT for patients with a PSA rise post-radical prostatectomy (46). However, when considering those patients with a Gleason 8 or higher disease, or those patients with a pre-ADT PSA doubling time (PSADT) of <12 months, there was an improvement in bone scan progression-free survival for those with a PSA of <5 ng/mL as opposed to >5 ng/mL, or for those with a PSA of >10 ng/mL as opposed to those with a PSA of <10 ng/mL. It is possible that lead-time bias represents the explanation for this finding. Given the lack of randomization here, one cannot view these data as being definitive but the finding that men with a PSADT of more than one year and a Gleason of 7 or less did not benefit from early ADT may be important.

Taken together, although ADT and radiation yields results that are superior to radiation alone in both intermediate and high risk disease, the use of early hormonal therapy for those with other disease states is considered controversial at best and no clear consensus can be drawn from the literature for those with a PSA rise after definitive therapy.

Intermittent versus continuous hormonal therapy

The use of hormonal therapy in an intermittent or continuous fashion is a current debate in our literature. For patients who have had a PSA recurrence after definitive radiation without evidence of metastatic disease, at 6.9 years of follow-up, both the intermittent and continuous therapeutic approach using ADT were not distinct when it comes to OS (47). However, there are improvements seen in the several quality of life parameters for patients treated with an intermittent approach, consequently many people now regard intermittent hormonal therapy as standard of care for individuals who have a non-metastatic PSA recurrence. Though this study convincingly shows that intermittent and continuous ADT showed no significant difference in OS for this population, the more important question regarding the timing of ADT (when should it

begin) was not settled by this study (48).

A large SWOG trial addressed patients who were treated for initial metastatic disease with an intermittent versus continuous ADT regimen but unfortunately the conclusions were equivocal (49). In a non-inferiority analysis, the intermittent arm had a HR slightly worse (HR: 1.1; 95% CI: 0.99-1.23) but the confidence intervals overlapped both 1.0 and the pre-specified upper boundary of 1.2 thus the study concluded that intermittent ADT in this setting was not non-inferior. There was much about this trial that was suboptimal and notably there were little difference between the intermittent versus continuous regimens in terms of overall quality of life. While most individuals continue to regard continuous ADT as the standard of care for metastatic patients intermittent may be a reasonable alternative.

Non-metastatic CRPC (mCRPC)

No definitive studies demonstrate any agents offer survival advantage for patient with non- mCRPC. Modest improvements in bone-scan free survival were reported for denosumab therapy as compared to placebo but OS was not distinct and the incidence of osteonecrosis of the jaw was significantly higher in denosumab treated patients (50).

Overview of mCRPC

The summary and sequence of overall FDA approvals in mCRPC can be seen in *Tables 4,5*. The first drug to prolong survival in this setting was docetaxel in 2004. Prior to that, various FDA approvals involved pain or other non-OS endpoints. The progress in metastatic castrate resistant prostate cancer has been phenomenal since 2010 when two drugs, sipuleucel-T and cabazitaxel where both approved after demonstrating a prolongation of OS. Additional trials demonstrating prolongation of OS have subsequently been demonstrated for abiraterone, enzalutamide and radium. It is possible to classify these trials into different categories based on whether they were "front line" or post-docetaxel. The cabazitaxel approval in 2010 was in the post-docetaxel space, the first abiraterone approval in 2011 was in the post-docetaxel space, as was enzalutamide in 2012. Abiraterone was given a second approval for those individuals treated with for asymptomatic disease in the pre-docetaxel space in 2012. Sipuleucel-T in 2010 was approved in the asymptomatic or minimally symptomatic setting without regard for prior docetaxel treatment.

Table 4 FDA approvals in metastatic CRPC by year of approval and key endpoints

Agent	Year FDA approval	Key endpoint/setting	Class of drug
Estramustine	1981	Response	Estrogenic action
Strontium-89	1993	Bone pain	Radiopharmaceutical/beta emitter
Mitoxantrone/prednisone	1996	Pain	Chemotherapy/anthracenedione
Samarium-153 EDTMP	1997	Bone pain	Radiopharmaceutical/beta emitter
Zoledronic acid	2002	Skeletal related events	Bisphosphonate
Docetaxel/prednisone	2004	Survival	Chemotherapy/taxane
Sipuleucel-T	2010	Survival	Autologous cellular immunotherapy
Cabazitaxel/prednisone	2010	Survival	Chemotherapy/taxane
Denosumab	2010	Skeletal related events	Monoclonal/anti-RANK ligand**
Abiraterone/prednisone	2011	Survival	Androgen synthesis inhibitor
Enzalutamide	2012	Survival	Anti-androgen
Abiraterone/prednisone	2012	Radiographic PFS*/survival	Androgen synthesis inhibitor
Radium-223	2013	Survival	Radiopharmaceutical/alpha emitter

*PFS, progression free survival; **Receptor activator of NF-Kappa B.

Table 5 Key trials in mCRPC demonstrating a survival benefit

Trial	Disease state (all mCRPC)	Trial design and comparator arm	HR	Survival (months)
TAX 327 (51) N=1,006	With or without symptoms	Docetaxel/prednisone *vs.* mitoxantrone/ prednisone	0.76	18.9 *vs.* 16.5
IMPACT (52) N=512	Minimal symptoms	Sipuleucel-T *vs.* control	0.78	25.8 *vs.* 21.7
TROPIC (53) N=755	Post-docetaxel	Cabazitaxel/prednisone *vs.* mitoxantrone/ prednisone	0.70	15.1 *vs.* 12.7
COU-AA-301 (54) N=1,195	Post-docetaxel	Abiraterone/prednisone *vs.* placebo/ prednisone	0.65	14.8 *vs.* 10.9
ALSYMPCA (55) N=921	Bone-metastatic symptomatic both pre- and post-docetaxel	Radium-223/BSC* *vs.* placebo/BSC	0.70	14.9 *vs.* 11.3
AFFIRM (56) N=1,199	Post-docetaxel	Enzalutamide *vs.* placebo	0.63	18.4 *vs.* 13.6
COU-AA-302 (57) N=1,088	Asymptomatic pre-docetaxel	Abiraterone/prednisone *vs.* placebo/ prednisone	0.75	NR *vs.* 27.2

*BSC, best supportive care.

The latest approval, radium-223 was approved in 2013 in symptomatic prostate cancer without visceral metastases. There was no mention of the docetaxel treatment in the radium-223 label as patients with or without docetaxel treatment both had a prolongation in OS in a pre-specified stratified analysis.

There are now a total of seven trials that have been pivotal for FDA approval in the mCRPC space as shown in *Table 5*. These trials all reported HRs for OS between 0.63 and 0.78 (51-58). The OS was quite variable from trial to trial but considering that some of these trials were conducted predominately in asymptomatic patients with no prior therapy for CRCP (52,57), whereas others were conducted in patients who had progressed post-docetaxel (53,54,56), a direct comparison of survival cannot be performed.

Pivotal docetaxel trials

In 2004, the FDA approved docetaxel/prednisone for mCRPC. Two trials examined the efficacy of docetaxel in patients with metastatic castrate resistant prostate cancer and served as the basis for the FDA approval. The TAX 327 trial randomized 1,006 men with metastatic castrate resistant prostate cancer to either 12 mg/m^2 mitoxantrone every three weeks, 30 mg/m^2 of docetaxel weekly for 5 out of 6 weeks or to 75 mg/m^2 of docetaxel every three weeks (51). The every 3 weeks schedule of docetaxel demonstrated a survival advantage with a median survival of 18.9 months compared to 16.5 months in the mitoxantrone group and 17.4 months in the weekly docetaxel group. The SWOG 9916 trial randomized 674 men to either docetaxel at 60 mg/m^2 with estramustine every three weeks or to 12 mg/m^2 of mitoxantrone every three weeks (58). Docetaxel demonstrated a survival advantage with a median survival of 17.5 compared to 15.6 months for mitoxantrone. Progression of prostate cancer on docetaxel is an inevitability and presents one of the challenges for the clinician that has been more recently addressed by a series of trials and FDA approvals in the post-docetaxel space (53,54,56).

Immunology therapy: sipuleucel-T

Immunology therapy has been a debatable topic in all of cancer with considerable discussion and little promise until recent years. After initial submission of limited data, and a convoluted review process that did not involve the usual divisions at the FDA, sipuleucel-T was initially not approved. The trials initially submitted included two relatively small randomized trials which were considerably smaller than typical for FDA approvals. The sponsors then designed and implemented a much larger trial called D9902B or the IMPACT study which was conducted in patients with asymptomatic or minimally symptomatic mCRPC. There was no benefit in terms of progression free survival or radiographic response, but the group randomized to initial treatment with sipuleucel-T had better OS compared to the placebo group (52). It has been questioned whether the control group did worse than might have been anticipated however our review of the data do not support this concept and the control group in this study did no worse than patients in other analogous trials.

Abiraterone and enzalutamide

The approvals of abiraterone and enzalutamide challenged commonly held beliefs in metastatic prostate cancer—specifically, both are hormonal therapies that have shown activity in what has been termed castration resistant disease. Abiraterone works through selective inhibition of CYP17 lyase, and a phase I/II study of the agent highlighted significant activity of the drug in both the pre- and post-docetaxel setting. Two phase III studies of abiraterone ensued, encompassing both of these disease spaces. In the COU-AA-301 trial, a total of 1,195 patients with mCRPC and prior docetaxel therapy were randomized in a 2:1 fashion to receive abiraterone or placebo (both with prednisone) (54). The trial met its primary endpoint, demonstrating an improvement in OS with abiraterone therapy (14.8 *vs.* 10.9 months; P<0.001). Secondary endpoints, including time to PSA progression and PSA response, were also improved with abiraterone. In contrast to COU-AA-301, COU-AA-302 examined a cohort of patients with mCPRC who were docetaxel naïve (57). In this study, patients were randomized in a 1:1 fashion to either abiraterone or placebo (again with prednisone). The study had a co-primary endpoint of improvement in radiographic PFS (rPFS) and OS. Ultimately, PFS was improved with abiraterone (16.5 *vs.* 8.3 months; P<0.0001). Although OS was improved with abiraterone (35.3 *vs.* 30.1 months; P=0.0151), the difference did not meet the threshold established by the O'Brien-Fleming method (P=0.0035). Nonetheless, on the basis of the two studies noted herein, abiraterone has garnered FDA approval in both the pre-docetaxel and post-docetaxel setting.

The mechanism of enzalutamide differs significantly from abiraterone. Specifically, enzalutamide is a potent antiandrogen that inhibits nuclear translocation of the androgen receptor. With phase I/II data showing compelling activity in mCRPC, two phase III programs were launched. In the AFFIRM trial, 1,199 patients with mCPRC and prior docetaxel therapy were randomized in a 2:1 fashion to receive enzalutamide or placebo (55). The study was stopped after a planned interim analysis, where it was determined that enzalutamide was associated with an improvement in OS (18.4 *vs.* 13.6 months; P<0.001). Secondary endpoints such as PSA response and soft tissue response were also improved with enzalutamide. Results from the second phase III study of enzalutamide are highly anticipated—in the phase III PREVAIL study, docetaxel-naïve patients with mCRPC were randomized to enzalutamide or placebo.

The clinical trajectories of abiraterone and enzalutamide have moved in parallel, creating a quandary for investigators.

Given the results from COU-AA-301 and AFFIRM, would it be preferable to use abiraterone/prednisone or enzalutamide in the docetaxel refractory patients? Notably, radium-223 and cabazitaxel (discussed elsewhere in this manuscript) are also options in this setting. Furthermore, if the noted PREVAIL (pre-docetaxel) enzalutamide study is positive, the oncologist is left with additional choices five valid options for first line therapy in mCRPC—sipuleucel-T, docetaxel, enzalutamide, radium-223, and abiraterone.

Cabazitaxel

Cabazitaxel represents the only cytotoxic therapy to demonstrate an OS advantage post-doctaxel (46). The TROPIC trial randomized 755 men who had progressed post-docetaxel were randomized to either 12 mg/m^2 of mitoxantrone every three weeks or to the novel taxane cabazitaxel at 25 mg/m^2 every three weeks (53). Median OS was 15.1 months in the cabazitaxel group and 12.7 months in the mitoxantrone group. The use of cabazitaxel represented the first therapy FDA approved for patients whose prostate cancer has progressed post-docetaxel. Febrile neutropenia was 7.5% and caution with regard to treatments in patients with borderline counts or performance status is advised. Given that cabazitaxel was approved in the post-docetaxel space, as was enzalutamide and abiraterone, one might question which drug is best for which patient in this setting. Thus far, we have no comparative trials so conclusions are limited.

Radium-223

The radium-223 approval was based on the ALSYMPCA trial which randomized 921 patients with an OS primary endpoint (55). Inclusion criteria specified at least 2 bone metastatic lesions on bone scan and the presence of some symptoms. Those with visceral disease were excluded. Patients were required to be post-docetaxel, have refused docetaxel, unfit to receive docetaxel, or did not have docetaxel available. Randomization was to intravenous radium at 50 kBq/kg or placebo for six doses with a 2 to 1 randomization. All patients received "best standard of care". The "best standard of care" consists of whatever hormonal treatments might be appropriate in the mind of the investigator (ketoconazole, estrogens, dexamethasone, etc.) but no concomitant chemotherapy, experimental agents, or other radiopharmaceuticals were allowed.

The pre-specified interim analysis was positive for OS and the placebo group patients were subsequently allowed to cross over to radium-223. An updated OS analysis was presented to the FDA, with median OS at 14.9 months in the radium treated group and 11.3 months in the placebo treated group (49). The HR was 0.695 and the P value was 0.00007. There was also a reduction in symptomatic skeletal events which consisted of radiation to bone, surgery to bone, pathologic fracture, or spinal cord compression. Overall the treatment was well tolerated with a 6% incidence of grade 3/4 thrombocytopenia being the most significant finding; 2% of the patients had grade 3/4 neutropenia.

One of the many challenges regarding radium-223 is an understanding of how best to optimize and integrate this novel therapy into the overall treatment paradigm. The initial clinical trial was conducted prior to the approval of enzalutamide or abiraterone and whether or not combinations of these novel hormonal agents would have provided additive value to radium-223 is untested. Phase I trials with radium-223 and docetaxel have been conducted (59) and phase II trials are now underway utilizing the 50 kB/kg radium dose q six weeks in combination with 60 mg/m^2 of docetaxel q three weeks. Looking at combination therapies with radium-223 may be quite interesting. It is also unclear whether or not the optimal dose and schedule of radium-223 was utilized in ALSYMCA and trials will examine various alternative doses and durations of radium therapy in hopes of defining what may or may not be more optimal doses and schedules.

Selecting appropriate therapies in the mCRPC patient

Front line therapies include docetaxel, sipuleucel-T, abiraterone/prednisone, and radium-223. Therapies available in the post-docetaxel space are abiraterone, enzalutamide, cabazitaxel, and radium-223. The sequence of therapies remains an area of debate but given there are no direct comparisons in clinical trials, the debate is more conjectural than data driven. Some agents are only currently approved post-docetaxel, such as enzalutamide and carbazitaxel—so those agents have a quite defined space. Given that many patients do not receive docetaxel, the issue of how to address these non-docetaxel patients in terms of second-line therapy is not at all clear. The radium-223 trials were the only trials with eligibility criteria that included those who were unfit for docetaxel or for those that refused docetaxel.

Table 6 Summary of retrospective experiences documenting the activity of enzalutamide followed by abiraterone (and vice versa)

Author	N	Sequence	Description of results
Loriot *et al.* (62)	38	Enzalutamide → abiraterone	• All patients had prior docetaxel. Abiraterone efficacy: median PFS: 2.7 months; 3 patients (8%) with PSA response (>50%); 7 patients (18%) with ≥30% PSA decline
Noonan *et al.* (63)	30	Enzalutamide → abiraterone	• All patients had prior docetaxel. Abiraterone efficacy: median duration of treatment: 3.3 months; 3 patients (10%) with ≥30% PSA decline; no radiographic responses
Schrader *et al.* (64)	35	Abiraterone → enzalutamide	• All patients had prior docetaxel. Enzalutamide efficacy: median duration of treatment: 4.9 months; 28.6% PSA decline >50%; 48.6% with no PSA response

There are several tremendous challenges with regard to making appropriate choices as to which drug we should administer to each patient. We currently have very little data with regards to making appropriate drug choices guided by anything but clinical parameters. Our much studied biomarkers have yet to adequately inform clinicians regarding appropriate steps to take in individual patients. This is a major challenge in our field.

The presence or absence of prior docetaxel treatment is important to consider given some FDA approvals are specifically in this space. Performance status is always critical, as is the location of the metastatic lesions. Poor performance status patients should not receive cytotoxic chemotherapy as a rule. Are the metastatic lesions in the bone, viscera, both, or neither? Taking into account the pace of the disease progression influences clinical thinking. In addition the presence or absence of focal pains (which may be amenable to palliative external beam radiation therapy) is important to assess. Tolerance or intolerance of prior therapies, hematopoeitic function, and the availability of clinical trials are also important to consider (as are various laboratory parameters). Patients' preferences as always are part of the issue, as are out of pocket costs. Many therapies are not administered because out of pocket costs are prohibitive. Cytotoxics such as docetaxel and cabazitaxel required good performance status/blood counts/liver functions. Sipuleucel-T should be restricted to good performance patients with minimal pain and preferably a relatively low burden/pace of the disease. Radium is for patients with bone-metastatic disease and neither radium nor sipuleucel-T are suitable for patients with extensive visceral disease. Out of pocket costs drive many decisions for oral drugs particularly in the United States.

The post-abiraterone/post-enzalutamide space

The question of what to do with patients who have failed abiraterone for mCRPC is currently subject to debate. Utilization of docetaxel has been viewed by many as being standard for patients who have not previously received any chemotherapy but results are mixed at best. The de Bono group has published data to indicate that docetaxel activity is diminished in patients' post-abiraterone (60). There are no large trials in this setting so conclusions must be tempered until more data are available.

Fizazi and colleagues studied cabazitaxel/prednisone in patients who had received abiraterone and reported relatively high PSA response rates (61). These data have only been published in abstract form so there is much we more to learn about response durability and characteristics of the treated patients.

Minimal data are available for enzalutamide post-abiraterone (*Table 6*). One series, recently published retrospective analysis indicates that the response to enzalutamide post-abiraterone/post-docetaxel is blunted relative to those patients treated post-docetaxel alone (64). One study noted that 28.6% of men had a PSA decline of >50%. Further, 48.6% of men had no PSA response at all. This is much lower than expected. In the phase I/II trials, 56% of post-docetaxel patients had a PSA decline of >50% and only 17% had no PSA response (65). This German series did not assess PFS in a traditional sense so PFS data are limited.

The finding of *any* responses to enzalutamide post-abiraterone is of interest and implications of this observation are several. It should be clearly noted that post-abiraterone patients are a major challenge in our field. It may be that more androgens are present in the post-abiraterone state than appreciated and this concept is supported by finding

that some urinary androgens can be still be detected despite abiraterone use (66). It is also possible that some non-androgenic steroids can engage the androgen receptor (AR) and that enzalutamide can block this interaction. After CYP17 inhibition, progesterone and its metabolites are increased (58). Given that synthetic progestin withdrawal can be associated with PSA declines (67), we suggest that progestin/AR interactions might be relevant. It is possible that enzalutamide blockade of the putative progestin/AR interactions could be growth-inhibitory. It is known that selected AR mutations can recognize progesterone as an agonist (68) lending plausibility to this hypothesis. Alternatively, it may be that simply post-abiraterone withdrawal, that androgen-synthesis resumes and that simply that intratumoral androgens are effectively blocked by enzalutamide.

Two studies have examined abiraterone effects post-enzalutamide (and also post-docetaxel). Both of these small case series indicated a high degree of cross-resistance between enzalutamide and abiraterone with PSA responses (>50% declines) being less than 10% and the median PFS being less than 4 months (62,63).

Taken together, it is clear that cross-resistance between abiraterone and other agents is an issue and understanding this cross-resistance and devising methods to over-come it, is a top priority in the field of CRPC research. Space limitations preclude the complete discussion on this topic but AR splice variants may also be partially responsible for cross resistance in some instances (69). Devising methods to block ligand-independent AR signaling is a key challenge for progress in CRPC.

Limitations of sequencing therapies in CRPC

We are currently in the "sequencing era" where we administer drug A then drug B and then drug C for patients with mCRPC. It is unusual in other cancers to choose this strategy. In Hodgkin's disease, at curable malignancy, we utilize four drug regimens to cure. In prostate cancer we have only begun to explore combination therapy and this will be a tremendous challenge going forward, particularly given the cost of the various therapies involved. Regardless, combination therapies will likely be necessary to continue to improve patient outcomes.

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Footnote

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Sequence of treatment in locally advanced and metastatic renal cell carcinoma

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Abstract: The spectrum of drugs that have shown activity in advanced or metastatic renal cell carcinoma (RCC) has led to a debate on the optimal sequence of treatments. There is agreement on recommending targeted agents as the standard of care in this disease. Uncertainty, however, remains on the best first-line drug choice. Physicians and patients may select sunitinib, bevacizumab in combination with interferon-alpha (IFN-α), pazopanib, or—in poor risk patients—temsirolimus. There are also a variety of therapies with proven efficacy on hand in the second-line setting: sorafenib, pazopanib, axitinib, and everolimus. While most randomized RCC trials assessed progression free survival (PFS) as primary endpoint, some agents were shown to improve median overall survival (OS), and given in sequence they have extended the life expectancy of RCC patients from 13 months in the cytokine era to over 30 months. Despite the progress made, there are sobering aspects to the oncologic success story in RCC, as the new treatments do not obtain an objective response or disease stabilization (SD) in all patients. There are also as yet no predictors to select patients who might benefit and those who are primary resistant to specific drugs, and ultimately almost all patients will experience disease progression. Bearing inevitable treatment failure in mind, availability of further drugs and switching therapy while the patient is in a condition to continue pharmacotherapy is essential. Of note, depending on the setting, only 33-59% of patients receive second-line treatment. In this review we present data on first-, second-, and third-line treatment in RCC, and discuss the difficulties in their interpretation in the context of treatment sequence. We summarize biological aspects and discuss mechanisms of resistance to anti-angiogenic therapy and their implications for treatment selection.

Keywords: Algorithms; carcinoma; renal cell; molecular targeted therapy

Introduction

Generally, in the treatment of solid tumours, the most effective drug, which provides the best response rate (RR), progression free survival (PFS) and possibly overall survival (OS), is the treatment of choice in the first-line setting. However, there are exceptions to this rule and concessions are made, especially when toxicity concerns come into play, e.g., in elderly patients (1), or when disease stabilization (SD) is a valid treatment objective (2). The fundamental dichotomy in solid tumour oncology of tumour response, a time-tested marker of therapeutic efficacy, and disease progression, an essential sign of treatment failure, has recently been challenged (3). Randomized clinical trials assess new treatments in comparison with established therapies in superiority or non-inferiority studies and are aimed to establish a position in the hierarchy of available treatments.

The concept of sequencing treatment is relatively new and there is little literature on this topic in solid tumours. Sequencing therapies may be discussed as a distinction from combining treatments, with examples in colorectal cancer (CRC) (4,5) and a Cochrane review in breast cancer (6). Alternatively, sequencing may be evaluated in the context

of treatment order (7) or as a combination of both questions (8). The prerequisites for discussing the sequence of anti-cancer treatment are the availability of several active drugs and the indication that certain treatments may be more or less active before or after another. This alludes to the topic of drug resistance and overcoming resistance mechanisms (9).

Colorectal cancer

In CRC only one randomized trial compared folinic acid, 5-fluoruracil, and irinotecan (FOLFIRI) followed by folinic acid, 5-fluoruracil and oxaliplatin (FOLFOX) or the reverse; however both sequences FOLFIRI → FOLFOX and FOLFOX → FOLFIRI achieved a prolonged survival and similar efficacy (10). The fact that a substantial proportion of patients (26% and 38%) did not receive second-line therapy demonstrated the importance of the choice in first-line therapy.

Prostate cancer

Many new treatments have recently been assessed and licensed in metastatic castration resistant prostate cancer (CRPC) (11-16) and sequence of drugs has become an issue (17). Studies indicate that CRPC with acquired resistance to first-generation androgen receptor (AR) inhibitors maintain reliance on AR signalling for survival (18) and are sensitive to subsequent therapy with second-generation AR inhibitors such as enzalutamide. However, the glucocorticoid receptor confers resistance to AR inhibitors by bypassing AR blockade and mediates enzalutamide resistance. This novel mechanism of escape from AR blockade through expansion of cells primed to drive AR target genes via an alternative nuclear receptor upon drug exposure may therefore be relevant for drug sequencing (19). In contrast, prior treatment with the androgen synthesis inhibitor ketoconazole did not have an impact on the clinical outcomes of patients with CRPC who received subsequent docetaxel-based therapy (20,21).

Renal cell carcinoma

The introduction of sorafenib as first targeted therapy in metastatic renal cell carcinoma (RCC) (22) was the beginning of a rapid process which led to the development of other vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitors (TKI), the monoclonal

VEGF-directed antibody bevacizumab and mammalian target of rapamycin (mTOR) inhibitors for the treatment of locally advanced and metastatic RCC. Crucial for this progress was the understanding of the role of angiogenesis in general and the VEGF- and mTOR-pathways (23). Currently, seven drug or drug combinations are licensed for the treatment of metastatic RCC. Several guidelines recommend targeted agents as standard treatment for metastatic RCC (24-26). The sequence for using these therapies is an ongoing matter of debate and several reviews have been published on this topic over the past years (27-31).

Sequential treatment in RCC is of interest as complete responses (CR) to treatment with TKIs are rare and TKIs usually do not produce long term remissions: patients relapse when therapy is discontinued (32,33), and resistance to treatment inevitably develops during therapy (34). However, the life expectancy of RCC patients has been extended to over 30 months (35) from 13 months in the cytokine era (36). Until 2004 treatment options for RCC were limited and usually immunotherapy was used: interleukin-2 (IL-2) (37) and interferon-alpha (IFN-α) (38), or a combination of both (39,40), depending on patient characteristics, availability of drugs and familiarity with the toxicity management (41). Outcome for the majority of patients was poor (42).

The sequencing question becomes relevant when multiple treatments are developed in a short period of time and new drugs are licensed before others have found a definite place in the armamentarium of therapies. It also gains importance when no direct comparison of drugs is possible due to the delay from trial conception to publication: new drugs become available while others are being evaluated in studies. Sequencing is especially relevant when prior treatment with a certain agent compromises efficacy of a subsequent therapy or enhances the treatment effect.

Some patients will receive several lines of treatment and will obtain a repeated treatment response or at least stable disease. In these patients the order of treatments may be of less relevance compared to other patients, who have aggressive and rapidly progressing disease and need treatment with the most active drug at the beginning. Debating treatment sequence is ultimately an expression of a success story with an embarrassment of riches in the treatment of RCC (43).

In this review we focus on clear cell RCC owing to the fact that only limited data is available on the treatment of patients with non-clear cell RCC and the optimal treatment remains unclear (44).

320

Fischer et al. Sequence of treatment in RCC

Table 1 First-line treatment

Risk group	Standard	Option
Good or intermediate	Sunitinib [I, A]	High-dose IL-2 [III, C]
	Pazopanib [I, A]	Bevacizumab + low-dose IFN-α [III, B]
	Bevacizumab + IFN-α [I, A]	
Poor	Temsirolimus [I, A]	Sunitinib [II, B]
IFN-α, interferon-alpha.		

First-line treatment (*Table 1*)

In the first-line treatment of metastatic RCC five drugs and drug combinations are currently licensed. Two pivotal trials assessing the TKI sunitinib (45) and the combination of bevacizumab and IFN-α (46) in comparison to standard IFN-α treatment in patients with Memorial Sloan Kettering Cancer Center (MSKCC) favourable and intermediate risk (47) were published in 2007. A concurrent three arm randomized trial evaluated the mTOR inhibitor temsirolimus as single agent compared to temsirolimus in combination with IFN-α compared to IFN-α as single agent in MSKCC poor risk patients (48). While the mTOR inhibitor was demonstrated to prolong OS, the VEGFR-targeting agents showed statistically significant improvement in PFS, which was the primary endpoint of these trials. The median OS of 26.4 months with sunitinib (49) and 23.3 months with bevacizumab and IFN-α (50) were unprecedented at the time. Rini *et al.* performed a CALGB trial with bevacizumab and IFN-α compared to IFN-α, which produced similar results (51,52) as the European trial.

The multi-TKI pazopanib was first tested in a randomized placebo-controlled phase III trial, with 54% treatment naive and 46% cytokine pre-treated patients (53,54). Due to the promising activity, and the favourable toxicity profile, a cross-over trial assessing treatment preference for pazopanib versus sunitinib was performed (55). The results were published a few months prior to data on treatment efficacy from a non-inferiority trial (56). In summary, pazopanib and sunitinib were found to be equally effective in terms of PFS, RR and OS (57), while quality-of-life favoured pazopanib. Despite the favourable safety and quality-of-life profiles for pazopanib relative to sunitinib, treatment was discontinued due to adverse events in 24%

of patients on pazopanib compared to 20% on sunitinib. There is also concern on the validity of the non-inferiority design, given that results of the intention-to-treat analysis differed from the per-protocol analysis (58).

The randomized phase III trial with tivozanib, a potent and selective VEGFR-TKI with a relatively long half-life, failed to show an improvement in OS despite prolonged PFS for tivozanib compared to sorafenib (11.9 *vs.* 9.1 months) in a mixed population of treatment naïve and cytokine pre-treated patients. Median OS reached 29.3 with sorafenib and 28.8 months with tivozanib, respectively (59). The authors postulate that differential use of second-line therapies confounded OS. They hypothesize that the trend toward longer OS in the sorafenib arm compared to tivozanib is related to the greater proportion of patients in the sorafenib arm who received second-line targeted treatment (63% *vs.* 13%). In addition, the one-way cross-over design allowed patients who had progressed on sorafenib to switch to tivozanib (61%). In essence, this is a sequential trial of two agents (sorafenib → tivozanib) compared with one agent (tivozanib) (60). Important in the context of sequencing treatments: two consecutive targeted agents are associated with a longer OS than treatment with only one line of targeted therapy (61) and absence of PD after first and second-line targeted therapy may characterize long-term survival (62). An alternative hypothesis to explain the trend toward longer OS on the sorafenib arm is that sorafenib is more effective than tivozanib for improving OS (63). This would not have been expected, since the first-line comparison of sorafenib versus IFN-α demonstrated comparable PFS for the two agents, however no OS data was published (64).

Another trial comparing first-line treatment with the potent and selective second-generation VEGFR inhibitor axitinib and sorafenib was performed in Asian patients. Sorafenib was chosen as the comparator because it was available in the regions where the trial was performed (65). Somewhat surprisingly, the trial was negative and axitinib did not significantly improve PFS (10.1 months) *vs.* sorafenib (6.5 months). An accompanying comment proposes that no significant difference in efficacy was shown because the study was underpowered and the benefit of sorafenib might have been underestimated (66). The striking difference in outcome for Eastern Cooperative Oncology Group performance status (ECOG) 0 (7.1 months difference in median PFS with axitinib *vs.* sorafenib) and ECOG 1 (no difference in PFS) might be attributed to the fact that the majority of patients was recruited in Eastern Europe,

Table 2 Second-line treatment		
Prior treatment	Standard	Option
TKI	Axitinb [I, B]	Sorafenib [II, B]
	Everolimus [II, B]	
Bev + IFN-α	Sunitinib [III, B]	
TKI, tyrosine kinase inhibitors; IFN-α, interferon-alpha.		

where resource limitations and local practice standards may have affected the type of patient enrolled, or patient management.

In our view, neither single agent IFN-α (36) nor subcutaneous IL-2 play a role in the treatment of RCC nowadays. This is especially relevant for patients with MSKCC intermediate or poor risk, due to the significant toxicity in these patients (40). However, infusional IL-2 is a treatment option in selected patients and centres, considering the long term survival of some RCC patients on this therapy (67).

Second-line treatment (*Table 2*)

There are four important phase III trials in the second-line setting of RCC. Treatment Approaches in Renal Cancer Global Evaluation Trial (TARGET) tested treatment with sorafenib versus placebo in patients who were progressing on standard therapy. At the time, standard therapies were mainly cytokines: most patients had received IL-2, IFN-α, or both before progression and enrolment. Nine hundred and three patients were randomized; primary end point of the trial was OS. The first PFS analysis revealed a significant benefit for sorafenib with a PFS of 5.5 *vs.* 2.8 months for the placebo group. Following these results, study-group assignments were to offer sorafenib to all patients in the placebo group. OS analysis showed a tendency towards longer survival for treatment with sorafenib. However, statistical significance was not reached, mainly due to cross-over from placebo to sorafenib (68). Secondary analysis, censoring post-cross-over placebo survival data, reached statistical significance showing better OS for patients treated with sorafenib (69).

The RECORD-1 (renal cell cancer treatment with oral RAD001 given daily) trial compared everolimus to placebo in RCC patients pre-treated with sunitinib, sorafenib or both. Median PFS was significantly longer with 4.9 months for patients treated with everolimus compared to 1.9 months for patients randomized to receive placebo (70,71). Approval of

everolimus was based on the results of the trial. A criticism can be made that this trial was not a pure second-line study. In fact, most patients had received more than one previous treatment-line, including IFN-α, IL-2 and bevacizumab. Twenty-six percent of patients in both treatment arms had been pre-treated with two VEGF-TKIs, namely sunitinib and sorafenib. Therefore, one may accept this trial as a rationale to consider everolimus as third-line option after treatment with two lines of anti-VEGF directed therapy. It is noteworthy that subgroup analysis revealed a benefit for patients in the everolimus arm who were pre-treated with only one VEGF-TKI compared to those pre-treated with two previous VEGF-TKIs (PFS 5.4 months for everolimus *vs.* 1.9 months for placebo after one previous VEGF-TKI; PFS 4.0 months with everolimus *vs.* 1.8 months with placebo after two previous VEGF-TKIs) (72).

There are two randomised phase III trials comparing different VEGF-TKIs and VEGF-TKI versus an mTOR inhibitor in the second-line, respectively.

The AXIS (comparative effectiveness of axitinib versus sorafenib in advanced RCC) trial randomized 723 patients who had progressed after first-line treatment with sunitinib, bevacizumab plus IFN-α, temsirolimus or cytokines to receive axitinib or sorafenib in the second-line. PFS was significantly longer for patients assigned to axitinib (6.7 *vs.* 4.7 months for sorafenib) (73). Although OR rate was also significantly better for axitinib, no significant OS benefit could be shown (74).

In the INTORSECT (Investigating Torisel As Second-Line Therapy) trial, patients who had progressed after treatment with sunitinib were randomized to receive the mTOR inhibitor temsirolimus or the TKI sorafenib. Five hundred and twelve patients were included and stratification according to duration of prior sunitinib therapy was performed. Although no significant difference in PFS was observed, OS was significantly longer for patients treated with sorafenib compared to those treated with temsirolimus (16.6 *vs.* 12.3 months). Subgroup analysis showed that median OS with sorafenib was only longer in comparison to temsirolimus for patients whose duration of pre-treatment with sunitinib was >180 days (17.8 *vs.* 14.4 months). For patients responding <180 days to sunitinib, no significant difference was observed (11.4 months for sorafenib *vs.* 10.1 months for temsirolimus) (75). Interpreting these results, one may assume that patients, who responded to anti VEGF-therapy in the first-line, should receive a VEGF-TKI in second-line. However, subgroup analysis should always be interpreted with caution and as OS is

322

Fischer et al. Sequence of treatment in RCC

Table 3 Third-line treatment

Prior treatment	Standard	Option
2 TKIs	Everolimus [II, B]	
TKI + mTOR inhibitor	Sorafenib [I, B]	Other TKI [IV, B]
		Re-challenge [IV, B]
TKI, tyrosine kinase inhibitors; mTOR, mammalian target of rapamycin.		

generally shorter in both treatment arms for patients with sunitinib response <180 days, one may also conclude that patients showing little benefit from first-line VEGF-TKI generally have a worse prognosis.

There is a phase II study analysing antitumour activity of sunitinib in patients pre-treated with bevacizumab. Twenty-three percent of patients showed a PR with sunitinib, and SD as best response was seen in 59% of patients. Median OS was 47.1 weeks (76). These data support the assumption of clinical benefit from sequential anti-VEGF directed therapy in patients with advanced and metastatic RCC.

Third-line treatment (*Table 3*)

In 2015 only limited data exist for the choice of third-line treatment in patients with metastatic RCC. Treatment selection is based on the treating physician's individual experience and availability of drugs rather than on scientific evidence.

An Italian retrospective study (77) analyzed sorafenib as third-line treatment after sequential therapy with sunitinib and mTOR inhibitors (everolimus or temsirolimus). A total of 34 patients were included. Median PFS was 4 months, and median OS 7 months. There were no treatment interruptions due to toxicity. Response to sorafenib was better in patients who had already responded to sunitinib in the first-line whereas no activity was seen in patients without previous benefit from sunitinib.

Although not directly comparable, one may assume that a median PFS of 4 months with third-line sorafenib is a sign of drug activity, when taking data from the RECORD-1 trial into consideration: patients receiving placebo had a median PFS of only 1.9 months (70). Most of these patients had received more than one previous treatment-line. Therefore one may presume that sorafenib is superior to placebo in the third-line setting.

So far, only one randomized prospective trial concerning

third-line therapy in patients with metastatic RCC has been conducted (78). Patients who had failed previous treatment with one VEGF-targeted therapy and one mTOR inhibitor were randomized to receive either sorafenib or the VEGF and fibroblast growth factor (FGF) receptor inhibitor dovitinib. The rationale for selecting dovitinib was derived from the hypothesis that adaptive resistance to anti-VEGF therapy may be caused by activation of the FGF pathway (79). Therefore, it was hypothesized that such a mechanism could be overcome by adding a TKI with FGF inhibiting properties. However, no differences regarding PFS or OS were observed between the two treatment arms.

In a small retrospective analysis, 40 patients with everolimus resistant RCC were treated with a VEGFR-TKI (80). All patients had received first-line VEGF-targeted therapy (sunitinib, sorafenib or bevacizumab and IFN-α) and this was associated with a median PFS of 11.3 months. A subset of ten patients was treated with a second-line TKI. Treatment with everolimus was associated with a median PFS of 5.9 months. Subsequent treatment after everolimus was associated with SD in 22 patients (55%) and PR in 4 patients (10%), whereas eleven patients had PD (28%). The median PFS on therapy after everolimus failure was 5.5 months. This data suggests that VEGF-resistance remains transient in nature, at least in initially susceptible patients.

Re-challenge

Based on the hypothesis that re-challenge of patients with a previously used VEGF-targeting agent could be a rationale strategy for tumour control, a retrospective review was undertaken to describe the experience of re-challenge with sunitinib in metastatic RCC (81). The investigators identified 23 patients who were re-challenged with sunitinib. The initial median PFS among these patients had been 13.7 months and was in line with the registration study (45). At re-challenge, median PFS was 7.2 months. Upon re-challenge, 22% of patients achieved a PR, while 74% had SD as their best response. Patients with a >6 months interval between sunitinib treatments had better PFS with re-challenge compared to patients who started the re-challenge within 6 months of discontinuing their initial treatment.

In another study, the efficacy of sunitinib re-challenge was assessed in two German centres. Thirteen patients received sunitinib (median PFS 21 months) and were subsequently treated with an mTOR inhibitor; upon disease

progression they received sunitinib again. This approach resulted in a median PFS of 6.9 months and consisted of two (15%) PR and ten (77%) SD (82). These two retrospective analyses and several case series serve as a proof of concept and have just recently been summarized and discussed in a review article (83).

Twelve patients who had previously received VEGF-targeted treatment and an mTOR inhibitor were re-challenged with a second mTOR inhibitor. Both sequences everolimus → temsirolimus (n=7) and temsirolimus → everolimus (n=5) were used. Six of 12 patients (50%) responded to everolimus and four of 12 patients (33%) responded to temsirolimus, however only one patient responded to both agents and three patients to none. Median treatment duration for everolimus → temsirolimus and temsirolimus → everolimus sequences were 10.3 and 5.8 months, respectively (84). No patient responded to temsirolimus re-challenge after response to everolimus as the first mTOR inhibitor, whereas patients who did not respond to everolimus as the first mTOR inhibitor may still respond to a re-challenge with temsirolimus (2/7). Despite structural similarities of both mTOR inhibitors and the same mode of action, the two drugs have distinct clinical pharmacokinetic and pharmacodynamic profiles, which may contribute to differing responses in patients. Due to the small sample size no definitive conclusions can be drawn from this data. In settings where several drugs are available, re-challenge is of limited interest. However, in countries with less treatment choices this topic might still be of relevance.

Trials assessing sequential treatment

There is only one randomized trial assessing a treatment sequence in RCC. In this phase II trial 471 patients were either assigned to first-line mTOR inhibitor everolimus followed by the TKI sunitinib upon disease progression (everolimus → sunitinib), or fist-line sunitinib followed by everolimus (sunitinib → everolimus) (35). Only 45% and 43% of the patients crossed-over and received second-line treatment, respectively. The primary endpoint, PFS non-inferiority of first-line everolimus compared with first-line sunitinib, was not met: the median PFS was 7.9 months for first-line everolimus and 10.7 months for first-line sunitinib. The median combined PFS was 21.1 months for everolimus → sunitinib and 25.8 months for sunitinib → everolimus. Feasibility for the combined PFS end point had not been established previously. Median OS was longer for sunitinib

→ everolimus (32 months) compared to everolimus → sunitinib (22.4 months). The hypothesis of the investigators that similar combined PFS lengths would be achieved by both sequences and that everolimus would be better tolerated than sunitinib as the first-line therapy was not confirmed.

In a retrospective French analysis, outcome of patients with either sunitinib followed by sorafenib (sunitinib → sorafenib) or sorafenib followed by sunitinib (sorafenib → sunitinib) was assessed (85). Of note, the majority of the 90 patients had received prior cytokines. The treatment durations were 61 weeks for sorafenib → sunitinib (33 weeks → 28 weeks) and 49 weeks for sunitinib → sorafenib (27 weeks → 22 weeks), respectively. These data confirm absence of absolute cross-resistance between sunitinib and sorafenib. They do not, however, guide on the optimal treatment sequence, especially in patients without prior cytokine exposure.

Biological aspects

Discussing optimal treatment strategies for advanced and metastatic RCC demands a closer look at biological aspects underlying this disease. Inactivation of the von Hippel Lindau (VHL) gene, a tumour suppressor gene, is crucial in the development of the disease. VHL encodes a protein, which supports degradation of hypoxia-inducible factor (HIF). Inactivation of VHL therefore leads to higher levels of the transcription factor HIF, which promotes transcription of several genes such as VEGF, platelet-derived growth factor (PDGF) and transforming growth factor alpha (TGF-α) (86,87). These are important factors for angiogenesis. Induction of chronic angiogenesis is crucial in the development of cancer and has been described as one of the "hallmarks of cancer" (88). RCC is a highly vascularized tumour type, thus targeting angiogenesis seems a promising treatment strategy.

However, some patients are primarily resistant to these targeted treatments and almost all show tumour progression over a longer period of time, even if there has been a tumour response to treatment in the beginning.

In patients with lack of response to VEGF-targeted therapy, primary resistance needs to be differentiated from inadequate dosing. TKIs can cause a diversity of adverse events such as hypertension or hand-foot-syndrome, which may lead to dose modifications due to intolerable toxicities. Moreover, most cancer patients are of older age and receive co-medication with several other drugs. This bears potential

324

Fischer et al. Sequence of treatment in RCC

for cytochrome P interactions and inadequate drug exposure. Animal models (89) and a meta-analysis (90) could show that increased exposure to sunitinib is associated with improved clinical outcomes and that increasing sunitinib dose can partly overcome resistance in xenografts and patients. There are two phase II trials assessing feasibility of dose escalation in patients treated with sorafenib (91). Some patients obtained a response upon dose escalation after early progression to standard dose (92). Axitinib dose titration in previously untreated patients was evaluated in a randomized phase II trial against placebo titration, as retrospective population pharmacokinetic data suggest axitinib plasma exposure correlates with efficacy in metastatic RCC (93). In fact, the greater proportion of patients in the axitinib titration group achieving an OR supports the concept of individual axitinib dose titration (94).

Taken together, adequate dosing of the antineoplastic drug and optimal management of potential side effects should be ensured before treatment strategy is changed due to suspected resistance, especially if tumour response has been observed in the beginning and dose reductions have taken place.

The challenge of adherence has been recognized in oncology practice (95). However, limited data is available on adherence to targeted therapies and efforts towards better patient education are warranted including dedicated staff for monitoring outpatient anticancer oral therapy (96).

PD is often defined by Response Evaluation Criteria in Solid Tumors (RECIST), which may not be an optimal determinant of resistance to targeted agents (97). Targeted therapies can induce central necrosis, alter tumour vascularity, and retard tumour growth without reducing tumour size. Taking these changes into account, Choi criteria have been examined in the context of targeted therapy in RCC (98,99). In summary, switching to second-line treatment should be prompted by objective criteria along with clinical judgment. Sonpavde *et al.* propose a formal evaluation of continuing the same agent in patients with RECIST progression unaccompanied by symptoms (28).

Under the circumstances of true tumour progression despite adequate dosing, a central question is whether to maintain the therapeutic target or to change the mechanism of action of the antineoplastic drug, i.e., changing to another VEGFR-TKI or mTOR inhibitor, respectively.

Mechanisms of resistance to anti-angiogenic therapy and implications for further therapy

Bergers and Hanahan (100) have reviewed possible

mechanisms of adaptive resistance to anti-angiogenic therapy. One of these mechanisms is up-regulation of alternative pro-angiogenic pathways. First clues for this hypothesis came from animal models in which higher mRNA expression levels for different pro-angiogenic factors were observed after blockage of VEGFR-signalling in pancreatic neuroendocrine cancer cells (80). Further studies showed up-regulation of pro-angiogenic factors such as PDGF and FGF (101) after angiogenesis inhibition. Moreover, the hypoxic environment caused by anti-VEGF therapy may lead to activation of the mTOR-pathway which integrates information about nutrients and growth factors and holds a central role in cell growth, cell cycle progression and coping with metabolic stress (102,103).

There is also growing evidence that the tumour microenvironment is crucial in adaptive resistance to anti-angiogenic therapy. For example, lower oxygen levels in tumours through VEGF-inhibition seem to lead to recruitment of vascular progenitor cells from the bone marrow. Experimentally induced ischaemia in tissues was shown to increase recruitment of bone marrow-derived cells and endothelial progenitors partly through elevated levels of HIF1 alpha (104,105). These progenitors may be able to maintain sufficient tumour angiogenesis even when VEGF-signalling is blocked.

Other studies could show that pericytes also seem to be of importance in acquiring resistance to anti-angiogenic therapy. Increased and thick coverage of vessels with these endothelial support cells was observed after VEGF-inhibition and may help to keep tumour vessels functioning (106,107).

Further investigations raise the hypothesis that cancer cells adapt to anti-angiogenic therapy by showing a more invasive phenotype and migrating more aggressively into normal tissues to ensure sufficient oxygen supply (108).

Other studies suggest an epithelial-to-mesenchymal transition (EMT) with acquisition of a sarcoma-like phenotype as a mechanism of escape from VEGF-inhibition. For example, Hammers *et al.* (109) described the case of a patient with initially pure clear cell RCC and response to sunitinib. After progression of the disease, a skin metastasis was excised and histologically showed EMT. After implantation into mice, clear cell histology as well as sensitivity to sunitinib was surprisingly restored. These observations underline importance of the tumour microenvironment for achieving resistance to anti-angiogenic therapy.

Taking into account all these possible mechanisms of

acquiring resistance, certain considerations regarding optimal treatment sequence in metastatic and advanced RCC arise.

Activation of the mTOR pathway as a potential resistance principle creates the rationale for a change in therapeutic strategy after treatment with a first-line VEGFR-TKI. Blocking up-regulated mTOR signalling with an mTOR inhibitor such as everolimus or temsirolimus seems promising. Clinical proof of concept comes from the RECORD-1 trial, which showed significantly longer PFS for patients treated with everolimus in comparison to those on placebo after first-line treatment with a VEGFR-TKI (70).

Further arguments supporting a change of treatment principle occur considering the tumour microenvironment as described above. Epithelial-to-mesenchymal transition as a resistance mechanism to anti-VEGF therapy was reversed and sensitivity to sunitinib restored after excision and transplantation of a metastasis into mice (109). This observation argues for the concept of "drug holidays" to achieve a resetting of the original tumour microenvironment and re-establishing VEGF-dependency. Therefore, switching to a different therapeutic target in second-line therapy seems reasonable and may restore sensitivity to anti-VEGF therapy as a potential third-line option.

Observation of a more invasive tumour phenotype after anti-VEGF therapy further supports the concept of changing treatment mode (108,110). It should been taken into account that prolonged anti-angiogenic therapy may even be detrimental.

On the other hand, there is evidence arguing against a change of treatment principle. It is known from *in vitro* studies that treatment with mTOR inhibitors alone leads to tumour stimulating feedback mechanisms. mTOR contains two different complexes, the rapamycin-sensitive complex (Raptor, mTORC1) and the rapamycin-insensitive complex (Rictor, mTORC2). Available mTOR inhibitors for treatment of RCC such as everolimus und temsirolimus as well as the original macrolide rapamycin (sirolimus) only inhibit activation of the Raptor complex. It has been shown experimentally that inhibition of Raptor leads to increased stimulation of AKT/PKB due to Rictor (111,112). AKT/PKB is a protein kinase which implements a central role in regulation of cell growth and division, apoptosis and protein metabolism. This may even cause tumour growth and progression and limits the value of sole mTOR inhibition as a therapeutic principle. One further resistance mechanism has been proposed: a negative feedback loop activating the mitogen activated protein kinase (MAPK) signalling

cascade, a separate oncogenic pathway (113). MAPK feedback activation was found to be PI3K-dependent (113).

In addition to mTOR, other pro-angiogenic factors such as PDGF and FGF have been shown to be up-regulated as a consequence of anti-angiogenic therapy. Therefore, patients progressing under anti-VEGF therapy may still show benefit from a VEGFR-TKI if the spectrum of inhibition is widened, for example by switching to a less-selective multi-kinase inhibitor such as sorafenib, which also inhibits PDGFR, c-KIT and Raf. This hypothesis is supported by results of the randomized phase III INTORSECT trial: sunitinib-resistant RCC patients were randomly assigned to treatment with either sorafenib or temsirolimus. Although no statistically significant difference in PFS could be observed, OS was longer for patients treated with sorafenib (75).

Interestingly, a third-line trial failed to show superiority of dovitinib, an inhibitor of both VEGFR and FGFR, over sorafenib in patients pre-treated with one anti-VEGF line and one line of an mTOR inhibitor (78). In her comment to this trial, M. Schmidinger raised the hypothesis that the timing of adding divotinib had been wrong rather than FGF as a target. Most patients in the trial (92%) had received a VEGF-inhibitor followed by an mTOR inhibitor. VEGF-inhibitor resistance has been suggested as being a temporary phenomenon due to changes of the tumour microenvironment. An "anti-VEGF drug holiday", for example during mTOR inhibition, may restore dependency on VEGF-signalling and attenuate up-regulation of the FGF pathway. Therefore, it might have been more reasonable to analyse efficacy of a combined VEGF- and FGF-inhibitor directly after failure of VEGF-directed therapy than in the third line after additional failure of an mTOR inhibitor (114).

A further observation supporting maintenance of treatment with anti-VEGF therapeutics is the lack of complete cross-resistance regarding different anti-VEGFR TKIs (28). Results from the AXIS trial (73) showed that pre-treated patients of whom the majority received sunitinib as first-line treatment, demonstrated a significantly longer PFS when treated with axitinib in the second-line than with sorafenib. In terms of pharmacological activity, axitinib is a more potent VEGFR-inhibitor than sunitinib and sorafenib (IC50s 0.2 nM for axitinib, 80 nM for sunitinib and 90 nM for sorafenib). This creates a rationale for a treatment sequence weaker VEGFR TKI followed by stronger VEGFR TKI. Biologically, pre-treatment with a less potent drug of the same class may lead to a weaker selection pressure in tumour cells and therefore cause adaptive
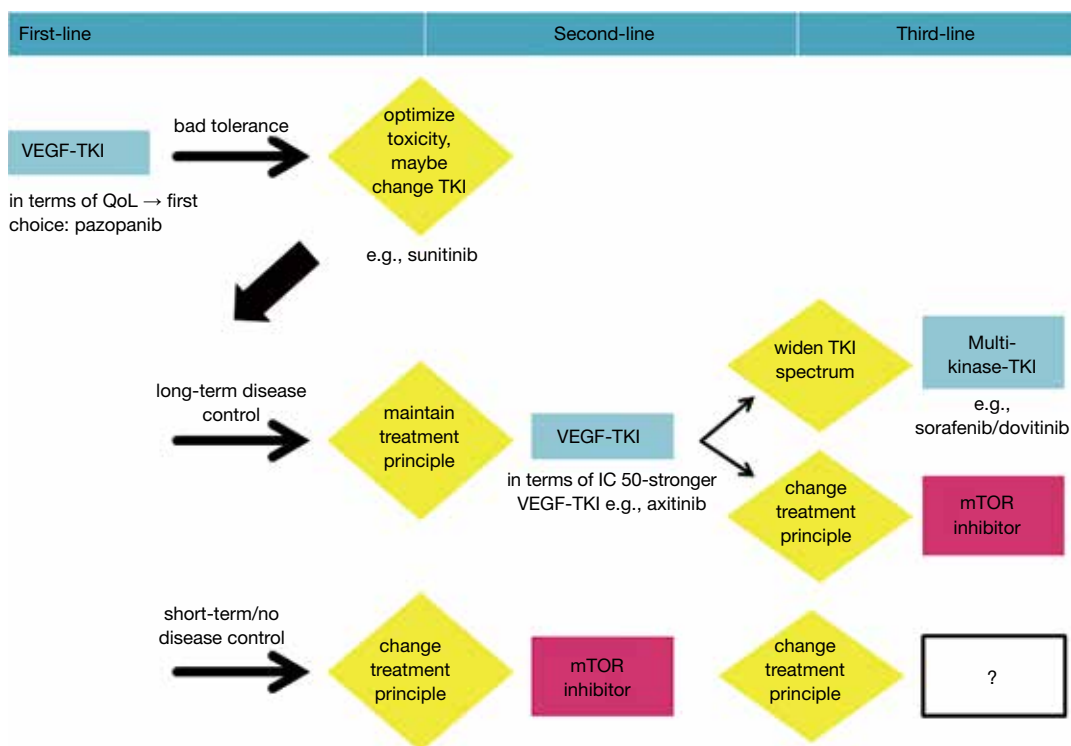
Figure 1 Possible treatment sequence. VEGF-TKI, vascular endothelial growth factor-tyrosine kinase inhibitors; mTOR, mammalian target of rapamycin.

mechanisms which can still be overcome using a drug with greater inhibitory activity but the same spectrum of action.

Gerlinger *et al.* performed multiregion genetic analysis on spatially separated samples from primary RCC and associated metastatic sites using exome sequencing, chromosome aberration analysis, and ploidy profiling. Phylogenetic reconstruction revealed branched evolutionary tumour growth, with 63% to 69% of all somatic mutations not detectable across every tumour region. They found ubiquitous alterations in the trunk of the phylogenetic tree, such as allelic-imbalance events on chromosome 3p (encoding VHL), 5q, 6q, and 10q. However, heterogeneity was observed for a mutation within an auto-inhibitory domain of the mTOR kinase and for multiple tumour-suppressor genes converging on loss of function (115). The importance of targeting ubiquitous alterations in the trunk of the phylogenetic tree is underscored by branched tumour evolution. The difficulties encountered in the validation of oncology biomarkers owing to sampling bias may be explained by intratumour heterogeneity (116). In addition, this heterogeneity may contribute to Darwinian selection of preexisting drug-resistant clones (117,118) and predict resistance to treatments (119).

Taken together, there are arguments supporting both treatment strategies. Maintaining anti-VEGFR directed therapy as well as changing treatment principle in second-line seem reasonable and can be justified on a biological level (*Figure 1*). However, to date, no prospective data exist addressing the issue whether one strategy is superior to the other. In the end, clinical reasoning is still crucial in finding the best treatment strategy for an individual patient. Comorbidities and spectrum of adverse events have to be taken into account. Moreover, the individual biology of the disease, determining the degree of aggressiveness, seems to be the most important factor of all as demonstrated by two cases (*Figures 2* and *3*).

Considering different courses of presumably the same disease, there seems to be a divide between patients with slow progression and repeated treatment responses, and those with an aggressive phenotype, rapidly succumbing to their tumour (120).

As to the latter, there may be those with intrinsic, pre-existing non-responsiveness to anti-angiogenic therapy. Bergers and Hanahan (100) envision a tumour phenotype intrinsically expressing a plethora of pro-angiogenic factors and therefore being indifferent to anti-VEGFR therapy.
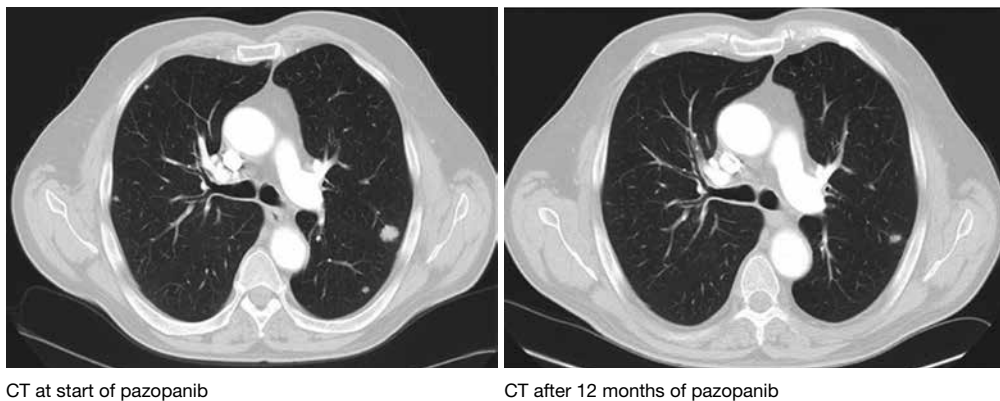
CT at start of pazopanib

CT after 12 months of pazopanib

Figure 2 Patient case 1. A 70-year-old male patient with primary metastatic RCC. The patient had a 10 cm renal mass and multiple pulmonary metastases. He underwent laparoscopic nephrectomy, histology showed clear cell RCC Fuhrman Grade 3, focally Grade 4. The patient had Memorial Sloan Kettering Cancer Center (MSKCC) intermediate risk (39) (1 risk factor). Treatment with the TKI pazopanib was initiated two months after surgery when progression of the lung metastases was seen. The patient had a dose reduction of pazopanib for nausea and hepatotoxicity. Nevertheless a PR was seen on CT. A recent CT scan shows sustained PR after 12 months of treatment. RCC, renal cell carcinoma; TKI, tyrosine kinase inhibitors; PR, partial remission.
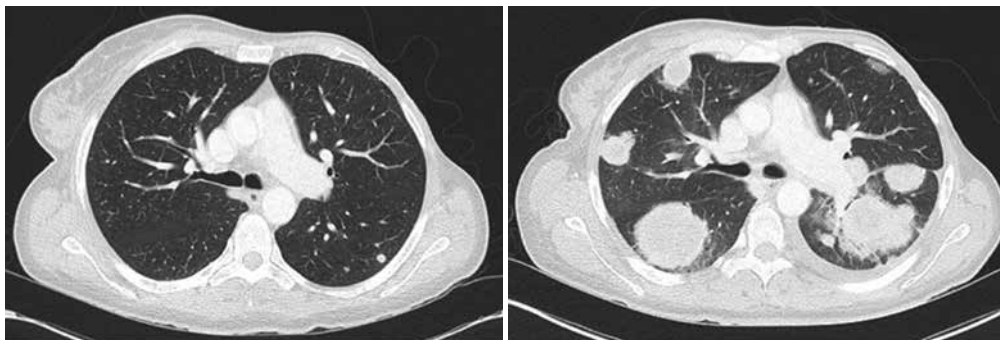
CT before nephrectomy

CT after nephrectomy

Figure 3 Patient case 2. A 48-year-old female patient with a history of breast cancer, which had been treated with surgery and adjuvant radiotherapy in 1997 in London. The patient presented with a 10 cm renal mass and disseminated lung metastases. She underwent nephrectomy, which revealed a clear cell RCC Fuhrman Grade 3, focally Grade 4. In addition, a wedge resection of a pulmonary metastasis was performed and confirmed RCC rather than metastatic breast cancer. The patient had MSKCC intermediate risk (2 risk factors). Postoperatively rapid disease progression was detected on a computed tomography (CT) scan 6 weeks after the initial CT and she was started on sunitinib. After the first 4-week cycle of treatment the patient had symptomatic and objective disease progression with bilateral pleural effusions, enlarged pulmonary metastases, a chest wall infiltration, and a newly diagnosed vena cava thrombosis. The patient was started on the mTOR inhibitor temsirolimus, which was available at the time. However, the patient died of progressive disease within 3 months of palliative nephrectomy. RCC, renal cell carcinoma; MSKCC, Memorial Sloan Kettering Cancer Center; mTOR, mammalian target of rapamycin.

This hypothesis is supported by an Italian retrospective study (77). In this analysis, patients did not show response to treatment with third-line sorafenib if there had already been lack of response to first-line sunitinib. This observation suggests existence of a primarily resistant phenotype concerning anti-VEGF therapy.

Furthermore, pre-existing inflammatory cell mediated vascular protection could be seen as another mechanism of intrinsic resistance to anti-angiogenic therapy. Animal studies could show pre-existing infiltration of inflammatory

328

Fischer et al. Sequence of treatment in RCC

myeloid cells expressing pro-angiogenic factors in murine transplant tumours non-responsive to anti-VEGF directed therapy (104). The RECORD-1 trial (70) could also identify inflammation (elevated neutrophils) as an independent prognostic factor for shorter PFS and OS.

At the opposite end, we see patients showing long-term disease control with first-line anti-VEGF therapy. It is tempting to assume that those patients should best continue anti-VEGF directed therapy in the second-line. However, results from a European retrospective study (121) suggested that long-term first-line VEGFR-TKI responders may benefit from both, further VEGFR-TKI or mTOR inhibitors in the second-line. Taken together, for these patients the sequence of therapy may only be of minor relevance.

Conclusions and future perspective

There is increasing evidence of the central role of the VEGF/VEGFR-pathway in the development of RCC and good rationale for inhibition of this pathway due to the frequent mutation of the VHL tumour suppressor gene in ccRCC also in sporadic forms of the disease. This molecular hallmark renders RCC particularly dependent on angiogenesis and thus susceptible to angiogenesis inhibition with targeted agents (83). On the basis of this biologic understanding many new drugs have been developed for the treatment of RCC. In this review we present the clinical trials on targeted therapy in RCC. We point to the challenge in interpreting the data and in deriving the optimal treatment sequence. Trial design in RCC in the past was not only driven by scientific rationale but also by the interest of pharmaceutical companies to obtain marketing authorization. In fact, some drugs were used as a comparator in clinical setting not supported by previous evidence and not reflecting current daily practice.

Sequencing treatment is exclusively relevant to patients who are offered a second- or third-line treatment and who remain well enough to receive this treatment. Retrospective French data show that only 59% of patients received second-line treatment after sunitinib, 52% after sorafenib, and 79% after bevacizumab, respectively (122). Following first-line VEGF-targeted therapy 33% of 645 patients received second-line VEGF-targeted therapy or mTOR inhibiting agents (123). Similarly, 13% of patients received third-line treatment in an Italian retrospective analysis of targeted therapies (124). The data suggests that MSKCC risk groups and first-line therapy may be predictive factors

for receiving second-line treatment. PFS was shown to be similar in the second- and third-line settings in a retrospective analysis of RECORD-1 patients (125). Hence, is the sequence relevant after all or is it merely a matter of favourable risk and access to drugs? And how important are toxicity management issues and correct assessment of disease progression?

The challenges are ahead. Novel immunotherapeutic agents have entered the field in RCC (126) and require integration in treatment algorithms and rethinking of the treatment sequence.

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330

Fischer et al. Sequence of treatment in RCC

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332

Fischer et al. Sequence of treatment in RCC

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Advances in the treatment of testicular cancer

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Abstract: Germ cell tumors (GCT) are relatively uncommon, accounting for only 1% of male malignancies in the United States. It has become an important oncological disease for several reasons. It is the most common malignancy in young men 15-35 years old. GCTs are among a unique numbers of neoplasms where biochemical markers play a critical role. Finally, it is a model of curable cancer. In this review we discuss cancer epidemiology, genetics, and therapeutic principles. Recent advances in the management of stage I GCT and controversies in the management of post chemotherapy residual mass are presented.

Keywords: Neoplasms germ cell tumor; chemotherapy; chemotherapy adjuvant; neoplasm staging; orchiectomy; patient selection; treatment outcome; lymph node excision; incidence

Despite their infrequency, accounting for only 1% of male malignancies in the United States, germ cell tumors (GCTs) have become an important oncological disease for several reasons. GCT is the most common malignancy in young men, 15-35 years old, and thus, has the potential to greatly shorten a man's productive years. Second, GCT is amongst a unique group of neoplasms in whom biochemical markers play a critical role. In GCT's serum tumor markers are an integral part of patient management as part of diagnosis, staging, risk assessment, evaluation of response to therapy and detection of relapse. Finally, GCT is a model of curable cancer, and a triumph of modern oncology. Current chemotherapy protocols and surgery yield cure rates exceeding 95% (1).

Epidemiology

GCTs affect young males with a median age at diagnosis of 34. The age-adjusted incidence rate in the United States is 5.6 per 100,000 men per year based on data from the Surveillance, Epidemiology and End Results (SEER) program between 2007 to 2011. The SEER database tracks incidence and survival data from specific geographic areas representing 26% of the U.S. population.

It is estimated that 8,820 new cases of testis cancer were diagnosed in the U.S. in 2007, while only 380 (4%) patients died of their disease. The lifetime risk of developing testis cancer is approximately 0.4% with an estimated 227,406 men living with testis cancer in the U.S. as of 2007. Improved survival over the last thirty years is attributed to the development of cisplatin combination chemotherapy. A high cure rate coupled with the young age at diagnosis has resulted in a growing population of testicular cancer survivors.

For unknown reasons, the incidence of GCT, particularly in Caucasian populations, is increasing globally. The lifetime risk of GCT's in U.S. Caucasian men is estimated to be 1 in 230. In countries with the highest rate of GCTs, such as Demark, lifetime risk exceeds 1%. The SEER data indicates that between 1975 and 2004, the age-adjusted incidence rate of testicular cancer for males aged 15-49 years increased from 2.9 to 5.1 per 100,000 (2). This increase was more pronounced in seminoma compared to nonseminomatous germ cell tumor (NSGCT). The trend appears to be influenced by a birth cohort effect, where people born in a specific time interval show different risk compared to the period immediately preceding or following. For example, men born in Denmark and Norway during World War II have a lower risk of testicular cancer than either previous or subsequent birth cohorts (3).

Overall, rates of testicular cancer in industrialized nations are five times higher than those in less developed regions of the world. Further, considerable differences can be noted between neighboring countries and even within regions of the same country. For example, Denmark, Norway and Switzerland report age-standardized rates of roughly 9.5 per 100,000, while in Lithuania, Estonia, Spain and Latvia cancer incidence approaches 2 per 100,000 (4). Immigrant populations tend to carry the risk of their country of birth in the first generation, whereas, the risk of the second generation immigrants shifts toward the adopting population incidence (5). These observations coupled with the young age at presentation suggest an environmental risk factor acting in-utero or early in life. No specific etiological factors have yet been identified.

The incidence of testicular cancer varies with respect to race with the highest rate in Caucasian populations. The U.S. incidence among white men historically has been five times that of African American men (4). Recently, rates of testicular cancer for African American (6) and Hispanic young adults (7) living in the U.S. appear to be increasing. In contrast, Pacific Islanders, Asian, American Indian and Alaskan Natives have an intermediate risk. The native Maori of New Zealand are an exception with one of the highest incidence of testicular cancer in the world, exceeding even the local white European population (8).

Life expectancy of men beyond the second year post-diagnosis of testicular cancer is nearly identical to the general population (9); however, potential long-term risk exists for these patients. The contralateral testis may produce a second primary GCT in 2-5%. Late relapse of GCT affects approximately 3% of patients with NSGCT (10). Non-germ cell cancers are becoming an increasing problem following treatment of GCT. The 40-year cumulative incidence of a second malignant neoplasm may reach approximately one in three (11). In addition, survivors are at increased risk of developing delayed cardiovascular disease (12) as well as other treatment-related complications including neuropathy, nephro, oto, and pulmonary toxicity. Finally, sexual dysfunction and sub-fertility post treatment represent significant long-term morbidity in this young patient population (13).

Family history

The most consistent chromosomal anomaly in GCT is a gain of the short arm of chromosome 12—i(12p). Genetic syndromes linked to GCTs include Klinefelter syndrome which is associated with primary mediastinal GCT, and Down's syndrome in which an increased rate of testicular seminoma is observed.

It is estimated that 1.4% of men with newly diagnosed GCT have a positive family history. This rate exceeds the degree expected by chance alone. Sons of men with testicular GCT have a four- to six-fold increased risk, while siblings of men with testicular GCT have an increased risk of eight- to ten-fold (14). The International Testicular Cancer Linkage Consortium is collaboration between multiple centers that holds the largest database of familial GCT published to date. A total of 985 patients from 461 families have been studied thus far. Clinical and pathologic characteristics were similar to those generally described for non-familial cases. However, an increased prevalence of testicular microlithiasis on sonography was found in men with familial testicular cancer and their relatives (15).

Unlike other hereditary cancers most GCT families consist of only two affected cases making genetic studies more difficult. Efforts are underway to find susceptibility genes for GCT within this unique group of familial cancers. Whether familial clusters of GCT are due to inherited mutations or simply reflect a shared environmental risk factor remains to be proven.

Therapeutic principles

In general, patients presenting with testicular cancer are divided into seminoma or nonseminomatous germ cell tumors (NSGCT). Management is based on volume of disease assessed using radiological staging and tumor marker level after orchiectomy. In low volume disease the goal is to decrease treatment related morbidity while maintaining a high cure rate. In patients presenting with advanced disease, especially those belonging to the intermediate and poor risk category, the goal of treatment is to improve response to chemotherapy with acceptable patient morbidity.

Seminoma

Seminoma represents approximately 60% of testicular GCTs. The incidence of testis tumors has risen over the last decade mostly due to seminomas (16). At presentation 80% of cases are stage I. Seminoma cases have a comparatively better prognosis than non-seminoma and stage III are very uncommon. Clinical research in GCT and seminoma in particular has led to a significant change in management. In

the 1970's and 1980's treatment was based on radiation as this tumor is very radio-sensitive, however, the late effects of radiation and success of chemotherapy as curative treatment have changed the treatment algorithms. Currently, stage I patients are typically managed by surveillance alone, and stage II by a balance of radiation and chemotherapy.

Stage I

The main option is observation, where patients are followed by a careful schedule and treatment is opted for only in those who present with retroperitoneal or metastatic disease during follow-up (17). Outcome is excellent with almost 100% survival.

Radiation

Previously, radiation was given after diagnosis of stage I disease to prevent relapse. Most series published from single institutions reported very high survival rates and relapses were mainly outside of the radiation field—lungs, mediastinum and left supraclavicular fossa (18,19). The classical radiation fields followed areas of documented nodal involvement from surgical studies of modified retroperitoneal lymph node dissection (RPLND) templates, on the right in the peri-caval and interaorto caval areas down to the common iliac vessels, and on the left, periaortic from the renal vessels to the bifurcation of the common iliac. The lower border on both sides is placed at a level roughly mid pelvis covering the common iliac nodes yet sparing the bladder and prostate (20). The most important prospective studies in this setting showed that 20 Gy in 2 Gy daily fractions is ideal, though, carboplatin has an equivalent curing effect (21,22).

The main side effects of radiation are sterility, cardiovascular disease and second malignancies (12,23-25). Shielding of the contralateral testis has a protective effect and large studies have shown that modern radiation fields do not hamper sperm counts in the long run (26). A large National Cancer Institute (NCI) study documented the chance of second malignancies to be twice as high as healthy counterparts (27).

Surveillance

Surveillance of seminoma patients in stage I is now increasingly preformed. Disease relapse while on surveillance is seen in 15-20% (17,19,28), and is confined mainly to the retroperitoneum. Some groups tried to use a model based on high risk for relapse (primary testis tumor >4 cm and rete testis involvement) to direct management to radiation or carboplatin. Nonetheless, using this approach is not sufficiently accurate and 65% of patients may receive

unnecessary treatment (29).

Most relapses appear in the first 2 to 3 years after diagnosis (30). As such, the tendency would be for close follow up early on to identify relapse early in its course. In the past this entailed a CT scan every 2 months in the first year and every 3 months in the second year; a not insignificant radiation exposure. As expected, such intense imaging has been scrutinized due to the potential danger of secondary malignancies. Most current guidelines recommend CT scanning every 6 months for the first 2 to 3 years. Despite the heightened attention to cumulative radiation exposure, diagnosis of relapse at an earlier stage with a smaller size of nodal disease allows for cure by radiation alone, whereas a higher disease load or relapse outside of the retroperitoneum necessitates use of chemotherapy.

Chemotherapy

Single agent carboplatin is the accepted alternative to radiation and surveillance (31). One or two cycles of carboplatin have reported relapse rates of 1.8-8.6% (17). The Medical Research Council (MRC) compared one cycle of carboplatin to adjuvant radiotherapy in nearly 1,500 patients. Updated results showed a 5-year relapse rate of 4% for radiotherapy and 5.3% for chemotherapy (32).

Management of relapse

Low volume retroperitoneal disease (i.e., less than 5 cm) may be cured by radiation. Large bulky disease or involvement of other organs is better treated by chemotherapy. Most cases may be cured by three courses of bleomycin, etoposide and cispatin (BEP) or four courses of EP. Rare cases of failure of primary chemo may be salvaged by local radiation or second line chemo therapy.

Stage II

Data accumulated in studies managing stage II seminoma show that for tumor size up to 5 cm radiation is an acceptable treatment modality with a 5-year relapse rate of up to 9%. Bulkier disease is best treated by chemotherapy with relapse rates of 6-13.5% (33-36). Recent studies as in SWENOTECA have shown the superiority of chemotherapy also in lower stages—seminoma IIa/b (37). The primary consideration for choice of therapy is chemotoxicity in older age patients where radiation may have fewer side effects. Radiation fields in this setting are similar to stage I, limiting pelvic radiation to the level of the acetabulum.

A residual mass after radiation or chemotherapy is a unique challenge. In contrast to NSGCT post-

chemotherapy residual disease where teratoma or cancer may be frequently found, most residual seminoma masses harbor fibrosis or necrosis. PET-CT may reliably indicate the presence of active tumor; therefore a negative PET-CT may allow observation even in large redial masses. Some centers advocate resection of all masses larger than 3 cm (38), though, this may be a difficult undertaking due to the desmoplastic reaction and adherence to the main blood vessels.

NSGCT

Clinical stage I (CSI)

Clinical stage I accounts for 50-60% of non-seminomatous testicular tumors. It is long known that the risk of occult metastatic disease (not identified on imaging) is dependent on the presence of lymphovascular invasion (LVI) in the tumor (39-41). LVI is present in about 30% of cases and the risk of recurrence is about 50% with LVI versus 15-20% without LVI (42,43). Another less accepted risk factor is embryonal predominance, with controversial data among different studies (41-43). Recurrences occur most commonly in the retroperitoneum, with the majority diagnosed within 2 years of orchiectomy (42,44). Management options for CSI NSGCT include surveillance, RPLND, and adjuvant chemotherapy.

Surveillance

The rationale for surveillance among patients with CSI NSGCT is that studies have shown that approximately one in four patients will recur and require salvage treatment (39-41). This is the group that actually would benefit from adjuvant therapy, whereas most patients will not benefit. Active surveillance became an option in the 1980's when Read *et al.* demonstrated that cisplatin-based chemotherapy could cure almost all recurrences (45). When studies revealed the importance of LVI as a prognostic factor for recurrence, risk-adapted approaches with surveillance or adjuvant treatment were implemented (46,47). At present, some centers advocate surveillance for all CSI NSGCT, consequently no patient will be treated unnecessarily; however, 50% of those with LVI and 15% of the patients without LVI will later need salvage treatment (41,46,47).

RPLND

Although not frequently used today, the advantage of RPLND is that it represents both a diagnostic and a therapeutic procedure. RPLND remains the most accurate means of staging patients with CSI NSGCT; roughly 50% to 70% will be pathologic stage I. In these patients, RPLND is purely diagnostic with the added benefit of a simpler follow up. Because retroperitoneal recurrence is rare with properly performed RPLND, abdominal CT scan may be omitted after negative RPLND. In the case of pathologic stage II disease RPLND is curative in 50% to 90% of patients, thus selected patients may avoid adjuvant chemotherapy (24,48).

Adjuvant chemotherapy

As noted previously, 50% of LVI positive patients will relapse, therefore adjuvant treatment would spare half of this group from a recurrence requiring three to four courses of chemotherapy and possibly post-chemotherapy surgery (PCS) for a residual tumor. Conversely, the other half would receive adjuvant chemotherapy 'unnecessarily'. The main argument against adjuvant chemotherapy is its lack of improved overall survival and its association with long-term side effects including infertility, secondary malignancies, and increased risk for cardiovascular disease, impaired kidney function, hearing impairment, and peripheral neuropathy (48-50).

One way to reduce toxicity of adjuvant chemotherapy is to reduce the number of cycles used (51,52). The German testicular study group published data in 2008 from a randomized study on 382 patients with CSI NSGCT. Patients were randomized to RPLND (in the community) or BEP ×1 without regard to LVI. This was a non-inferiority study with a median follow-up of 4.7 years and a primary endpoint of recurrence rate. The recurrence rate was 1% and 7.9% for patients treated with BEP ×1 and RPLND, respectively. About 40% of each group were LVI-positive (53). The main criticism of this study is that RPLND was performed in less skilled hands as evidenced by unacceptably high in-field recurrence rates.

SWENOTECA—the Swedish-Norwegian testicular cancer group now comprises all centers treating testicular cancer patients in Sweden and Norway. Based on the results from earlier treatment protocols a new risk-adapted treatment protocol for CSI NSGCT was initiated in 1995.

During the period of 1995-1997, 232 patients were accrued to the SWENOTECA III protocol. CSI NSGCT LVI⁻ patients were randomized to cisplatin, vinblastin, bleomycin (CVB) ×1, or surveillance. LVI⁺ patients were treated with CVB ×2 and data was collected prospectively. The recurrence rate among the CVB ×1 patients was higher than expected and as such, the study was terminated early (54).

SWENOTECA VI randomized low-risk patients (LVI⁻) to surveillance or BEP ×1, and high-risk patients (LVI⁺) to

Table 1 International germ cell consensus classification prognostic groups in patients with metastatic disease treated with first line chemotherapy

Risk group	NSGCT	Seminoma	5-year survival*
Good prognosis	Primary site: testis or RP and metastases: nodal or pulmonary and marker level: S1	Primary site: all and metastases: nodal or pulmonary and marker level: any LDH, any hCG	Seminoma 86%; NSGCT 94%
Intermediate prognosis	Primary site: testis or RP and metastases: nodal or pulmonary and marker level: S2	Primary site: all and metastases: non-pulmonary visceral and marker level: any LDH, any hCG	Seminoma 72%; NSGCT 83%
Poor prognosis	Primary site: mediastinal or metastases: non-pulmonary visceral or marker level: S3	No patients classified as poor prognosis	NSGCT 71%

NSGCT, nonseminomatous germ cell tumor; S1, a-fetoprotein (AFP) <1,000 ng/mL, and human chorionic gonadotrophin (hCG) <5,000 mIU/mL, and lactic dehydrogenase (LDH) <1.5 upper limit of normal; S2, AFP =1,000-10,000 ng/mL, or hCG =5,000-50,000 mIU/mL, or LDH =1.5-10 upper limit of normal; S3, AFP >10,000 ng/mL, or hCG >50,000 mIU/mL, or LDH >10 upper limit of normal; RP, retroperitoneum; *, survival data for seminoma patients based on IGCCCG study (57). Survival for NSGCT patients is based on a more recent meta-analysis (58).

BEP ×2 or BEP ×1. Yearly assessments of the total cohort were performed and low relapse rates with BEP ×1 were noted. Accordingly, the protocol was amended to treat high-risk patients with BEP ×1. In 2009 results with a median follow-up of 4.7 years were reported. A total of 313 patients were treated with one course of adjuvant BEP (157 LVI⁺, 155 LVI⁻ and 1 LVI unknown). The relapse rate was 3.2% for LVI⁺ and 1.3% for LVI⁻ (47).

Recently, the expanded data from a total of 517 patients (258 LVI⁺, 255 LVI⁻ and 4 LVI unknown) treated with one course of adjuvant BEP between 1998 and 2010 was reported (44). The median follow-up was 7.9 years. The data confirmed the SWENOTECAs earlier reported low relapse rates as well as excellent overall- and cause-specific survival. Only one patient died because of progressive cancer and there were no treatment related deaths. Five of the 12 relapses (42%) were cured by RPLND alone, and only 1.4% (7/517) of the patients actually required salvage chemotherapy. These findings confirm that one course of adjuvant BEP reduces the risk of relapse by 90-95% in all patients. No recurrences occurred later than 3.3 years post-treatment and as such, follow-up can safely be reduced to 5 years (55).

The optimal treatment strategy for CSI NSGCT is controversial. To date, there are no randomized trials that demonstrate superiority of surveillance or adjuvant treatment. Further, cure approaches 100% regardless of treatment strategy. Thus the main issue is how to best minimize treatment related toxicity. As noted earlier,

chemotherapy increases the risks of cardiovascular damage resulting in hypertension, cardiac events, and decreased kidney function. Impaired hearing, metabolic late effects, hypogonadism and increased risk for secondary cancers are also associated with adjuvant chemotherapy. As well, there is a clear dose-response relationship associated with increased cycles of chemotherapy. For stage I NSGCT, results from the SWENOTECA study show that adjuvant therapy can be safely reduced to just one course of BEP, resulting in a reduction in relapse rate of 90-95%. This lower dose of chemotherapy may mitigate many of the long-term consequences of therapy.

Clinical stage II (CSII) and III (CSIII)

In metastatic NSGCT, the degree of marker elevation before chemotherapy correlates with prognosis. The International Germ Cell Cancer Collaborative Group (IGCCCG) has incorporated serum concentrations of human chorionic gonadotrophin (hCG), AFP, and lactic dehydrogenase (LDH) into a prognostic classification system with high, intermediate, and low risk disease (*Table 1*), and treatment is tailored according to the risk assignment. Systemic therapy for metastatic GCT consists of cisplatin-based chemotherapy. For good risk disease, the accepted standard is three courses of BEP or four courses of EP. Standard therapy for intermediate and poor risk disease remains four courses of BEP.

Depending upon the patient population selected, roughly 70% of patients treated with first line chemotherapy

will have complete radiographic and biochemical response. In the remaining 30% a residual mass will persist after chemotherapy, most commonly in the retroperitoneum (58,59). These patients will then undergo post-chemotherapy retroperitoneal lymph node dissection (PC-RPLND) because of possible residual teratoma (40%) or active cancer (10%) (60).

Controversies in PC-RPLND

The excision of all masses after chemotherapy, within and outside the boundaries of the retroperitoneum, is integral to the cure of NSGCT (61). It is well recognized that incomplete resection or surveillance of a residual mass after chemotherapy risks relapse (62,63). Therefore, any patient who has a mass larger than 1 cm in the retroperitoneum should undergo surgery. The proper extent of PCS resection and the need for PC-RPLND in patients achieving complete remission remains controversial (64,65).

At most centers, the management of patients achieving a complete radiographic response to systemic chemotherapy is observation. However, studies have documented the incidence of residual teratoma in sub-centimeter retroperitoneal nodes following chemotherapy to be 20-30% (66-68). Therefore, some institutions have adopted a policy of PC-RPLND in all patients, including those achieving complete radiographic response.

A study from Indiana University analyzed 141 consecutive patients who achieved complete remission following first-line induction chemotherapy (64). All patients were observed and did not undergo immediate PC-RPLND. Patients who had intermediate or poor prognosis disease constituted 23% of the cohort. After a median follow-up of 15.5 years, 12 patients experienced a relapse, of who four died of their disease. Amongst these four, the relapse occurred within the first year of chemotherapy; in two of them, the retroperitoneum was the site of relapse. Altogether, of the 141 patients, six (4%) relapses occurred in the retroperitoneum. The estimated 15-year cancer-specific survival rate was 97%.

The data suggest that patients that relapse in the retroperitoneum on observation (4%) remain curable. It is unlikely that a different strategy could result in a higher 15 years cancer-specific survival. In other words, there is no evidence that immediate PC-RPLND would prevent those rare relapses in exchange for subjecting all patients to the morbidity of PC-RPLND. Two other North American

studies similarly support the safety of observation (69,70). The current European and Canadian guidelines endorse this data and favor observation for patients achieving complete radiographic remission, whereas in the NCCN guidelines either immediate PC-RPLND or observation are appropriate.

The second area of controversy is the extent of PC-RPLND. The extent of primary RPLND for stage I NSGCT has changed considerably over the last three decades from a full bilateral suprahilar dissection to a unilateral nerve sparing template without compromising cure. Unlike primary RPLND, in the management of post-chemotherapy retroperitoneal disease there has been no such reduction in surgical boundaries with full bilateral dissection considered standard therapy.

In the 1970's and 1980's lower stage metastatic disease was more often treated with primary bilateral template RPLND in an attempt to avoid chemotherapy which had considerable morbidity at the time. With the improvement of antiemetic, growth factors and supportive care, the toxicity of chemotherapy has decreased. Today only select patients with limited retroperitoneal disease who have normalized serum tumor markers may be considered for primary RPLND (71). Patients with teratoma or non-germ cell component in their primary tumor can benefit the most from primary RPLND (72).

Based on early experience from primary RPLND, retroperitoneal mapping studies have accurately documented the lymphatic spread of metastasis (73,74). Metastases right of the vena cava were rare in patients with a left-sided primary (3-7%), but crossover metastases left of the aorta were more common in patients with a right sided primary (8-19%). Theoretically, had chemotherapy been administered before surgery, it would not have changed the retroperitoneal distribution of the residual tumors, suggesting that template crossover after chemotherapy is not generally expected in low-stage left-sided tumors. Our group has shown from our experience with bilateral PC-RPLND that patients with left-sided primary tumors and clinical stage IIA or IIB disease at presentation did not have metastasis right of the aorta (75). In these patients it may be safe to perform a left modified dissection. Beck *et al.* (76) and Heidenreich *et al.* (77) have further shown that a modified unilateral PC-RPLND (either right or left) may be safe in select patients with low volume retroperitoneal disease (less than 5 cm), restricted to the primary landing zone of the affected testicle.

340

Ehrlich et al. Advances in the treatment of testicular cancer

Acknowledgements

Footnote

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Upper urinary tract disease: what we know today and unmet needs

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Purpose: Upper tract urothelial carcinoma (UTUC) is a rare and poorly investigated disease. Intense collaborative efforts have increased our knowledge and improved the management of the disease. The objective of this review was to discuss recent advances and unmet needs in UTUC.

Methods: A non-systematic Medline/PubMed literature search was performed on UTUC using the terms "upper tract urothelial carcinoma" with different combinations of keywords. Original articles, reviews and editorials in English language were selected based on their clinical relevance.

Results: UTUC is a disease with specific epidemiologic and risk factors different to urothelial carcinoma of the bladder (UCB). Similarly to UCB, smoking increases the risk of UTUC and worsens its prognosis, whereas aristolochic acid (AA) exposure and mismatch repair genes abnormality are UTUC specific risk factors. A growing understanding of biological pathways involved in the tumorigenesis of UTUC has led to the identification of promising prognostic/predictive biomarkers. Risk stratification of UTUC is difficult due to limitations in staging and grading. Modern imaging and endoscopy have improved clinical decision-making, and allowed kidney-sparing management and surveillance in favorable-risk tumors. In high-risk tumors, radical nephroureterectomy (RNU) remains the standard. Complete removal of the intramural ureter is necessary with inferiority of endoscopic management. Post-RNU intravesical instillation has been shown to decrease bladder cancer recurrence rates. While the role of neoadjuvant cisplatin based combination chemotherapy and lymphadenectomy are not clearly established, the body of evidence suggests a survival benefit to these. There is currently no evidence for adjuvant chemotherapy (AC) in UTUC.

Conclusions: Despite growing interest and understanding of UTUC, its management remains challenging, requiring further high quality multicenter collaborations. Accurate risk estimation is necessary to avoid unnecessary RNUs while advances in technology are still required for optimal kidney-sparing approaches.

Keywords: Upper tract urothelial carcinoma (UTUC); risk factors; predictive tools; ureteroscopy; radical nephroureterectomy (RNU); chemotherapy

Introduction

Upper tract urothelial carcinoma (UTUC) accounts for 5% of all urothelial carcinomas with an estimated annual incidence of 1 to 2 cases per 100,000 inhabitants (1).

Sometimes considered the twin of urothelial carcinoma of the bladder (UCB) (2), UTUC is now considered a distinct entity by SIU and EAU guidelines (3). The annual number of UTUC-related publications has almost tripled over the last 10 years (*Figure 1*). First symposia
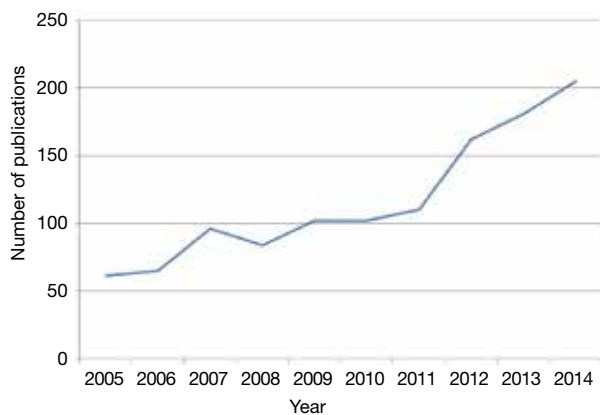
Figure 1 Annual number of publications related to "upper tract urothelial carcinoma".

and working groups such as the Upper Tract Urothelial Carcinoma Collaboration, French Collaborative National Working-Group on UTUC, and Canadian Upper Tract Collaboration reflect growing interest for UTUC.

Basic research and collaborative efforts have contributed to improve our knowledge on the natural history of UTUC. Improvements in technologies and extrapolation from UCB management, have greatly contributed to progress in UTUC management. The low incidence of the disease is, however, a limit for studies with high level of evidence. Prediction models have been developed to help physicians with evidence based personalized clinical decision making (4).

The objective of this review was to provide insights in current advances in UTUC tumorigenesis, risk stratification and treatment, and to highlight unmet needs of UTUC as we know it today.

Methods and evidence acquisition

A non-systematic Medline/PubMed literature search was performed using a combination of the terms "upper tract urothelial carcinoma" with different keywords. To select relevant articles, reviews and editorials from English literature, the following keywords were used for the needs of the different sections of the manuscript: (I) "epidemiology", "risk factors", and "biology"; (II) "staging" and "risk stratification"; (III) "conservative treatment", "nephro-ureterectomy" and "lymphadenectomy"; (IV) "neo-adjuvant treatment" and "adjuvant treatment". Time period included articles published between January 2000 and January 2015. Additional informative articles were

collected by cross referencing the bibliography of previously selected articles.

Evidence synthesis

Soil and seed

Specific risk factors

UTUC has long been considered the twin tumor of UCB. Therefore, past and current practices are mainly derived from UCB management. However, several epidemiologic and basic research studies have clearly demonstrated UTUC presents specific anatomical, biological, clinical and pathological features (2). These tumors are less frequent than UCB, more invasive at diagnosis, and have a male to female ratio of 2:1 (2).

UCB and UTUC share common risk factors like smoking and exposure to aromatic amines. Specific risk factors have been identified in UTUC. It has been recently shown that UTUC is associated with Balkan endemic nephropathy, a disease linked to aristolochic acid (AA) exposure leading to DNA-adducts with specific genetic signatures such as a *TP53* A to T transversion (5-7). Epidemiologic studies estimated that one third of the Taiwanese population has been exposed to AA, as it is an integral part of Chinese herbal medicine (8). This exposure also exists in China, other parts of Asia, as well as in ayurvedic medicine in India. Genetic features could also represent specific risk factors to develop UTUC even with a relatively low environmental risk exposure to known carcinogens.

Some UTUC have a hereditary predilection belonging to the hereditary nonpolyposis colorectal carcinoma (HNPCC) tumors spectrum (9). Alterations of mismatch repair genes, responsible for HNPCC, could also be involved in sporadic UTUC as a potential initiating event (10,11). It has been recommended to test all patients with UTUC who are less than 60 years old, have a personal history of an HNPCC-associated cancer, a first-degree relative <50 years of age with HNPCC-associated cancer, or two first-degree relatives with HNPCC-associated cancer, to identify hereditary cancers that have been misclassified as sporadic cancers (3). Studies on genetic variations in the population have also identified specific genetic polymorphisms associated with a higher risk of UTUC (12,13). Sasaki *et al.* reported DNA repair gene polymorphisms could have a prognostic value since more than two variant alleles in these genes was associated with a significant better overall survival (OS) and cancer specific survival (CSS) after

radical nephroureterectomy (RNU) (14). Such genetic risk markers of UTUC could help identify patients who have an increased risk of developing UTUC but also those who are more likely to harbor biologically aggressive disease.

Molecular biomarkers

Several tissue, blood, genetic or urine based biomarkers, such as microsatellite instability (MSI) of the tumor, p53, E-cadherin, HIF1alpha, and ki-67 have been proposed to help in the prognostication of UTUC (15). Krabbe *et al.* reported in 475 patients treated with RNU that Ki-67 was an independent prognosticator of recurrence free survival (RFS) and CSS for high grade tumors (16). Bagrodia *et al.* similarly demonstrated that both PI3K and cyclin D, two mTOR biomarkers, were associated with adverse pathologic results and worse oncological outcomes in a cohort of 620 patients who underwent RNU or partial ureterectomy (17).

To date, none of these potential biomarkers have been integrated to clinical practice or predictive models. While there are many challenges to the stepwise assessment of new biomarkers before integration into clinical care (18), in UTUC, biomarkers are mainly needed to help risk stratify the disease in order to identify patients who may benefit from kidney-sparing management, neoadjuvant chemotherapy (NC), or extended lymphadenectomy. These initial studies were done on RNU specimens, so they help understand the biological potential of these biomarkers post-operatively but not in the pre-RNU setting. After RNU, adjuvant chemotherapy (AC) is not established for the reasons we discussed below. The validation of these new molecular and genetic characteristics may help physicians better appraise patient and tumor identities to guide clinical decisions and design a personalized approach to some cases. Still, biomarkers are urgently needed in the pre-RNU setting. Biomarkers that can be evaluated in small tissue samples obtained by endoscopic biopsy may help overcome the shortcomings of current staging in UTUC through refined biomarker-guided risk stratification.

"Plant anatomy and morphology"

Imaging and biopsy

Imaging and ureteroscopic biopsy now play a critical role to define stage and grade of UTUC, which are the most accurate independent factors of outcome (15). However, despite technological advances, current modalities yield limited samples that preclude optimal staging and grading. Multi-detector computed tomography urography (MDCTU) with images during excretory phase (10-15 min) is the standard technique used for staging today (3). Its accuracy to stage the tumor ranges from 59% to 88% (19,20). Assessment of nodal involvement by MDCTU is even less accurate since only 60% of the patients with positive lymph nodes (LNs) at LN dissection (LND) are considered N+ on preoperative imaging (21). Nevertheless, if invasion is seen on MDCTU, it indicates at least muscle invasive disease (22). In addition, hydronephrosis has also been associated with invasive disease which may not benefit from kidney-sparing management (23).

Flexible ureteroscopy has revolutionized preoperative evaluation of UTUC allowing to visualize all upper urinary tract and to perform tumor biopsy. There are anatomical and instrumental limitations to sample the tumor adequately (24). Even when the biopsy can be properly analyzed, the accuracy of biopsy to define T stage is limited. Smith *et al.* reported a stage discrepancy between final RNU pathology and endoscopic biopsy in 38% of the cases (25). Biopsy is more efficient regarding grading assessment with an accuracy ranging from 69% to 91% when compared with RNU pathology (26). Biopsy grading can enhance T staging evaluation: 68-100% of grade 1 biopsies are associated with ≤ pT1 tumors while 62-100% of biopsies with grade 3 correctly predict muscle invasive stage (≥ pT2) (26).

To improve T staging by imaging and compensate the paucity of current pathological data from biopsy, new modalities of acquisition and evaluation have been developed. Matin *et al.* tested a promising endoluminal ultrasound (US) (27). In this pilot study, seven patients with UTUC underwent RNU after endoluminal US evaluation to stage the tumor. PPV and NPV for invasive disease status were 66.7% and 100%, respectively. Other technologies such as optical coherence tomography and confocal laser endomicroscopy are under evaluation (28,29). Preliminary reports suggest multiparametric MRI, especially ADC, could also be useful tools for staging and grading the tumor (30,31). Sassa *et al.* evaluated 11C-choline positron emission tomography-computed tomography (PET/CT) for primary diagnosis and staging of UTUC and demonstrated encouraging results, especially regarding nodal evaluation. In this study, among 12 patients with UTUC on final pathology and pre-operative PET/CT, 11 had choline tumor uptake on pre-operative PET/CT. LN or distant metastases were diagnosed in five patients on pre-operative PET/CT and all metastatic sites displayed choline uptake (32).

To improve the quality of biopsies, new instrumental

methods have been tested and showed that tumor removal using baskets could better determinate tumor grade in some cases (33,34). New technologies such as narrow band imaging (NBI) and high definition digital ureteroscopy can also help better characterize tumor characteristics (35).

Predictive models

To overcome current limited accuracy of imaging and biopsy sampling and to combine all available data to improve outcome prediction, multi-institutional clinical research groups have developed preoperative predictive models to guide clinical decision-making (36). Favaretto *et al.* proposed a model based on the combination of data from imaging (local invasion and hydronephrosis) and ureteroscopy (tumor location and high grade at biopsy) (22). Margulis *et al.* combined grade, architecture and tumor location (37). These models were able to predict non organ confined disease with an accuracy of 70% and 77%, respectively. Brien *et al.* proposed a simple model based on the presence of hydronephrosis, high grade at biopsy and positive cytology. The positivity or negativity of all three features was able to predict the muscle invasion with 89% PPV and 100% NPV (38).

To date, guidelines propose a risk stratification on low risk and high risk tumors based on pre-operative parameters to guide therapeutic management of patients with UTUC (3,39). This decision making definition relies on relatively small studies and experts' opinion. These predictive models represent evidence based data that may be integrated in treatment decision algorithms. However, no external validation of these models has been published yet. Therefore, large and multicenter external validation of these models are the first step before considering their use in the management of UTUC.

"The best harvest"

Kidney sparing approach using flexible ureteroscopy

Kidney sparing management of UTUC was historically limited to imperative indications (renal insufficiency or solitary functional kidney). The previously described concept of "low risk" tumors, the high percentage of pTa tumors at time of RNU, and the development of flexible ureterorenoscopy and novel instrumentations lead to a shift of the indications to elective cases (when the contralateral kidney is functional) (40). The tumor has to be resectable and with a low risk of recurrence and progression, and the patient has to understand that a close follow up is necessary (3). This can be achieved with flexible/semi-rigid ureteroscopy today. Open and percutaneous resection of tumors of the renal pelvis or calices have almost disappeared (3). Distal ureteral segmentectomy remains, however, an option for tumors of the distal ureter or in case of ureteroscopic failure (41).

Recently, using the Surveillance, Epidemiology, and End Results (SEER) database, Simhan *et al.* reported similar CSS with RNU and kidney sparing procedure (KSP), including ureteral segmentectomy and endoscopic KSP (42). Patients treated with KSP were older with a greater proportion of grade 1 tumors and underwent segmental ureterectomy in 62.5% of cases. To date, oncological outcomes of endoscopic KSP with percutaneous resection and/or flexible ureteroscopy tumor ablation have been compared to RNU in nine non-randomized studies (43-51). A recent meta-analysis included eight of these studies and revealed no difference in terms of OS and CSS between both strategies (52). These studies were all retrospective with small cohorts and limited follow-up. Selection bias was clearly a major limitation since most tumors in the KSP group were unifocal, <2 cm and low grade, in contrast with a higher incidence of invasive tumors in the RNU group. Local recurrence rate, a major issue in endoscopic conservative management, ranged from 6% to 71% in these heterogeneous cohorts. Results were so variable that no reliable RFS meta-analysis could be performed. Yakoubi *et al.* partly related the high heterogeneity among studies to differences in expertise of endoscopy between centers (52). Progression rate, another major concern regarding conservative management, remains unclear because of the inability to accurately grade and stage UTUC. Grade and stage migration during follow up has been estimated to reach 19% and 14%, respectively, and varied widely according grade at first biopsy (26). A delayed RNU is finally performed in 28-43% patients initially treated endoscopically (26). A major issue to address is the oncologic impact of such delayed radical treatment. Two studies compared delayed RNU after endoscopic KSP to immediate RNU and reported similar oncologic outcomes (53,54). However, these results should be considered with caution due to small populations and short follow-up.

Many improvements with digital ureteroscopes such as NBI and photodynamic diagnosis are currently under evaluation (35). These new technologies could help better diagnose UTUC but also perform a complete tumoral ablation during endoscopic KSP. Despite the lack of prospective randomized studies, the differential indications

for KSP versus RNU seem reasonable based on the available evidence in order to provide optimal risk-based therapy for the individual patient.

Radical nephro-ureterectomy

Because of the limits of KSP and since more than 60% of tumors are invasive at presentation, RNU still remains the standard treatment for the majority of UTUC (3). To ensure negative margin, complete removal of the ureter including a bladder cuff is mandatory during RNU. In high risk UTUC (pT3N0, pT4N0 and/or N+ and/or M+), positive margins have been identified as an independent prognostic factor for CSS and OS (55). Lughezzani *et al.* showed that avoiding bladder cuff excision increased cancer specific mortality (CSM), especially in high risk UTUC (56). Several approaches have been proposed to perform bladder cuff excision with no difference in RFS, CSS, and OS between transvesical, extravesical, or endoscopic approaches in a large multicenter study of 2,681 patients treated with RNU (57). However, endoscopic approach was associated with a higher risk of intravesical recurrence. Recently, Kapoor *et al.* reported an improved overall and intravesical RFS with open intravesical excision of the distal ureter compared with endoscopic but also extravesical approaches (58).

Similarly to other fields of urology, laparoscopy and robotic assistance have been adopted to perform RNU. Robotic assisted RNU is still in its infancy and comparative studies are scarce (59,60). Conversely, many studies have compared laparoscopic RNU (LRNU) to open RNU (ORNU), and a recent meta-analysis reported similar oncologic outcome (61). Caution should be advocated especially in locally advanced disease since LRNU is generally performed in favorable-risk patients (62). Indeed, Fairey *et al.* reported that LRNU may be associated with poorer RFS compared to ORNU in a study of 849 patients (403 ORNU *vs.* 446 LRNU) (P=0.06) (63). In the only randomized controlled trial, Simone *et al.* found CSS and metastasis free survival were significantly different between the two procedures for pT3 tumors, in favor of ORNU (P=0.039 and P=0.004, respectively) (64). However, this and other studies were limited by their small size and other potential biases of selection or expertise, but one main limitation may be the use or extent of LND during LRNU.

The importance of LND remains a question of debate, yet all the evidence shows improved outcomes with higher number of LN removed, specially in LN negative patients (65). Capitanio *et al.* reported that LND was not

commonly performed during ORNU and LRNU [42% and 24% of cases, respectively (62)]. Guidelines advocate LND in RNU for two reasons: (I) improve prognostication; (II) a potential therapeutic effect (3). Indeed, LN status is one of the most powerful predictor of CSS in patients treated with RNU, possibly guiding treatment decision for follow-up scheduling and AC (66). Roscigno *et al.* estimated that removal of eight LNs was the critical cut off to reach a prognostic significance and a 75% probability to correctly stage the patients (67). Therapeutic effect remains, however, unclear. A potential survival benefit in patients who underwent a LND during RNU has been reported in several monocentric studies with small cohorts (68-70). Two retrospective studies in large cohorts of patients reported this benefit could only be valuable in muscle invasive or locally advanced UTUC (71,72). Indeed, the risk of LN involvement is limited in Ta T1 UTUC, probably less than 5% (65,73). Recently, Yang *et al.* included 6,000 patients in a meta-analysis and confirmed a benefit of LND only in the group of patients with muscle invasive tumors (74). One question that remains unclear in these studies is the template for LND. Kondo *et al.* proposed a template for LND according to tumor location in the upper two-thirds of ureter, or in the lower third of the ureter (68). The former implies a dissection of iliac vessels, the latter a dissection of the aorta or the vena cava that could limit its performance minimally-invasively.

Therefore, prospective comparative studies are mandatory to assess the oncologic outcomes according to surgical approach and the extent of LND. Futures studies with RNU should match patients for grade and stage, but also for surgical approach. Strict definitions of the extent of LND using predefined templates will be necessary to make evidence-based recommendations.

"Prevent growth and regrowth"

Local instillations

One major concern with each management is the prediction, prevention and treatment of disease recurrence. Urothelial carcinoma can either recur in the bladder, contralateral ureter and/or in the ipsilateral ureter if KSP has been attempted.

After KSP, recurrence rate in the upper tract can be reported in up to 70% of the cases (52). Instillations of topical agents in the upper tract have been proposed to decrease this risk. Different approaches have been reported (percutaneous nephrostomy, retrograde

catheterisation and vesico-ureteral reflux) with bacille calmette guerin (BCG) and mitomycin C (MMC) (75,76). BCG instillations for carcinoma in situ (CIS) may be the only one with sufficient evidence today. Only one study compared BCG instillation and RNU for CIS in 11 and 6 patients, respectively, and reported no significant difference in 5-year RFS and CSS (77). Topical instillations with BCG and MMC have been also reported as therapy after endoscopic management of Ta/T1 UTUC. Rastinehad *et al.* performed the largest comparative study with adjuvant antegrade BCG therapy after percutaneous resection and demonstrated no benefit in terms of recurrence and progression rates (78). These studies were retrospective, mostly non comparative, and included small cohorts treated mainly by percutaneous resection. These limitations preclude any conclusion regarding the use of instillations in the upper tract for UTUC after conservative treatment. Therefore, new studies should investigate its efficacy but also the best way to administrate it in the era of flexible ureteroscopic management.

Instillation of post-operative topical agents in the bladder have also been proposed to decrease the risk of intravesical recurrence after RNU. Indeed, 30% to 50% of patients will develop UCB during the first 5 years after RNU for UTUC (79). O'Brien *et al.* demonstrated, in a prospective multicentre randomized study, that a single post-operative intravesical dose of MMC after RNU decreased the relative risk of bladder tumor by 40% within the first year (80). In a phase II trial using intravesical pirarubicin (THP) within 48 h after RNU, Ito *et al.* reported similar results (81). Xylinas *et al.* developed a tool to identify the patients most likely to benefit from immediate post RNU intravesical chemotherapy (82). No study on the role of early post operative bladder instillation has been yet published after endoscopic management. Therefore, high level of evidence regarding the usefulness of post operative instillation of MMC after RNU now exits but further evaluation is needed to conclude on its efficacy after KSP management, another area of high likelihood of benefit.

Chemotherapy

Systemic NC before radical cystectomy has demonstrated survival benefit in patients with T2-4 N0 M0 UCB with high level of evidence (83). To date, no level 1 evidence exists to state on the role of peri-operative chemotherapy in UTUC. A recent review and meta-analysis identified ten studies that investigated the role of chemotherapy in

an adjuvant setting (84). All but one were retrospective studies. These studies harbored many potential biases with most patients who received AC having worse prognostic factors and more likely to have LN metastasis. Conversely, patients receiving AC may have better renal function and performance status. Meta-analysis demonstrated only a statistically significant benefit for OS and disease free survival (DFS) among the three studies using cisplatin-based AC (HR, 0.43; 95% CI, 0.21-0.89; P=0.023). Furthermore, recent studies suggested that AC may only benefit high risk patients with pT3-4 UTUC and LN involvement (85,86). With potential benefit restricted to cisplatin based chemotherapy in locally advanced disease, the impact of AC appears limited since most patients with UTUC will experience renal function loss after RNU, becoming ineligible (87). Even before RNU, only 49% of patients have a glomerular filtration rate that would allow cisplatin based chemotherapy. This rate decreases to 19% after RNU.

Potential use and efficacy of chemotherapy in a pre-operative setting is, therefore, a critical issue. To date, two prospective studies assessed the role of NC in patients with urothelial carcinoma but only recruited 21 patients with UTUC. These studies suggested NC could be associated with a significant downstaging. The small cohorts and the inaccuracy of current methods to pre-operatively stage the tumor limit any conclusion (84). Results from four larger retrospective and comparative studies that specifically evaluated NC in UTUC have been published so far (88-91). Matin *et al.* reported outcomes of 43 patients with high grade UTUC who received NC compared to a historical cohort. A significant higher pathologic downstaging and a complete response in 14% of patients were observed in the NC group (89). In a recent study, use of NC in 31 patients was associated with a significant improvement of OS and CSS compared to a cohort of 81 patients who underwent RNU alone (91). Upper Tract Urothelial Carcinoma Collaboration group reported as well outcome in a large cohort of 313 patients including 18 patients with biopsy proven LN involvement who received NC and demonstrated favorable oncologic outcomes in this group: 5-yr DFS and CSS rates of 49% and 44%, respectively (88). Considering these two last studies, a recent meta-analysis reported a CSS benefit of 59% with NC (HR, 0.41; 95% CI: 0.22-0.76; P=0.005) (84). These retrospective data suggest that all eligible patients should be proposed cisplatin combination chemotherapy in UTUC. Which patients are most likely to benefit from NC remains

to be defined. Patients with clinically suspect LN should receive definitive chemotherapy and a RNU in case of response. However, the level of evidence of the studies does not allow any firm conclusion. Further prospective trials are needed to assess the role of peri-operative chemotherapy in UTUC. One randomized controlled phase 3 trial, the peri-operative chemotherapy versus surveillance in upper tract urothelial cancer (POUT) trial, is ongoing (92). This trial will randomize 345 patients undergoing RNU for UTUC between adjuvant platin-based chemotherapy and surveillance. Results from phase 2 trials that investigate impact of neoadjuvant gemcitabine in patients with high grade or T2-T4 N0/X M0 UTUC before RNU will probably help us further to define the place of perioperative chemotherapy in UTUC.

Predictive models

The lack of randomized trials and reliable preoperative staging and grading evaluation leads to complex decision making in UTUC management. Intense collaborative and multi-institutional efforts resulted in propositions of predictive tools. To date, three pre-operative models have been proposed to predict muscle invasive and non-organ-confined UTUC (22,23,37,38). We previously discussed the usefulness of these models to decide between KSP and RNU. Other predictive models using pre-operative data represent promising tools. Two models based on imaging, urine cytology, or neutrophil count demonstrated significant ability to predict RFS and CSS (93,94). To manage the potential risk of renal function loss after RNU, several prognostic factors have been identified and corresponding predictive models constructed to identify patients that would not be suitable for post-operative chemotherapy (95-97). These prediction tools could help physicians identify patients who may benefit from neoadjuvant medical treatments in UTUC or LND during surgery.

Several postoperative prognostic risk factors after UTUC have been identified and were combined to propose post-operative prediction tools (36). Jeldres *et al.* were the first to propose a post-operative nomogram for UTUC. Within a cohort of almost 6,000 patients from the SEER database, a model based on age, tumor stage, tumor grade, and LN status predicted 5-year CSS with a discrimination of 75.4%. Since this first nomogram, four new models have been published. The French collaborative group and international UTUC collaboration proposed their own models to predict CSS (98,99). Both cohorts (3,387 patients) were combined and the model predicted 5-year

CSS with 80% discrimination (100). Recently, Seisen *et al.* proposed a model including only patients without NC from both collaborative groups (101). However, to date, only one external validation focusing on the French collaborative group model has been published (102). Xylinas *et al.* recently published a predictive model of intravesical recurrence after RNU (82). Based on age, gender, previous history of bladder cancer, tumor location, tumor stage, presence of concomitant CIS, and LN status, the model discrimination was 68%. With this model, considering a risk of intravesical recurrence of 15% at 2 years to perform post operative instillation would spare 23% of the patients while not preventing only 0.3% of intravesical recurrences. These models could be particularly relevant to help physicians identifying patients whose disease is more likely to recur and therefore benefit from adjuvant therapy.

Conclusions

Ten years of intense collaborative efforts in basic and clinical research have made the natural history of UTUC more comprehensible and predictable. Current management is based, however, on low level evidence and there are many challenges to face in the future. There is a need to clarify the role of KSP management, topical agents, LND, and perioperative chemotherapy. New further collaborative efforts are mandatory to propose ambitious multi-institutional studies with preferentially prospective design.

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Footnote

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Indications for biopsy and the current status of focal therapy for renal tumours

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Abstract: The increased detection of small renal masses (SRMs) has focused attention on their uncertain natural history. The development of treatment alternatives and the discovery of biologically targeted drugs have also raised interest. Renal mass biopsies (RMBs) have a crucial role as they provide the pathological, molecular and genetic information needed to classify these lesions and guide clinical management. The improved accuracy has improved our knowledge of the behaviour of different tumour histologies and opened the potential for risk-adapted individualized treatment approaches. To date, studies have demonstrated that percutaneous ablation is an effective therapy with acceptable outcomes and low risk in the appropriate clinical setting. Although partial nephrectomy (PN) is still considered the standard treatment for SRM, percutaneous ablation is increasingly being performed and if long-term efficacy is sustained, it may have a wider application for SRMs after biopsy characterization.

Keywords: Biopsy; focal therapy; renal tumours; surveillance

Introduction

The incidence of kidney cancer has been increasing, largely due to the increased use of imaging and the incidental detection of small renal masses (SRMs) (1,2). Not all SRMs are malignant and those that are, demonstrate heterogeneous pathology and behaviour. These features are not reliably predictable with conventional imaging or biomarkers (3). Early efforts to predict malignant pathology and tumor grade using tumor size and other clinical variables (such as age, gender, smoking history and presence of symptoms) are inaccurate, which limits their clinical utility (4,5). The presence of an enhancing renal lesion in CT imaging has traditionally been considered by the majority of the urologists as sufficient indication of surgery. Increasingly, renal tumour needle biopsy is being performed to characterize SRMs to assist in treatment decisions, as not all are malignant (3,6). Active surveillance and focal therapies are increasingly being considered as alternatives to partial and radical nephrectomy in selected patients. A multidisciplinary approach with experienced urologists, pathologists and radiologists is optimal for an accurate diagnosis and individualized renal mass management.

Renal mass biopsy (RMB) techniques, accuracy and safety

At least 20% of the SRMs are benign and these do not always require treatment. RMB/renal tumour biopsy (RTB) is increasingly being used to characterize renal masses.

RMB techniques

Several developments have been made in biopsy technique, imaging approaches, pathology evaluation and genetic testing in a way to improve renal mass characterization.

RMBs are performed as an outpatient or short-stay procedure using ultrasound or CT guidance with local

anesthesia (7). MRI could also be used. There is no data to suggest superiority of one image guidance method over another (8). In experts' opinion, the imaging should be chosen based on the availability, expertise, patient and tumour characteristics (9). Preference should be given to US to limit exposure and cost (9).

The technique has evolved, but basically RMBs are performed using two methods; fine-needle aspiration (FNA) and needle core biopsy. The accuracy of FNA for the diagnosis of malignancy is inferior to that of core biopsies (10). Cytologic evaluation only permits diagnosis of tumour histology and grade in a minority of cases (11,12). Some authors imply that the two techniques can provide complementary results, increasing diagnostic rates and accuracy but we rely on needle cores (9,13).

We believe that co-axial sheathed needles are superior for needle core biopsies although there is variation in practice (7). The coaxial technique allows multiple biopsies through one tract with an increased likelihood of sampling the tumour with each pass, although the site sampled will not be as geographically distributed throughout the tumour. The use of the coaxial techniques appears to reduce the risk of tumour seeding along the needle track (14,15). The use of an 18-gauge needle is associated with low morbidity and provides sufficient tissue for diagnosis in the majority of cases. Needle cores provide a greater diagnostic yield and better accuracy for diagnosing malignancy and histological type in comparison with FNA. There is no consensus about the ideal number and location of core biopsies. It is accepted that at least two good quality cores (non-fragmented, >10 mm in length) should be obtained, and necrotic areas should be avoided to obtain a diagnostic specimen in up to 97% of cases (8,9,11,12,14-16). Regardless of the number of cores, the quality of the tissue retrieved seems to be the most important variable for biopsy success (17). Further studies are needed to define the ideal number, site and length of RMB, mostly based in the new upcoming methodologies for tumoral heterogeneity characterization.

RMB accuracy

A diagnostic rate of 80-94% including the identification of renal cell carcinoma (RCC) subtypes has been reported in larger series (6-8). Many studies published before 2001, reported false negative or non-diagnostic rates up to 25% (13,18). Of diagnostic biopsies, a benign diagnosis is obtained in 20-30% of cases.

One of the main concerns about RMB is the rate of non-diagnostic samples. A non-diagnostic biopsy is not a surrogate for benign pathology (8). These could represent samples with insufficient tissue, normal kidney parenchyma, or other situations where the renal mass cannot be described. Rates of non-diagnostic samples range between 0 and 47% and are more frequent in small and cystic lesions (3,8). Several authors have reported higher diagnostic rates for repeat core biopsies (83%) after a first non-diagnostic one (3,6,8,19).

Overall tumour size in particular, including the size of solid components of cystic tumours, and location correlate with diagnostic yield (3,7,20). The risk of cyst rupture with potential local seeding of tumour cells limits the role for biopsy in Bosniak IV cysts with enhanced solid areas (12,21,22). We manage tumours <1 cm in diameter by initial active surveillance until they reach 1 cm before an attempt to biopsy with increased diagnostic rate (8,12,23). Sampling error, tumour necrosis and tumour heterogeneity are responsible for most false-negative biopsy results (22).

Although tumour heterogeneity is a potential cause of sampling errors, concordance with dominant surgical pathology has been reported in approximately 100% of cases, and erroneous diagnosis of benign *vs.* malignant after adequate biopsy specimen is now rare (3,14,24). The infrequent hybrid tumours are difficult to adequately define using RMBs (25). It is possible to miss the malignant portion of a hybrid tumor and misclassify the lesion as being benign. Hybrid tumours have previously been reported to be present in up to 18% of oncocytomas diagnosed following RMB, although this has not been our experience (25,26).

The relative accuracy of grading renal cell cancers with percutaneous biopsy is controversial, with reported accuracy rates ranging from 43% to 75% (14). Grade concordance at surgery has traditionally been reported as low when evaluated grade by grade (12,27). However, in our recent series of 496 biopsied masses, when Fuhrman grades are pooled into low- and high-grade, the concordance is as high as 96.1% (6). Millet *et al.*, also found an increase in concordance and accuracy when combining low and high Fuhrman grades (as high as 93%) (27). Intratumoral grade heterogeneity has been reported in 5-25% of renal tumours, this may lead to an underestimation of the genetic complexity of a tumor when single-biopsy procedures are used (28).

Experience is required for pathological interpretation of biopsy specimens (29). Another limitation of RMB may occur in patients with multifocal renal lesions (both unilateral and bilateral) with possible discordance between different

358

Leão et al. Role of biopsy and focal therapy for small renal mass

tumours. Knowing the histology of one tumour does not necessarily reveal information about the histology of the other synchronous tumours (30). Thus, in patients with multiple lesions in which RMBs are being considered, each lesion should be biopsied to identify their respective histology.

Experienced multidisciplinary centers are more likely to achieve diagnostic outcomes with biopsy (3). Multidisciplinary expertise in urology, pathology and imaging is crucial to exploit the diagnostic yield of renal tumour biopsies.

At a genomic level, Gerlinger et al., using whole-exome sequencing, found that somatic genetic mutations are not present ubiquitously within the primary tumor in cases with metastases (31). It is not surprising that intratumoral heterogeneity might be of concern clinically but the rate of clinically significant genomic alterations is still undetermined (32).

In the new era of personalized medicine, we believe that intratumoural heterogeneity is matter of concern. Potential tumour heterogeneity presents a considerable therapeutic challenge. A single tumor biopsy, currently the standard of tumor diagnosis, despite the high diagnostic rate, may not be representative of the landscape of genomic abnormalities in a tumour. Further studies and new markers will help us understand the role of heterogeneity in renal masses in treatment and follow-up.

Alternatives to needle biopsy are an appealing concept. New biomarkers and fluid biopsy (using blood and urine) are exciting prospects. Recently, Morrissey et al., presented data updating their experience with the clinical utility of the urine biomarkers, aquaporin-1 (AQP1) and perilipin-2 (PLIN2), to screen for RCC (33). Elevated AQP1 and PLIN2 levels are associated with the presence of RCC and have potential utility in both general population screening and the SRM management setting to distinguish malignant from benign (33). Frantzi et al. reported a marker model based on urinary peptides, as a tool for the detection of RCC in selected patients at risk (34).

Safety

Major complications of biopsy are rare (<1%). A small amount of bleeding (minimal perirenal and subcapsular hematoma) is common (85% to 91%) based on post-biopsy CT imaging but haemorrhage necessitating blood transfusion is rare (13,18). The risk of bleeding appears to be greater with larger (<18-gauge) needles. Possible tumour seeding along the needle track is the greatest concern and is frequently raised by patients and physicians based on older literature and internet sources. However, only few cases of tumour seeding along the needle track have been reported and all were prior to 2001, probably due to improved techniques trough a coaxial guide or cannula (35-38). Other potential complications of RMB include infection, pneumothorax (<1%), and arteriovenous fistula (7).

Indications for RMB

The use of renal biopsies was historically indicated to diagnose secondary, metastatic renal tumours as well as benign non-tumour pathology such as renal abscess (28).

There is increasing acceptance in many centres that RMB should be offered to most, if not all (as we do) patients presenting with a SRM including those who are potential candidates for surgery or ablative therapy (pre-treatment) as well as active surveillance (39,40). Other indications include post ablative therapy for suspected recurrence, confirmation of a complete ablation and RCC subtype characterization of the primary in the setting of metastatic disease to select the optimal biological systemic therapy (particularly when a cytoreductive nephrectomy is not indicated). The role for biopsy of larger localized tumours (> T1b) is controversial but may be used increasingly when partial nephrectomy (PN) is being considered to rule out high grade tumours with theoretically higher risk of local recurrence and the not so rare, large oncocytoma or fat-poor component AML.

There are few contraindications for RMB. The only absolute one is un-correctable coagulopathy. Relative contraindications for RMB might include those in patients with short life expectancy who are not candidates for any surgical, ablative or medial treatment, as the results would not alter the management strategy.

Impact of RMB in clinical management

Treatment decision making for SRM's is an increasingly frequent and challenging clinical problem. The selection of the optimal treatment modality is based on patient age, clinical assessment of patient comorbidities and tumour characteristics (3). Non-adopters of routine RMBs have long argued that results will not affect the clinical management. However, recent study results suggest that this is not the case.

RMB can decrease the SRM surgical and ablation rate if benign disease is observed. Despite improvements in imaging, benign lesions cannot be accurately identified.

Frank *et al.* verified that 30% of renal lesions <4 cm that were removed by surgery (between 1970 to 2000) were benign (41). Rothman *et al.* proved that among patients with localized renal lesions, 84% of renal masses with <4 cm in size are low grade lesions (42). In the Toronto cohort, we have demonstrated that nearly 41% of our cohort avoided definitive treatment following biopsy either because they were found to have a benign tumor, favourable histology for active surveillance or because the RMB confirmed the presence of metastatic disease of another primary origin. Similarly, Maturen *et al.* have shown that biopsies can significantly impact clinical management in 60.5% of their cohort, which was defined as a change between surgery and no surgery (43). Oncocytomas and fat-poor angiomyolipomas with component are lesions that can frequently be misdiagnosed on imaging (27,44,45). In other series of percutaneous biopsies, surgery was avoided in 16-17% of patients after a histologic benign diagnosis (11,12,14).

Patients' life expectancy and performance status (Charlson Comorbidity Index) are predictors of overall survival (OS) (46). Thus, the perception that active treatment for SRMs may not improve OS as led to the development of conservative and minimally invasive treatments for selected elderly and surgically high-risk patients (47). For these patients, the characterization of their renal lesions is crucial and RTB can provide useful information.

RMB are also very useful for active surveillance protocols. The concept of active surveillance arose from the knowledge of the natural history of renal masses. In general, small low-grade lesions are indolent and non-harmful in the short term at least. The active surveillance protocols are built on tumour kinetics concept where non-growing or slow-growing tumours are amenable to follow-up with abdominal imaging and symptom evaluation. However, although tumour kinetics provide important information, the assessment of growth rate alone is not sufficient to determine malignancy. We have demonstrated that the initial growth rates of histologically benign and malignant lesions are not significantly different (48).

Minimally invasive treatments, such as radiofrequency ablation (RFA) and cryoablation (CA), are frequently used for SRMs in older patients with comorbidities and high surgical risk. Pre-ablation renal biopsy is often the only source of pathology in patients undergoing thermal ablation of a SRM. We recommend RMB before the treatment decision to reduce the risk of unnecessary ablation. Pre-ablation biopsy was shown to have a high diagnostic yield of 94.2% in a multicentre series of RFA (49,50). Routine post ablation biopsy is not consistently done, however persistence of viable tumor after the procedure is possible and imaging may not detect viable tumor after thermal treatments (51,52). Weight *et al.* demonstrated that 46.2% of renal tumours with a post-ablation positive biopsy after RFA exhibited no enhancement on post-treatment CT or MRI (52). These results should stimulate urologists to define protocols for thermal ablation where pre-ablation and post-ablation biopsies are considered to monitor treatment success.

Another indication for RMB with potential impact is in metastatic RCC. The use of targeted therapies has increased the interest in renal tumours histologic characterization. The new era of targeted therapy enable urologists and medical oncologists to precisely target oncologic disease based on their histologic and genetic features. It is known that 20-30% of RCC present with metastatic disease and similar proportion of patients will develop metastases after surgical treatment of localized disease (53). Percutaneous biopsies can assess the presence of adverse prognostic factors and the histologic subtypes, both useful for selecting specific systemic treatment. Targeted therapies demonstrate different response rates in different histologic subtypes. Sunitinib and sorafenib showed low clinical responses when treating papillary lesions, though the efficacy of mammalian target of rapamycin (mTOR) inhibitor, temsirolimus, may be more effective among non-clear cell lesions and papillary subtype than in clear cell lesions (54,55).

We support adoption of RMB in the management of all solid, contrast enhancing SRMs (3).

Potential for histological, molecular and genetic characterization of renal tumours using biopsy material

Relevant information from RMBs with respect to biological aggressiveness is of great potential clinical value when making treatment recommendations. Sarcomatoid de-differentiation or histological necrosis correlates with decreased recurrence-free survival (56,57). It has been known for some time that carbonic anhydrase IX has prognostic implications for patient with localized and metastatic disease (58). However, subsequent advances in translational research have enabled increasingly relevant information from tissue sampling. Diagnostic and prognostic information can be obtained not only with immunohistochemistry (IHC), cytogenetic and molecular

analysis but also gene expression profiling (3).

An IHC antibody panel, including CD10, parvalbumin, a-methylacyl-coenzyme A racemase (AMACR), cytokeratin 7 (CK7), S100A1, cathepsin K, and carbonic anhydrase IX (CAIX) and others, seems to be the most promising (3). Some other studies have used RNA based assays; this molecular diagnostic algorithm increased the overall accuracy for histotype diagnosis from 83.3% to 95%, with sensitivity and NPV for diagnosing the clear cell variant at 100% (59).

Fluorescence in situ hybridization (FISH) studies, analyzing chromosomal abnormalities have shown to improve the accuracy of IHC. The addition of cytogenetic information to histology alone increased to 94% the diagnosis accuracy (59).

Several molecular and genetic tissue markers have been investigated as potential prognostic factors for RCC, including markers typically associated with renal cell carcinogenesis and progression [von Hippel-Lindau, hypoxia-induced factor 1 alpha (HIF-1α), VEGF, CAIX, pS6, phosphatase and tensin homolog] and markers described in other malignancies (p53, Ki67, CXCR3, CXCR4, matrix metalloproteinases 2 and 9, IGF II mRNA binding protein, epithelial cell adhesion molecule, vimentin, fascin, livin, survivin) (60). Microarray technology has demonstrated some ability to differentiate tumours by gene expression profiling (61). There is evidence that gene-expression profiles obtained with high-throughput microarray technology can identify histologic subtypes of RCC and predict clinical outcomes of the disease. Lane *et al*. recently identified a 44-gene expression profile that was able to distinguish two groups of ccRCCs with significantly different clinical behavior (62).

Overall, the results of studies with available molecular and genetic tissue markers are promising.

Clinical nomograms and their utility in SRM management

Clinical nomograms have been proposed to predict SRM malignancy prior to surgery as a substitute for RMB. Combining individual descriptors of the nephrometry score with patient characteristics (age, gender), Kutikov *et al*. developed a nomogram that could accurately define malignant RCC histology and high-grade features (5). Recently externally validated, these models represent the most accurate preoperative predictors of malignant potential of localized renal tumours to date, and their

accuracy for predicting tumor grade may match that of percutaneous core biopsy (63,64). Although early efforts have been encouraging, the role of statistical modeling for risk prediction during AS is likely to evolve and expand in the future (65).

Focal therapy for SRMs

During the last 20 years, minimally invasive and nephron sparing surgical approaches have become widely available. PN, more commonly done laparoscopically or robotically, remains the gold standard treatment for cT1 SRMs that are RCC (SRM^{RCC}). However, focal ablative therapies, CA and RFA are increasingly used. Microwave ablation, laser ablation and high-intensity focused ultra-sound are alternatives energy sources for ablation but are generally considered experimental techniques.

Patients considered candidates for percutaneous image-guided renal tumor ablation are typically evaluated jointly by an interventional radiologist and a urologist in our centre. We regularly perform pre-ablative biopsy and up to 37% of biopsied SRMs in this setting are benign oncocytomas or lipid-poor angiomyolipomas (41,66). In addition, pre-ablative biopsies can provide the interventional radiologists with a better understanding of how the patient will tolerate the ablation, the optimal position for ablation, the best percutaneous approach to the lesion, and how much IV sedation and analgesia might be required (67).

There is general consensus that ablative techniques are ideal for many SRM patients who are unfit for surgery, who are not candidates for active surveillance or who prefer these methods. Presently, the European Association of Urology (EAU) guidelines state that no recommendation can be made for RFA or CA due to the low quality of available data (19). The American Urological Association (AUA) guidelines state that ablation in general should be offered as an option but is not as a standard for high-risk patients (68). General limitations for focal ablative techniques are lesion dimensions (success is inversely related to size), lesion location (proximity with abdominal organs or vessels) and patient morbidities (malformations limiting access to the lesions, coagulopathies).

The advantages of CA include real-time imaging of the therapeutic ice-ball, uniformity of the ablation zone, the use of multiple probes simultaneously, outpatient therapy and repeat therapeutic cycles at the same setting. It is relatively safe which has encouraged the acceptance of this modality as an alternative to PN (19).

CA is performed using either a percutaneous or a laparoscopic-assisted approach (under vision) with no difference in terms of overall complications (69). In a survey of 64 institutions performing ablative procedures for SRMs, Patel *et al*. identified laparoscopic CA as the most commonly performed ablative procedure (70). However, the percutaneous approach is associated with a shorter hospital stay and less morbidity (69).

Local disease control occurs in 85% to 99% of cases (lower than with PN) (71). The overall complication rate after laparoscopic CA ranges from 10% to 20%, and are generally minor. Bleeding (5%), urinary leakage (<0.5%) and adjacent organ injury (0.6%) are reported complications (72-77). Renal function is generally not affected by CA (72,73). Compared to PN, CA is associated with shorter operative times, less blood loss, shorter length of hospital stay but a higher risk of local and metastatic disease progression (78).

Percutaneous CA is generally done with CT guidance but US can also be used. Local control ranges from 84% to 97% (72,75,79,80). The results for local disease control are similar to CA techniques. Complication rates are around 20% (Clavien I and II) and are usually bleeding and hematoma (81). As in the laparoscopic approach, with percutaneous CA, measurable renal function is expected to be unaffected (82). One advantage of percutaneous ablation relative to laparoscopic is lower cost (83). Patients submitted to CA are followed by CT imaging with local recurrence or residual lesions appearing as enhancing lesions (84).

Recent data demonstrate that CA is a reasonable option for older patients, patients with several morbidities, solitary kidney or renal impairment, or patients in whom surgery is not felt to be feasible. Additional indications for CA are treatment of local recurrence, *de novo* tumours following ipsilateral PN or even metastatic lesions (85,86).

Zlotta *et al*. first used RFA in RCC patients in 1997, and demonstrated that this technique could be performed without damage to the surrounding healthy kidney (87). Since then, this methodology is increasingly used in urology departments and is currently the most commonly used and studied mode of ablation (67). RFA can be performed laparoscopic or percutaneously (guided either by US or CT) and is usually recommended for patients with lesions <3-4 cm, according to the EAU and AUA guidelines respectively, although some authors have reported successful treatment for pT1b lesions (88). There are no difference in terms of complication rate and type when comparing RFA performed laparoscopically with the percutaneous approach. RFA complications are generally minor but occur in up to 29% of patients (19).

As in other ablative techniques, clinician concern is related to the ability of these methods to achieve good oncological outcomes. A meta-analysis showed a 12.3% risk of local recurrence after RFA (89). However, more recent work shows a better oncological outcomes with local recurrences ranging from 2.5% to 9% for lesion <4 cm (90,91). A systematic review by Katsanos *et al*. showed that RFA of SRMs produces oncologic outcomes similar to nephrectomy and it is associated with significantly lower overall complication rates and importantly, less decline of renal function (92).

Recently RFA has been reported for the management of cT1b lesions with local control highly dependent on both tumour site and location (93). RFA is not recommended for central tumours with contact with the hilum, vessels or ureter due to heat sink effect (90).

Cryotherapy vs. radio frequency ablation

Two published studies comparing RFA and CA did not show significant differences in OS, cancer specific survival or recurrence-free survival (94,95). When considering local recurrence-free survival at five years, one study reported benefits for RFA and the other benefits for CA (94,95).

RFA is known to be effective in the treatment of small, peripheral renal masses (96,97). In contrast, CA appears more effective with tumours >3 cm or extending centrally into the kidney, though at the cost of increased complication rates (98).

A recent study evaluated the clinical outcomes of PN, percutaneous RFA, and percutaneous CA for the treatment of cT1 renal masses (93). Local control was similar among the three treatment groups, metastases-free survival was inferior for RFA, and OS was superior for PN. For patients with cT1b renal masses, local control and metastases-free survival were similar for PN and CA patients and OS favored PN patients (93). Kunkle and Uzzo reported that 12.9% and 5.2% of patients experienced local recurrence of their T1a tumor after RF ablation and CA, respectively, suggesting RF ablation to be the superior modality (89).

Microwave ablation is an experimental technique. It creates kinetic energy that is transformed into heat, leading to coagulation necrosis and cell death (99). Some studies have been published with success rates varying from 62% to 100% (100-103). In the Guan *et al*. study, a comparison between microwave ablation and PN showed that blood

362

Leão et al. Role of biopsy and focal therapy for small renal mass

loss, complications and postoperative decline of renal function is significantly better in the microwave ablation group (102). Recurrence-free survival at three years is not statistically different although the rates were 90.4% for microwave ablation and 96.6% for PN (102). Castle *et al.*, reported intra-procedural complications in 20% of patients, post-procedural complications in 40% of patients, and recurrence in 38% of patients followed to 17.9 months on average (101).

Laser interstitial thermotherapy, another experimental approach, utilizes an optical fiber inserted with US, CT or MRI guidance (104). Two different types of lasers have been used, Nd:YAG laser or diode laser, and both showed feasibility but further studies are needed for renal tumour treatment.

High-intensity focused ultrasonography (HIFU) is a therapeutic modality that induces heat by the absorption of focused ultrasound waves within targeted tissue, inducing cellular necrosis. Currently, there are two ways to perform HIFU treatment, extracorporeal or intra-corporeal. In contrast to CA and RFA, extracorporeal HIFU has the advantage of being a non-invasive treatment. It is also theoretically able to induce coagulation necrosis without damage in the surrounding healthy renal parenchyma and skin, because US beam intensities outside of the focal zone are much lower (105). There are no major complications related to HIFU (106). Marberger *et al.* performed a clinical phase II study which demonstrated that extracorporeal HIFU only covered 15-35% of the targeted lesion (107). Häcker *et al.*, also showed that the size of ablated lesions never reached the targeted volume (77). With intra-corporeal approach, different protocols have been used. Technical improvements have resulted in ablation zones reaching about 90-100% (108). A recent review of HIFU for RCC identified several limitations including technical and anatomic difficulties in delivery of HIFU beams to an SRM. Tracking the lesion during treatment showed non-uniform ablation, and clinical efficacies of only 57% to 67% (109). Additional technical advancements are necessary before HIFU is adopted in the treatment of SRMs.

Finally, the current limitations encountered when comparing RFA to CA also apply to the assessment of the newer ablative technologies.

Conclusions

SRMs are a heterogeneous group of benign and malignant entities (3). Although clinical judgment remains important, a risk stratification algorithm to help direct management

following RMBs has been proposed (110). Halverson *et al.* have demonstrated that biopsies were 96% sensitive and 100% specific in correctly assigning patients to intervention versus active surveillance. However, longer prospective studies will be required to validate this strategy. Despite new clinical evidence, there is no standard protocol for RMB. Generally, local practice patterns and research interests determine its use. Specific protocols for disease diagnosis, prognosis and follow-up are needed. Different protocols should address patient's clinical characteristics, histologic and molecular tissue characterization and treatments done.

With regards to tumour sampling, further research is needed to define the optimal number of cores and their location with an optimal biopsy pattern. It is crucial to obtain samples that allow a reliable and accurate evaluation of the tumour histology and grade and this should be addressed in future clinical research.

There are insufficient studies comparing outcomes among PN, RFA, and CA patients. Current AUA and EAU guidelines suggest the use of tumour ablation approaches in patients with several comorbidities, patients with genetic predisposition to develop multiple tumours, patients with bilateral tumours or solitary kidney.

Genetic and epigenetic studies are the next steps in tumour tissue evaluation. The ability to predict disease recurrence and determine disease aggressiveness is the key in the new era of personalized medicine. Molecular patterns within specific histological subtypes could soon be used to predict likelihood of recurrence (62). The capacity to stratify patients according to their disease phenotype will empower us to prescribe them the best possible and updated health care, ranging from active surveillance to targeted therapy passing through invasive and minimally approaches.

Known tumour suggests that a single biopsy specimen may not be representative of the landscape genomic alterations in a tumor. Probably the best future approach is to determine and identify baseline and common mutations in the stem of the phylogenetic tree of renal tumours.

Reliable diagnostic and prognostic serum and urine markers for RCC would greatly straightforwardness screening and management of patients with renal tumours by affording important diagnostic and prognostic information with a completely non-invasive approaches.

Footnote

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364

Leão et al. Role of biopsy and focal therapy for small renal mass

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366

Leão et al. Role of biopsy and focal therapy for small renal mass

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Active surveillance and focal therapy for low-intermediate risk prostate cancer

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Abstract: Low risk and many cases of low-intermediate risk prostate cancer, are indolent, have little or no metastatic potential, and are not life threatening. Major advances have been made in understanding who these patients are, and in encouraging the use of conservative management in such individuals. Conservative management incorporates the early identification of those 'low risk' patients who harbor higher risk disease, and benefit from definitive therapy. Based on the current algorithm of PSA followed by systematic biopsy, this represents about 30% of newly diagnosed low risk patients. A further small proportion of patients with low risk disease demonstrate biological progression to higher grade disease. Men with lower risk disease can defer treatment, usually for life. Men with higher risk disease that can be localized to a relatively small volume of the prostate may be candidates for focal, prostate sparing therapy. The results of active surveillance, embodying conservative management with selective delayed intervention for the subset who are re-classified as higher risk over time based on repeat biopsy, imaging, or biomarker results, have shown that this approach is safe in the intermediate to long term, with a 1-5% cancer specific mortality at 15 years. Further refinement of the surveillance approach is ongoing, incorporating MRI, targeted biopsies, and molecular biomarkers.

Keywords: Active surveillance; focal therapy; low risk prostate cancer; minimally invasive; conservative management; biomarkers

Introduction

The problem of prostate cancer overdiagnosis and overtreatment emerged shortly after PSA testing became widely adopted in North America and Europe beginning in the late 1980s. Enthusiasm for screening, despite evidence of overdiagnosis, continued unabated until 2012, when the US Preventive Services Task Force published a level D recommendation against screening (1), followed by several other respected national health policy organizations, including the Canadian Task Force on the Public Health Exam (CTFPHE) (2). The use of PSA for prostate cancer screening and early detection has declined over the last few years, reflecting the impact of these recommendations. Nonetheless, screening is still highly controversial.

The recommendations against PSA screening were largely due to concerns about overdiagnosis and overtreatment of non-clinically significant disease. As a result of these recommendations, the role of radical intervention for low risk cancer has been re-evaluated, and conservative management for low risk patients has been increasingly adopted by clinicians. The two approaches that reduce overtreatment and its attendant risks of adverse quality of life effects are active surveillance and focal therapy. They are complementary. Both share the principle that tissue preservation is important when it is possible. Men with lower risk disease can be managed with active surveillance, and defer treatment, in most cases for life. Men with higher risk disease that can be localized to a relatively small volume of the prostate may be candidates for focal

Table 1 Gleason 3 lacks the hallmarks of cancer

Characteristic of cancer	Gleason 3	Gleason 4
Expression of pro-proliferation embryonic, neuronal, haematopoietic stem cell genes, EGF, EGFR (7,8)	Not present	Overexpressed
AKT pathway (7)	No present	Aberrant
HER2/neu (8)	No present	Amplified
Insensitivity to antigrowth signals such as cyclin D2 methylation, CKDN1β (9-13)	Expressed	Absent
Resistance to apoptosis: BCL2 (14)	Negative	Strong expression
Senescence (15,16)	Present	Absent
TMPRSS2-ERG (17-20)	ERG normal	Increased
Sustained angiogenesis: VEGF (21)	Expression low	Increased
Other proangiogenic factors and microvessel density (18)	Normal	Increased
Tissue invasion and metastasis markers (CXCR4, others) (11)	Normal	Overexpressed
Clinical evidence of metastasis mortality (22,23)	Virtually absent	Present

therapy. The rationale and results of these two approaches will be reviewed in this article.

The natural history and molecular biology of low grade prostate cancer

Prostate cancer develops with age in the majority of men, including those from all races and regions. In Caucasians, the chance of harboring prostate cancer is approximately the same as one's age; thirty percent of men in their 30's, 40% in their 40's, 80% in their 80s (3). Most of these are microfoci (<1 mm^3) and low grade, particularly in younger men. The high prevalence of microfocal prostate cancer has been confirmed in autopsy studies of Caucasians, Asians, and other ethnic groups going back more than 50 years. A recent autopsy study in Japanese and Russian men who died of other causes showed that overall 35% of both groups had prostate cancer, and 50% of the cancers in Japanese men aged >70 were Gleason score 7 or above (3).

Genetic features of low grade prostate cancer

The two most common histologic patterns of prostate cancer are Gleason pattern 3 and 4. Importantly, as a result of several modifications of the Gleason system, a grade shift has occurred over the last 20 years. Many cases called Gleason 3 prior to 2005 are now called Gleason 7, particularly those with cribriform pattern histology. Thus Gleason 3 today is more 'benign' than in the past (4). The molecular hallmarks of cancer differ profoundly between

pattern 3 and 4, and represents an important basis for the dramatically different approach taken to these two types of cancer (5,6). The six original hallmarks of cancer described by Hanahan and Weinberg include unlimited replicative potential, sustained angiogenesis, local tissue invasion, insensitivity to antigrowth signals, metastasis, and replicative self-sufficiency. More recently, de-regulated cellular energetics and evasion of immune destruction have been added to this list. The genetic pathways responsible for these hallmarks of malignancy have been worked out in detail (*Table 1*). The Gleason score has a remarkable ability to disaggregate prostate cancer between genetically relatively normal and abnormal cells. There are many examples of this. Genetic pathways mediating apoptosis resistance, angiogenesis and the development of other pro-angiogenic factors, genes involved in regulating cellular metabolomics, and metastasis and invasion processes, are similarly overexpressed in Gleason 4 and normal in 3 (7-18,21,24). Proliferation pathway associated genes, including AkT and HER2neu, are expressed normally in Gleason 3 and abnormally in Gleason 4 (*Table 1*). There are exceptions; in particular, both pTEN (19) and TMPRSS2-ERG (20,25), commonly up-regulated and present respectively in most Gleason 4s, are altered in a significant proportion of Gleason 3. The likelihood of a pTEN deletion is much higher in Gleason 3 from a prostate with co-existent Gleason 4, indicated that pre-histologic genetic changes occur. It is not surprising that some genetic heterogeneity exists within a single histologic pattern. However, these isolated genetic alterations do not appear to

translate into an aggressive phenotype, with rare exceptions.

Metastatic potential

Several large clinical series have reported a rate of metastasis for surgically confirmed Gleason 6 (where there is no possibility of occult higher grade cancer co-existing in the prostate, and accounting for the metastasis) that approaches zero (22,23). Occult higher grade cancer is present in about 25% of patients whose biopsy shows only low grade cancer, and this likely accounts for the prostate cancer deaths reported in series of low risk patients managed conservatively

An alternative explanation for the exceptionally low rate of metastasis after surgery for Gleason 6 cancer is that the intervention is highly successful, and alters the natural history of the disease. However, if so, a reasonable expectation is that a few of the Gleason 6 cancers would have micro-metastasized prior to surgery, or to had a local recurrence with subsequent metastasis. This is rarely seen, if ever. An analogy is the results of surgical management of basal cell carcinomas of the skin, which are almost universally cured by surgical resection, and yet may become lethal if neglected. Further, if resection of a small basal cell carcinoma of the skin had the same effects on quality of life as a radical prostatectomy, dermatologists would plausibly also be advocating for conservative management in the 'low risk' cases!

One multi-center study of 24,000 men with long term follow-up after surgery included 12,000 with surgically confirmed Gleason 6 cancer (22). The 20-year prostate cancer mortality was 0.2%. A total of 4,000 of these were treated at MSKCC; of these, 1 died of prostate cancer; a pathological review of this patient revealed Gleason 4+3 disease (Scott. Eggener, personal communication). A second study of 14,000 men with surgically confirmed Gleason 6 disease found only 22 with lymph node metastases; review of these cases showed that all had higher grade cancer in the primary tumour. The rate of node positive disease in the patients with no Gleason 4 or 5 disease in their prostates was therefore zero (23). (In this study patients had, in most cases, a limited node dissection, and perhaps a more thorough resection might have identified more positive nodes; but the message is still unequivocal. Lymph node metastases with Gleason 6 are extremely rare).

Genetic analysis of cancer has demonstrated conclusively that pre-histologic mutations that confer an aggressive phenotype without altering the histology may occur. A recent genetic analysis of multiple metastatic sites from a patient who had extensive Gleason 4+3 pT3a N1 disease resected at age 47, and died 17 years later of metastatic CRPC, reported that the metastatic lesions appeared to derive from a microfocus of Gleason pattern 3 disease, rather than, as expected, from the high-grade cancers elsewhere in the prostate (26). This case report is a challenge to the view that Gleason pattern 3 does not metastasize. However, the report should be viewed in the context that it is a single case report; that the patient had Gleason 4+3 cancer with a small component of pattern 3, not Gleason 6 cancer which metastasized; and histological Gleason pattern 3, particularly when it coexists with higher grade cancer, may harbour pre-histological genetic alterations that confer a more-aggressive phenotype. It is has been proposed that in this case the low grade cancer occurred as a result of clonal re-differentiation from a higher grade cancer that metastasized, resulting in a common genetic phenotype (27). This is the conceptual basis for genetically based predictive assays that disaggregate low grade cancer into low and higher risk groups. Another case report from the same group described a patient on active surveillance who had annual biopsies for 12 years, all of which were negative or contained microfocal Gleason 6 cancer. Five years after the 12th biopsy the patient was found to have metastatic prostate cancer due to extensive Gleason 9 disease on repeat biopsy. Genetic characterization showed a complete switch of the molecular phenotype, indicating that the high grade cancer was unrelated to the original Gleason 6 disease (28).

Several new biomarkers have been approved by the FDA based on their ability to predict co- a higher risk of adverse pathologic findings in low grade prostate cancer patients. These include the Oncotype DX assay (Genome Health) which identifies a panel of genes linked to a more aggressive phenotype (29), and the Prolaris assay (30) (Myriad Genetics), which looks for abnormal expression of cell cycle related genes. The Decipher assay powerfully predicts for the risk of PSA failure after surgery (31). The Mitomics assay identifies the presence of a functional mitochondrial DNA deletion associated with aggressive prostate cancer (32). These tests interrogate the microfocus of Gleason 6 found on biopsy to identify the risk of those cells progressing to higher risk disease, as well as for the presence of higher grade cancer elsewhere in the prostate. That the biomarkers can achieve this confirms the inter-relationship of heterogeneous multi-focal cancers.

These molecular tests, performed on biopsy tissue, predict future biological behavior based on identifying genetic alterations in low-grade cancer cells. An unmet need is to better understand how to integrate the results of genetic biomarker tests and MRI. For example, the optimal management of the patient in whom results are discrepant (i.e., genetic test indicates high risk but MRI is negative) is currently unknown. Both diagnostic approaches are not perfect. MRI can miss small high grade cancers; the molecular assays may over or underestimate risk. These genetic tests are likely to be most useful for the low risk (higher volume of Gleason 6 than very low risk) and low-intermediate risk (small amount of Gleason 4 disease on biopsy) patients who wish a surveillance approach. A study of the impact of the Genomic Health GPS test showed that 25% of patients had their management changed as a result of the test, always in the direction suggested by the test result (33).

Multiparametric MRI is another powerful tool to identify the 'wolf in sheep's clothing', that is, the patient with low grade cancer on biopsy who harbours a large high grade cancer elsewhere in the prostate (34). These occult cancers are usually anterior, and a part of the prostate that is harder to target using TRUS guided systematic biopsies, but easily seen on MRI. One study showed that 100% of cancers >1 cm in men on surveillance who subsequently had surgery were anterior (35).

Appreciating that Gleason pattern 3 has little or no metastatic phenotype has altered our approach to patients with this cancer. Phrases, like 'pseudo-cancer', 'pseudo-disease', 'part of the aging process', and 'pre-cancer', are useful in counseling these men. They are reassuring, and accurately reflect the extremely indolent nature of the disease entity. The capacity to metastasize is not the sine qua non of cancer, and local invasion, which can occur with Gleason 6, does legitimize the term 'cancer' for this disease. However, changing the terminology away from 'cancer', the diagnosis of which has profound emotional and psychological implications for patients, would significantly reassure the patient and derail a headlong rush into aggressive treatment.

Active surveillance is appropriate for men of all ages, including young men (under 55). The benefits of avoiding erectile dysfunction and incontinence are greater in young men, and the risks of second malignancies occurring in the irradiated field as sequelae of radiation are also greater in men with a long life expectancy. About 40% of men in their 40s harbor microfocal have low-grade cancer (36).

Diagnosing this on a transrectal ultrasound (TRUS)-guided biopsy, does not mean that disease progression is inevitable. High volume Gleason 6 also does not preclude expectant management. However, those with high-volume Gleason pattern 3 have a considerably higher risk of harboring higher grade cancer. The volume threshold of Gleason 3 on biopsy at which point higher grade cancer is more likely to be present is not clear; it may be a continuous variable. One group recently identified this as more than 8 mm of total cancer on systematic biopsy (37). The management of patients with higher volume Gleason 6 is to rigorously exclude the presence of higher-grade cancer (based on MRI, targeted/template biopsies, and biomarkers). Patients confirmed to have only Gleason 6, even if higher volume, are unlikely to require treatment.

The benefits of PSA screening has been discounted by health policy bodies such as the USPSTF because of concerns about overtreatment and a high number needed to treat (NNT) for each death avoided. However, many believe that abandoning early detection will result in an increase in prostate cancer mortality. Selective treatment employing active surveillance would result in a decrease in the NNT for each death avoided. Thus, if widely adopted, active surveillance should eventually result in a re-appraisal of the benefits of PSA screening, and a greater acceptance of its value by policy makers such as the USPSTF. The result might be a re-acceptance of PSA screening, earlier identification of those with aggressive disease, lives saved, and an overall reduction in prostate cancer mortality (compared to no screening resulting from the perceived hazards of overtreatment). Less is more!

Who is a candidate for active surveillance? Low risk disease based on biopsy is widely defined as Gleason 6 and PSA <10 ng/mL. Most such newly diagnosed patients are stage T1c. This group includes around 45% of newly diagnosed patients in the USA and Canada, approximately 150,000 men per year. Low risk disease has been stratified into very low and low based on the number of cores, extent of core involvement, and PSA density (38). The Epstein criteria include patients with only one or two positive cores (counter-intuitively this is irrespective of the number of cores), no core with more than 50% involvement and PSA density <0.15. The Epstein criteria were based on those biopsy criteria which predicted for the Stamey definition of clinically insignificant disease (<0.5 cc of Gleason 6 prostate cancer). This definition is stringent, and would exclude many patients with low risk disease who would otherwise be excellent candidates. Informed by the genetic

characterization of Gleason pattern 3 and the clinical experience with Gleason 6, we believe that all 'true' Gleason 6 cancers (that is, without any occult Gleason 4 pattern)have an extremely low risk of metastasis. The major significance of higher volume disease is as a predictor of occult higher grade cancer. Higher volume disease may be manifested as extensive core involvement, a high PSA density, or a large lesion on MRI confirmed to be Gleason 6. In the absence of higher grade cancer, metastasis is exceedingly unlikely. Thus these patients require close scrutiny to preclude as much as possible co-existent higher grade disease, but do not necessarily require treatment in the absence of higher grade cancer. Biological progression to higher grade cancer may occur over time, and is higher volume disease is a predictor for this; but a possible risk of future grade progression should rarely drive current treatment decisions.

A high PSA density (PSA: prostate volume ratio) has been demonstrated in many studies to be a predictor for risk progression. A high PSA density in some surveillance candidates reflects PSA arising from a large occult cancer. Increased caution is warranted in these cases. In particular, this includes young men (age <55 years) who have extensive Gleason 6 cancer on biopsy. In these patients, uncertainty exists about. The risk of true tumour progression over time, as well as the risk of harboring occult high grade disease. It is reasonable to offer these men treatment. Where exactly to draw the line is a matter of clinical judgment.

Race plays a role. African Americans managed with surveillance have a higher rate of risk re-classification, and PSA failure when treated than Caucasian men (39). Black men who are surveillance candidates also have a higher rate of large anterior cancers than Caucasians (40). Japanese men younger than 60 have a lower rate of histological 'autopsy' cancer than Caucasian men. Thus the finding of low-grade prostate cancer in young Asian men is perhaps less likely to represent overdiagnosis. However, black and Asian patients diagnosed with low grade prostate cancer still include a majority of men who have little or no probability of a prostate cancer related-death during their remaining lives. Thus active surveillance is still an appealing option for those who have been appropriately risk-stratified. These higher risk patients are a group in whom improved imaging and biomarkers will likely have a major impact.

Further, the modification of the Gleason system in 2005 has resulted in a decrease in the number of newly diagnosed Gleason 6 compared to 7, and therefore a smaller proportion of prostate cancer patients classified as low risk and therefore fulfilling stringent criteria for surveillance.

There is an increasing recognition that patients with Gleason 3+4=7, where the component of pattern 4 is small (<10%) have a very similar natural history to those with Gleason 3+3, perhaps reflecting the stage migration phenomenon. A recent pathology study showed that men with Gleason 3+ < 5% pattern 4 on biopsy had exactly the same distribution of cancer grades on radical prostatectomy pathology as those with Gleason 3 only (41).

Principles of management

The clinical management of men on AS has evolved. Currently most experienced clinicians use the following approach: Patients have PSA performed every 3 months for the first 2 years, and then every 6 months indefinitely (until infirmity). A confirmatory biopsy must be carried out within 6-12 months of the initial diagnostic biopsy on which cancer was identified. This confirmatory biopsy targets the areas of the prostate that have been shown to harbor significant cancer in patients who are initially diagnosed with Gleason 6: the anterior prostate, base, and apex. These are the areas are typically under-sampled on the initial diagnostic biopsy. If the confirmatory biopsy is either negative or confirms microfocal Gleason 3+3 disease, subsequent biopsies are performed every 3-5 years until the patient reaches age 80, or has a life expectancy <5 years because of co-morbidity. The frequency of biopsy varies widely between groups. Some have performed annual biopsies for many years. This approach has been valuable in establishing the likelihood of biological progression over time, but represents an overly large burden of biopsies. Multi-parametric MRI should be performed on those patients whose PSA kinetics suggest more aggressive disease (usually defined as a PSA DT <3 years), whose biopsy shows substantial volume increase, or who is upgraded to Gleason 3+4 and surveillance is still desired as a management option. Identification of an MRI target suspicious for high grade disease should warrant a targeted biopsy; or, if the lesion is large and unequivocal, intervention.

About one third of patients will be reclassified as higher risk and in most cases offered treatment. The proportion risk-reclassified will depend on the inclusion criteria used for eligibility for surveillance. An inclusive approach, offering surveillance to all patients with Gleason 6 and PSA <15, for example, will include more patients with occult high grade disease than a narrower approach, restricting surveillance to those who meet Epstein criteria. However, the more stringent eligibility denies the benefits of AS to

many men with indolent disease who do not fit the Epstein criteria and thus are discouraged from choosing AS.

Most cases that are upgraded on the confirmatory or initial subsequent biopsy are upgraded based on re-sampling (about 25% of patients). Of those upgraded, more than 85% are upgraded to Gleason 3+4 only (42). In the Toronto cohort, the likelihood of upgrading increased by 1% per year from the time of the confirmatory biopsy (43). This is an estimate of the rate of spontaneous grade progression from Gleason 3 to Gleason 4.

The commonest cause of death in men on AS is cardiovascular disease. Death from prostate cancer is rare. In the most mature surveillance cohort (44), with a median follow-up of 8 years, the cumulative hazard ratio (or relative risk) of non-prostate-cancer death was 10 times that for prostate cancer. To date, the published literature on surveillance includes 14 prospective studies, encompassing about 5,000 men (44-58). Most of these studies have a duration of follow-up that is insufficient to identify an increased risk of prostate cancer mortality as a result of surveillance. For example, a Swedish study reported that the risk of prostate cancer mortality in patients managed by watchful waiting was low for many years, but tripled in patients who survived more than 15 years (59) ('Watchful waiting' meant no opportunity for selective delayed intervention, whereas about 30% of patients in the surveillance series have had radical treatment). In the Toronto experience, 70 patients have been followed for 14 years; 2.5% had late disease progression (with metastasis developing after 7 years), but there is no evidence of a sharp increase in mortality to date. Thus a critical question in this field is what the long term prostate cancer mortality will be beyond 15 years. It will be 5-7 years before the most mature cohorts have a substantial group of patients with more than 15 years of follow-up. *Table 2* summarizes the results of the 10 prospective series. The key outcome measures include the proportion of patients treated, overall, and cause specific survival. Overall, about one third of patients are treated; most series have few or no prostate cancer deaths. In the Toronto cohort, 1.5% has died of prostate cancer; the actuarial 15-year prostate cancer mortality is 5%. In the Hopkins series, which was restricted patients to those with Epstein criteria, and treated all patients with volume progression beyond Epstein, the 15-year CSM was 0.5% (60). Few of the other publications have significant numbers of patients followed more than 10 years.

All groups have relied on systematic TRUS-guided biopsies performed serially, at varying intervals. This technique has significant limitations. TRUS guided biopsy tends to under sample the anterior prostate, apex, and antero-lateral horn. Thus all groups stress the importance of a confirmatory biopsy to target these areas. Since prostate cancer in most cases starts early and takes 10-20 years to reach clinical significance, the delay of 6-12 months in finding occult higher grade cancer is unlikely to alter curability significantly. MRI has an obvious increasing role in the management of AS patients. There are two potential benefits: reassurance that no higher risk disease is present in those with no visualized disease; and, in the subset harboring higher grade disease, earlier identification of this cancer. With respect to the former the negative predictive value is the key metric. This has been reported to be 97% for a group of about 300 surveillance candidates at MSKCC, and similar figure of 95-97% reported by several other groups (61,62). The PPV of an MRI abnormality with a PiRADS (Prostate Imaging Reporting and Data System) score of 4 or 5/5 had a 90% positive predictive value for high-grade cancer. This abnormality is characterized by a lesion with a positive T2-weighted image, with both restricted diffusion and enhanced contrast. These lesions should trigger a targeted biopsy. If confirmed by further studies, this reliability would permit a level of confidence in a negative MRI that would allow it to replace the biopsy. This would decrease the number of men requiring biopsies (a major unmet need) and facilitate early identification of clinically significant disease earlier.

PSA kinetics are currently used as a guide to identify patients at higher risk, but not to drive the decision to treat. Until multiparametric MRI became available, men on AS with poor PSA kinetics (doubling time <3 years) were offered treatment. In the PRIAS multi-institutional AS registry, 20% of men being treated had intervention based on a PSA doubling time <3 years (45). PSA kinetics is sensitive but lack specificity. For example, in a report of the 5 men dying of metastatic prostate cancer in the Toronto cohort, all had a PSA doubling time <2 years (63). In a study of PSA kinetics in a large surveillance cohort, false positive PSA triggers (doubling time <3 years, or PSA velocity >2 ng/year) occurred in 50% of stable untreated patients, none of whom went on to progress, require treatment, or die of prostate cancer, emphasizing the lack of sensitivity of a rapid rise in PSA (64). Vickers, in an overview of all of the studies of more than 200 patients examining the predictive value of PSA kinetics in localized prostate cancer, concluded that kinetics had no independent predictive value beyond the absolute value of PSA (65).

Table 2 Outcomes of AS in large prospective series

Reference	n	Median follow-up (months)	% treated overall; % treatment free at time indicated	Overall/disease specific survival (%)	% BCR post deferred treatment
Klotz et al. (44)	993	92	30; 72 at 5 years	79/97 at 10 years	25% (6% overall)
Bul et al. (45)	2,500	47	32; 43 at 10 years	77/100 at 10 years	20%^
Dall'Era et al. (46)	328	43	24; 67 at 5 years	100/100 at 5 years	NR
Kakehi et al. (47)	118	36	51; 49 at 3 years	NR	NR
Tosoian et al. (48)	769	32	33; 41 at 10 years	NR/100 at 10 years	NR
Roemeling et al. (49)	273	41	29; 71 at 5 years	89/100 at 5 years	NR (31% of 13 RP positive margins)
Soloway et al. (50)	99	35	8; 85 at 5 years	NR	NR
Hardie et al. (51)	80	42	14;79 at 5 years	NR	0%
Patel et al. (52)	88	35	35; 58 at 5 years	NR	NR
Barayan et al. (53)	155	65	20	NR	NR
Ramirez-Backhaus et al. (54)	232	36	27	93 at 5 years	99.5%
Ischia et al. (55)	154	23	19; 45 at 10 years	NR/100	NR
Godtman et al. (56)	439	72	37; 45 at 10 years	81/99.5 at 10 years	86%
Thomsen et al. (57)	167	40	33; 60 at 5 years	NR/100	NR
Selvadurai et al. (58)	471	68	NR; 70 at 5 years	NR/99.5	NR

Active surveillance is an appealing approach for low risk patients, and an antidote to the widely recognized problem of overtreatment. Widespread adoption of surveillance would result in a reduction in the number NNT for each death avoided without the risk of increasing disease mortality. One hopes that a dispassionate re-assessment of PSA screening based on these improved metrics would lead to a re-consideration of the value of prostate cancer screening by organizations such as the USPSTF and the Canadian Task Force on the Public Health Exam. Ongoing improvements in diagnostic accuracy based on multiparametric MRI and genetic biomarkers should reduce the need for systematic biopsies, improve the early identification of occult higher risk disease, and enhance the ability to detect patients destined to have grade progression over time. A minimum standard currently is a confirmatory biopsy within 6–12 months. PSA should be performed every 6 months and subsequent biopsies every 3-5 years until the patient is no longer a candidate for definitive therapy. MRI is indicated for men with a grade or volume increase, or adverse PSA kinetics. Treatment should be offered for most patients with upgraded disease.

Focal therapy

A second, intermediate risk group, are also candidates for a tissue sparing approach. These are patients who either have small, unifocal Gleason 7 cancers, or larger Gleason 6 cancers confined to one lobe. Focal therapy is increasingly being advocated as a minimally invasive, less morbid alternative to conventional treatment. This is consistent with trends in surgical oncology in other tumour sites. Just as breast cancer, once treated routinely with radical mastectomy, is now widely managed with lumpectomy, tissue sparing treatment of small prostate cancers seems rational and appealing.

Focal therapy is based on the concept of the 'index lesion'. Although most prostate cancers are multifocal, many patients have a single substantial lesion; the multifocality usually consists of small foci of low grade cancer scattered throughout the prostate. While the index lesion has not been demonstrated to invariably be the most aggressive lesion, clinical evidence suggests this is usually (although not invariably) the case.

Patient selection is critical to a successful outcome. The ideal patient has an unequivocal solitary lesion on MRI,

confirmed as Gleason 7 on biopsy. Co-existent microfocal Gleason 6 disease elsewhere in the prostate is not a contra-indication. In the absence of the index lesion, the Gleason 6 microfoci would be managed conservatively. Selected small Gleason 8 cancers, in whom the rest of the prostate is normal on MRI, may also be managed in this fashion. The initial experience with focal therapy is impressive. A trifecta outcome, meaning continent, with normal erectile function and undetectable PSA, has been reported in 84%. The trifecta result with surgery and radiation are 40-50% (at centers of excellence) (66,67).

A key principle of patient selection is the use of an accurate technique to identify the presence or absence of aggressive prostate cancer. Uniquely in oncology, prostate cancer has been treated for many years without identifying the site of disease within the gland. Location was not critical if the entire prostate was removed or radiated, or if no treatment was offered. Advances in defining the location of disease now include MRI (68), image guided biopsy (69), and template prostate mapping (70). This has resulted in a reduction in under grading and risk assessment based on needle biopsy. Recent data on targeted biopsies have described a concordance rate of 95% with prostatectomy pathology, compared to 60% with systematic biopsies (69,71). This level of accuracy is paradigm shifting, in the sense that accurate assessment of extent of disease will permit treatment tailored to the location of this disease, rather than complete excision of the gland.

A diagnostic approach based on MRI with targeted biopsy will result in fewer men being biopsied and fewer cores per patient. The volume of disease on the core will increase dramatically as the needle is directed towards the lesion center. The traditional parameters of cancer core length, proportion of involvement, and risk will have to be re-calibrated based on targeted biopsies. For example, a Gleason 6 0.5 cc lesion, corresponding to Stamey's volume threshold for significance, if hit directly, could result in a cancer core length of 10-11 mm, or 75% of a 14-mm core. Based on having clinically insignificant disease by the most stringent criteria, such a patient should be managed with surveillance, but may be dissuaded from doing so by conventional risk stratification systems.

A targeted approach to biopsy based on imaging will also result in fewer men found to have clinically insignificant prostate cancer. Although such men may avoid the side effects of therapy, they still are subject to the anxiety attendant upon a cancer diagnosis, the 'survivor' label, and repeated diagnostic studies. Reducing overdiagnosis, even

if overtreatment is avoided, would be a major public health benefit.

In applying focal therapy, 3 different imaging requirements are present, each with different demands. Imaging is required (I) for patient selection, ie men with low-intermediate risk prostate cancer (Gleason ≤4+3, PSA <20 ng/mL, and ≤T2), with a target lesion confined to one lobe of the prostate; (II) for real time treatment guidance of the therapy to the targeted lesion; and (III) follow-up to confirm no residual or recurrent tumor. Focal therapy emerged as a plausible approach only with the availability of MP-MRI, beginning around 2009, which for the first time made accurate imaging of prostate cancer feasible.

The published series have modest numbers and short follow-up. A systematic review summarized this data (67). The results, summarized in *Table 3*, are as follows. Focal therapy is safe in the short term. The GU and rectal morbidity is low. No prostate cancer deaths or metastases have been reported, likely reflecting the absence of long term follow-up. Oncological outcomes are favorable, with freedom from disease recurrence of 80-85% (using a variety of definitions of recurrence) (*Table 4*). Re-treatment is required in about 10%. A change in treatment was applied in 5%. Longer follow-up is required to validate these findings.

Technique of focal therapy

A variety of techniques have been described, all involving the use of directed energy and image guidance. These include high intensity focused ultrasound (HIFU), MR guided ultrasound, laser ablation, cryosurgical ablation, focal photodynamic therapy, electroporation, various forms of radiation. Ultimately, which of these therapies becomes widely used will be a reflection of precision of treatment, morbidity, cost, and availability and convenience. The principles and methods used with these directed energies have been described previously. The experience with these technologies used for focal therapy is summarized in the table below, in chronological order.

Most of the focal therapy data lacks robust endpoints. In most published studies, follow-up biopsies were usually not systematic, and in most studies the majority of patients were not biopsied. This is a potential source of bias, in that PSA and MRI may misidentify as responders some patients with residual disease. In patients having a biopsy, the rate of positive biopsies ranged from 14% to 50%

Table 3 Types of focal therapy, with pros and cons

Items	HIFU	Laser	Cryo	PDT	Radiation
Energy	Thermal	Photothermal	Disruption of cell membranes, vascular occlusion	Light activated, O_2 dependent	DNA damage
Method	Transrectal	Nd:YAG	Transperineal	Transperineal	XRT, Brachy
Pros	Non-invasive; outpatient; morbidity low; ED 5%; incont 0-10%	Real time MRI thermal monitoring; short hospital stay; no photosensitizer	Real time monitoring; short stay; ED 10-35%; incont 0-5%	Short stay	Short stay Variable treatment intensity
Salvage therapy (RP, XRT)	Yes	Yes	Yes	Yes	Challenging
Cons	Unable to treat large glands; rectal toxicity possible; pre-op cytoreduction	Limited experience; anterior tumors difficult; lack of treatment planning	Anterior tumours, small prostates challenging; cytoreduction; cost	Limited experience; anterior cancers challenging; treatment planning; photosensitizer toxicity	Rectal toxicity; large prostates (brachy); cyberknife: cost
Local failure rate	8-23%	33-50%	4-23%	Variable	Variable
Monitoring	MRI/U/S	MRI thermometry	U/S/thermosensors	MRI/U/S	Variable

Table 4 Oncological results of focal therapy

Author	N	Energy	F/U (months)	BCR-FS (%)	Pos Bx (%)	Recurrence [%]
Durand (72)	48	Cryo	13	98 (Phoenix)	26	6/46 [13]
Bahn (73)	73	Cryo	44	NR	25	4/73 [6]
Ward (74)	1,160	Cryo	36	76 (ASTRO)	26	NR
Truesdale (75)	77	Cryo	24	73 (ASTRO)	45	NR
Onik (76)	48	Cryo	54	92 (ASTRO)	14	7/48 [14]
Lambert (77)	25	Cryo	28	84 (Phoenix)	43	2/25 [8]
Ellis (78)	60	Cryo	15	80 (ASTRO)	40	13/60 [21]
Ahmed (66)	42	HIFU		NR	23	5/42 [15]
Lindner (79)	12	Laser	6	NR	50	2/12 [17]

(*Table 2*). Further, most authors only biopsied the treated area. Biopsies of the untreated area were selective based on mpMRI.

It is predictable that a non-morbid, minimally invasive therapy for a slow growing disease will produce excellent short term clinical results. The key question is that of the long term durability of this therapy with respect to local recurrence and metastasis. This is unknown. The FDA, in a recent attempt to develop surrogate end points to evaluate focal therapy, concluded that neither PSA, biopsy showing absence of upgrading, or MRI changes, fulfilled the criteria for a valid endpoint for confirming the long term benefit of focal treatment. They did not identify a putative marker that would fulfill these criteria. Recently, the FDA has changed its requirements for approval of minimally invasive therapies. These devices must now demonstrate that they are effective and safe for tissue ablation, rather than demonstrating that the outcome of these treatments is equivalent to other therapies.

Although the idea of focal treatment is simple,

the application has many nuances and unresolved issues. Challenges include accurate visualization and characterization of significant cancer foci, establishing rational criteria for patient selection, precise localization of therapy matched to the targeted lesion and accurate and precise direction of the ablative energy into the area to be targeted, and post treatment surveillance strategies. Most importantly, establishing the effectiveness of focal therapy in altering the natural history of low-intermediate risk prostate cancer will require large series with long follow-up. This will take several decades and perhaps more.

Conclusions

Most, if not all Gleason 6 cancers lack metastatic potential. Conservative management in these cases, which represent about 45% of newly diagnosed prostate cancers in a screened population, is warranted as an initial strategy. The objective of management is early identification of occult higher grade cancer and long-term follow-up to identify the minority of patients who exhibit grade progression over time. Several large cohorts with follow-up of 10-15 years have confirmed the safety of this approach. In younger men with extensive Gleason 6 cancer, or those whose imaging identifies an index lesion, treatment may be warranted. Minimally-invasive therapies, including focal therapy based on precise mp-MRI targeting, have an emerging role in this context. With widespread adoption of this approach, the number NNT radically in a screened population for each death avoided will fall substantially. This will enhance the value and appeal of early detection for prostate cancer.

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Footnote

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Hormonal therapy and chemotherapy in hormone-naive and castration resistant prostate cancer

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Abstract: The management of advanced castration resistant prostate cancer (CRPC) has been rapidly changing and is still evolving. In the last years, there has been an increasing knowledge of prostate cancer biology. New therapeutic agents and approaches have been evaluated demonstrating benefits in survival and quality of life in patients with metastatic prostate cancer.

Keywords: Prostate cancer; chemotherapy; hormonal therapy

Introduction

Prostate cancer is the most common tumor in men in industrialized countries and the second cause of death in this population (1). Since the seminal work of Huggins in 1940, castration obtained with androgen deprivation therapy (ADT) has remained the cornerstone of treatment for patients with prostate cancer (2).

ADT comprising orchiectomy or a luteinising hormone-releasing hormone (LHRH) agonist or antagonist, with or without an antiandrogen, is the first line of treatment for men with metastatic prostate cancer (3,4).

ADT is capable of achieving castrate levels of testosterone (≤50 ng/dL), and most patients with metastatic hormone naive prostate cancer initially respond to this treatment, with both tumor and biochemical responses (5). However, the majority of patients will develop resistance to these traditional hormonal approaches and the median time to progression is about 18-24 months (3).

More than 20-40% of prostate tumors that progress on first line ADT may respond to second- and third-line hormonal treatments, suggesting the importance of androgen receptor (AR) signaling in the pathogenesis of prostate cancer (3-7).

AR signaling plays a central role in the biology of prostate cancer and it is necessary for the proliferation and survival of prostate cancer cells (8). The AR is a cytoplasmatic steroid receptor that binds specific ligands, the androgens. Androgenic steroids are the most important AR agonists including testosterone, dehydroepiandrosterone, androstenediol and androstenedione. Testosterone is the major circulating androgen and 90-95% is synthesized in the leydig cells of the testis, while 5-10% is derived from the adrenal glands. In prostate cells, the enzyme 5alfa-reductase converts testosterone to the active hormone, dehydrotestosterone, which binds the AR (9).

In the absence of androgens, the AR is bound to heat-shock proteins and remains primarily in the cytoplasm. Upon activation by androgens, the AR dissociates from heat-shock proteins and translocates into the nucleus, where it binds with co-activators and corepressors to androgen-response elements in the promoter regions of genes to activate their transcription. This interaction determines activation or repression of genes regulating development, differentiation and proliferation of cells (10).

There is increasing preclinical and clinical evidence that the AR remains active in castration resistant prostate cancer (CRPC). The persistence of AR signaling is key to prostate cancer progression and the AR represents the most important therapeutic target in the treatment of this disease, both in hormone sensitive and in castration resistant disease (11-13). The AR binds androgens activating specific DNA sequences with the transcription of androgen correlated genes determining the physiological effects of androgens.

The aim of this review is to summarize the current

knowledge concerning both hormonal therapy and chemotherapy in hormone naive and CRPC patients.

Hormonal therapy and chemotherapy in hormone naive patients

Chemotherapy treatment with taxanes is known to improve survival in metastatic disease in prostate cancer (14,15). Under investigation is whether the addition of hormonal therapies and chemotherapy to local treatments with radiotherapy or surgery, could improve outcomes in the management of high-risk localized prostate cancer.

Hormone chemotherapy in localized disease

High risk prostate cancer is a potentially lethal disease accounting for approximately 15% of all new diagnoses. Despite local treatments, one third of patients with high risk prostate cancer can experience a recurrence of disease and death from prostate cancer (16,17).

Even though the definition of high risk varies widely, the most significant validated predictive factors of disease relapse are clinical tumor stage, PSA level, Gleason score of the diagnostic biopsy specimen and nodal status (18-20).

There is growing interest in a multimodal approach to high risk localized prostate cancer combining local and systemic therapies and in this context chemotherapy may play an important role in disease control. Specifically, the benefit in overall survival in metastatic prostate cancer, has led to evaluation of the use of docetaxel in early stages of disease.

Multiple phase III trials are ongoing to investigate the impact of chemotherapy in the neoadjuvant and adjuvant settings of prostate cancer with or without hormonal therapy. Long term follow-up is required to assess the outcome of patients with localized prostate cancer and just a few of these trials have completed their planned accrual. We report the most important trials investigating the role of docetaxel in the neoadjuvant and adjuvant settings. Additional details about these trials are shown in *Table 1*.

Neoadjuvant trials

The use of neoadjuvant chemotherapy has been evaluated for high-risk prostate cancer. The combination of hormonal therapy and chemotherapy with docetaxel appears to be associated with downstaging of disease and is well tolerated.

There have been several phase III trials evaluating the benefit of chemotherapy prior to surgery associated with hormonal therapy, but currently results of these trials have not yet been reported. Two important current trials evaluating neoadjuvant docetaxel based (and estramustine) chemotherapy is described.

The Cancer and Leukemia Group B is currently conducting a phase III randomized trial (CALGB 90203) which is evaluating neoadjuvant chemotherapy and ADT prior to radical prostatectomy versus immediate radical prostatectomy in patients with high risk localized prostate cancer (stage T1 to T3a NX M0). In this trial 750 patients have been treated with 6 months of androgen deprivation plus eight cycles of neoadjuvant taxane based chemotherapy followed by radical prostatectomy with lymph node dissection compared to surgery alone. The primary end point of the trial is progression-free survival at 5 years (21).

The GETUG 12 trial, a French randomized phase III study, compared four cycles of neoadjuvant treatment with docetaxel and estramustine prior to local therapy plus ADT for 3 years versus local therapy and ADT for 3 years. In this trial 413 patients were included with locally advanced or high-risk prostate cancer treated locally with radiotherapy, in most cases (87%). The study showed a borderline significant improvement in progression free survival (PFS) in the combination arm compared with ADT alone (HR =0.75, 95% CI, 0.55-1.01; P=0.06). Another benefit was shown in PSA response that was significantly higher in the group treated with chemotherapy than in the patients treated with ADT alone. The combination of docetaxel and estramustine had an acceptable l toxicity profile (22).

Adjuvant trials

There are several large trials assessing adjuvant docetaxel based chemotherapy in patients with high risk localized prostate cancer treated with radical prostatectomy. None of the phase III trials available investigating the use of docetaxel-based chemotherapy after surgery, have reported mature results because longer follow-up is required (*Table 1*).

A prospective phase III RTOG 0521 trial was designed to assess the efficacy of adjuvant chemotherapy with docetaxel combined with ADT and radiotherapy. This study is investigating high-risk patients receiving ADT for a total of 2 years plus radiotherapy with or without adjuvant docetaxel chemotherapy for six cycles. The primary endpoint is overall survival (ClinicalTrials.gov; Identifier: NCT00288080).

TAX 3501 was a phase III trial evaluating immediate adjuvant therapy or active surveillance with therapy at the time of biochemical progression. High risk patients were

Table 1 Phase III trials in high risk prostate cancer (modified from K. Fizazi)

Study name	Local tumor treatment	Design of the study	Primary end point	Status
GETUG 12	XRT	Neoadj DE + ADT (3 years) *vs.* local therapy + ADT (3 years)	PFS	Accrual completed
CALGB 90203	RP	Neoadj D (6 cycles) + ADT (18-24 weeks) prior to RP *vs.* RP alone	PFS	Ongoing
RTOG 0521	XRT	Adj D+ ADT (2 years) *vs.* ADT (2 years)	OS	Accrual completed
TAX 3501	RP	Adj D (6 cycles) + ADT *vs.* ADT	PFS	Early accrual termination
VA # 553 CAP	RP	Adj D (6 cycles) *vs.* observation	PFS	Ongoing
AdPro	RP	Adj D (6 cycles) *vs.* surveillance	TTF	Accrual completed
AdRad	XRT	Adj D (6cycles) + ADT *vs.* ADT	PSA progression rate	Ongoing
DANA FARBER	XRT	Neoadj D (6 cycles) + ADT *vs.* ADT (6 cycles)	OS	Ongoing

XRT, external radiation therapy; ADT, androgen deprivation therapy; PFS, progression free survival; RP, radical prostatectomy; D, docetaxel; DE, docetaxel plus estramustine; TTF, time to treatment failure.

randomly assigned to receive observation, androgen therapy with leuprorelin acetate for 18 months or leuprorelin acetate plus docetaxel 75 mg/m^2 every 3 weeks for six cycles. The primary end point was progression-free survival. This trial was prematurely closed due to enrollment difficulties, leaving results insufficient and underpowered to detect significant differences in PFS (23).

A multi arm and multicenter trial conducted by the Medical Research Council called the Systemic Therapy in Advanced or Metastatic Prostate Cancer: Evaluation of Drug Efficacy (STAMPEDE) trial is the largest trial with a multistage design which is evaluating several drugs in combination with hormonal therapy in patients with high-risk localized or metastatic prostate cancer (ClinicalTrials.gov identifier NCT00268476). The purpose of the trial is to compare further treatments, including docetaxel, zoledronic acid, celecoxib, abiraterone, enzalutamide and radiotherapy (only among the patients with metastatic disease) in combination with ADT *vs.* ADT alone. Moreover the study is evaluating whether these second line treatments should be included earlier in the management of prostate cancer. The primary objective of the STAMPEDE trial is overall survival. The intermediate primary outcome is failure-free survival. The study started in October 2005 with five original experimental arms compared to the control arm.

In November 2011 a new arm was introduced assessing abiraterone, prednisone and ADT and accrual was completed in January 2014. Another new arm evaluating radiotherapy to the prostate for newly diagnosed metastatic patients was initiated in January 2013. Recently, in

January 2014 a new arm evaluating the combination of enzalutamide, abiraterone, and prednisone with ADT was initiated (24). The celecoxib arm was closed for lack of sufficient activity at the second interim analysis (25). The arms with zoledronic acid, docetaxel, and zoledronic acid with docetaxel have closed successfully their enrollment in March 2013.

Currently the total number of arms in the STAMPEDE trial is eight.

An interim analysis on survival outcomes in the control ADT arm showed an improvement in overall survival in this cohort of patients with newly diagnosed metastatic disease receiving standard of care therapy compared to previous reports in literature (26).

The final comparative survival results should emerge in mid-2015 and are eagerly anticipated.

Hormonal therapy and chemotherapy in metastatic disease

It has been recently demonstrated that the use of chemotherapy can improve outcomes in patients with metastatic hormone naive prostate cancer. It appears that some patients initiating hormonal therapy may actually be better candidates for cytotoxic therapy at this stage of disease than when their disease becomes castration resistant (27,28).

It has been controversial as to whether or not early chemotherapy in hormone naive patients would be beneficial. There have been arguments for and against this approach. In favor is the idea that attacking *de novo* testosterone independent clones early should allow ADT

to keep prostate cancer in remission longer. In addition, there is the possibility that some patients at the time of progression may be too frail to receive chemotherapy.

Alternatively, ADT may take cells out of cycle and make them less responsive to cytotoxics. The fact that some patients respond for long periods to ADT and never need chemotherapy is the other argument against early chemotherapy.

Since the early 80's several studies tried to clarify these differing viewpoints, investigating the addition of chemotherapy with hormonal therapy in patients with metastatic prostate cancer (29-34).

None of the trials reported positive results, concluding that androgen suppression remains the preferred first line treatment in metastatic prostate cancer and that there was no cytotoxic regimen with consistent activity against hormone-sensitive prostate cancer.

Over the years it has been noted that none of these trials included cytotoxic therapy shown to prolong overall survival in the setting of metastatic CRPC. The availability of active chemotherapy for CRPC has led to renewed interest and investigation of this topic with different agents in hormone sensitive disease.

The trial by Millikan *et al.* included 286 patients and compared ketoconazole and doxorubicin alternating with vinblastine and estramustine in addition to ADT *vs.* standard ADT. They showed no differences in time to progression to CRPC and in median survival between the two groups (35).

Another study conducted by Wang *et al.* compared the combination of mitoxantrone and ADT with ADT alone in 93 patients with locally advanced or metastatic prostate cancer. Overall survival and responses were significantly improved in patients with locally advanced disease treated with mitoxantrone, but patients with metastatic disease did not show benefit (36).

A French trial, GETUG-15, conducted by Gravis and colleagues investigated 385 patients affected by metastatic hormone sensitive prostate cancer with the combination of docetaxel and ADT (28). This study was the first to investigate an agent shown to prolong overall survival in CRPC and the rationale was that this agent should have more efficacies in men with initial metastatic prostate cancer than in patients with the castration resistant disease. Patients received up to nine cycles of docetaxel. At median follow-up of 50 months the majority of patients had what is today considered "low volume" disease and the results showed a significantly improvements in clinical PFS (cPFS)

and biochemical PFS without a significant difference in overall survival.

At ASCO GU 2015 updated results have been presented with a longer follow-up, of some 80 months, showing that the median overall survival was 46.5 months in the ADT arm and 60.9 months in the ADT + D arm (HR =0.9, 95% CI, 0.7-1.2). In a retrospective analysis using the same definition of high volume disease (HVD) as in the CHAARTED trial discussed below, the subgroup of patients with HVD showed a median overall survival of 35.1 months in the ADT alone arm compared to 39 months in the ADT plus chemotherapy arm (HR =0.8, 95% CI, 0.6-1.2). The outcomes in HVD patients were similar to those in the CHAARTED trial, however the trial showed a non-significant improvement in overall survival with ADT + D of about 4 months (37).

An important trial regarding the combination of chemotherapy and hormonal therapy in patients affected by metastatic prostate cancer is the CHAARTED trial (ChemoHormonal Therapy *vs.* Androgen Ablation Randomized Trial for Extensive Disease in Prostate Cancer), the ECOG-led phase III trial presented by Sweeney *et al.* at ASCO in 2014 (27). In this trial, early chemotherapy with docetaxel in combination with ADT in hormone naive metastatic prostate cancer patients was compared to ADT alone. In the study 790 patients received chemotherapy with docetaxel 75 mg/m^2 every 21 days for a maximum of six cycles plus ADT or ADT alone. Twice daily prednisone was not used. This trial emphasized the concept of HVD, in fact patients were stratified as high-volume or low-volume according to the extension of metastatic disease. High volume was defined as visceral metastasis (lung or liver) and/or four or more bone metastases (with at least one beyond the pelvis and vertebral column). At study initiation, only patients with high-volume disease were to be accrued, but the study was amended to also include low volume disease patients. Unlike the GETUG-15 trial, approximately two-thirds of patients in CHAARTED had HVD. The primary endpoint was overall survival while secondary endpoints were time to progressive disease and time to symptomatic progressive disease.

The association of chemotherapy and ADT in this trial was motivated by the hypothesis that testosterone independent cellular clones would be best treated early with cytotoxic chemotherapy (27). Moreover it has been reported in several studies, including that of the SWOG trial of intermittent *vs.* continuous therapy that the presence of high tumor burden with visceral disease and bone involvement beyond the axial skeleton is correlated with

poor prognosis and shorter survival (35,36,38).

In the CHAARTED study, the combination of docetaxel and ADT showed a benefit in overall survival of 14 months compared to ADT alone, with a median overall survival of 57.6 *vs.* 44 months (HR =0.61, 95% CI, 0.47-0.80; P=0.0003). In the HVD group, median overall survival was 49.2 months with docetaxel plus ADT compared with 32.2 months with ADT (HR =0.60, 95% CI, 0.45-0.81; P=0.0006), a 17-month difference in overall survival. In patients with low-volume disease, median overall survival has not yet been reached at the time of the analysis, at a median follow-up of 29 months. The CHAARTED trial results also demonstrated improvement in median time to clinical progression and in median time to the development of castration resistant status.

Of special interest, the median time to clinical progression in the docetaxel plus ADT group was 32.7 months as compared to 19.8 months in the ADT arm (HR =0.49, 95% CI, 0.37-0.65; P<0.0001). In addition, the median time to CRPC was 20.7 months in the combination arm compared with 14.7 in the ADT alone arm (HR =0.56, 95% CI, 0.44-0.70; P<0.0001). Of note, 129/174 (74%) of patients who progressed on ADT subsequently received docetaxel.

The adverse event profile was favorable and lower than previously seen in CRPC trials, reporting 6% of febrile neutropenia in patients receiving the chemo-hormonal regimen. There was one sudden death in the chemotherapy arm. Grade 3 non-hematologic toxicity was low, with 2% allergic reactions and 4% having fatigue. A total of 1% of patients experienced G3 toxicity of sensory nerves and 1% of motor nerves.

There are several critical points about this trial. First of all, the concept of high and low volume disease should be more profoundly considered, as there is evidence of this also from earlier studies from the SWOG (SWOG trials S8894 and S9346) and from the MD Anderson Hospital (35,36,38). This can dramatically change our first line treatment choices for patients with metastatic prostate cancer. Final publications of these data are awaited.

Hormonal therapy and chemotherapy in CRPC patients

CRPC is an aggressive disease that contains heterogeneous types of cells developing a variety of abnormal pathways to survive in a castrate environment. The biological heterogeneity of prostate cancer cells has become clear as there are different clinical subsets of patients, from indolent tumors to those that are aggressive and lethal with multiple metastases. The biological and clinical heterogeneity dictates the different therapeutic options in the management of CRPC.

Heterogeneity of castration resistance prostate cancer

Even though the AR plays a major role in the progression to CRPC, alternative pathways can have a role in stimulating prostate cancer cells, confirming the cellular heterogeneity in prostate cancer (39,40).

Prostate cancer cells can develop alternative AR independent molecular pathways for survival that bypass AR activation, including cancer stem cells, receptor tyrosine kinases and neuroendocrine differentiation (NE) (41). A potential mechanism for survival in the castrate environment is the presence of prostate cancer stem cells that continually supply the cancer cell population, despite therapy. These cells are not affected by ADT and can differentiate into androgen dependent and independent cells, leading to a heterogeneous phenotype of AR (42,43).

Activation of the PI3 kinase signaling pathway is critical for the survival of prostate cancer cells. PTEN is a tumor suppressor and has lipid phosphatase activity that metabolizes PIP3 (phosphatidylinositol triphosphate). The PTEN function is expressed primarily through negative regulation of the PI3K/Akt pathway. PTEN is inactivated in several types of cancers, including prostate cancer. Loss of PTEN function in prostate cancer can occur through several mechanisms, including deletion, mutation and methylation. These events can cause tumor cell survival through selective pressure caused by ADT (44-46).

Another potentially relevant pathway is NE of tumor cells in prostate cancer. The prevalence of NE cells in prostate adenocarcinoma varies from 30% to 100% and they do not express the AR. These cells may develop from a predominantly adenocarcinoma PSA secreting environment under the pressure of ADT. NE cells may contribute to the progression to CRPC through the production of neurosecretory products, such as parathyroid hormone-related protein, the neurotransmitter serotonin, the neuropeptide hormone bombesin, calcitonin, chromagranin A, neurotensin, and thyroid-stimulatory hormone (6,44,45). Patient with predominantly NE or small cell carcinoma should be treated with cisplatin based chemotherapy (47).

Chemotherapy treatment in CRPC

Systemic chemotherapy is one of the options for the treatment of metastatic CRPC. Taxanes represent the class of chemotherapeutic agents that have shown a benefit in terms of overall survival. In particular, docetaxel and recently cabazitaxel have become the currently standard first and second-line chemotherapy agents for the treatment of metastatic prostate cancer patients after ADT failure (14,15,45).

SWOG 99-16 and TAX327 trials are the most important randomized studies showing the benefit of chemotherapy with docetaxel in metastatic prostate cancer.

In the TAX327 trial, 1,006 patients were randomized to receive docetaxel (30 mg/m^2 weekly or 75 mg/m^2 every 3 weeks) plus prednisone or mitoxantrone 12 mg/m^2 every 3 weeks plus prednisone. This trial demonstrated a significant improvement in overall survival in the patient group treated with every 3 weeks docetaxel compared to mitoxantrone, leading also to an advantage in other secondary endpoints such as pain and quality of life (14).

In the SWOG 99-16 trial, patients with metastatic CRPC were randomized to receive estramustine, the combination of non-nitrogen mustard and estradiol and docetaxel *vs.* mitoxantrone and prednisone. This trial confirmed that docetaxel was associated with a significant benefit in overall survival. However, there was significant myelosuppression, DVTs and gastrointestinal toxicities correlated with the combination of docetaxel and estramustine. Thus, docetaxel plus prednisone has become the standard of care for the first-line treatment of progressive CRPC (15).

Cabazitaxel is a new taxane approved as a second-line treatment in metastatic CRPC following docetaxel therapy with a significant survival benefit compared to mitoxantrone. The TROPIC trial evaluated 755 patients treated with cabazitaxel plus prednisone compared to mitoxantrone plus prednisone. The primary endpoint was overall survival, which was 15.1 months in the cabazitaxel plus prednisone arm and 12.7 months in the mitoxantrone plus prednisone arm. Other secondary endpoints such as progression-free survival, safety, tumor response, time to tumor progression and PSA response rate were all improved in the cabazitaxel plus prednisone arm (48). An ongoing phase III study (Cabazitaxel *vs.* Docetaxel Both With Prednisone in Patients With Metastatic CRPC, FIRSTANA) is evaluating cabazitaxel as first-line cytotoxic therapy, randomizing patients with metastatic CRPC to receive docetaxel *vs.* cabazitaxel (ClinicalTrials.gov; Identifier: NCT01308567).

At the cellular level taxanes stabilize microtubules. The microtubules are dynamic elements of the cytoskeleton necessary for the many cellular events, such as mitotic synthesis and intracellular protein transportation (49,50). There is preclinical evidence that treatment with taxanes can interfere with AR activity in addition to blocking cell division, evidence that enables new insights into the therapeutic efficacy of microtubule-targeting drugs in prostate cancer. In prostate cancer cells, taxanes inhibit AR nuclear signaling binding cellular microtubules, blocking AR nuclear translocation and consequently transcriptional activity. Microtubules facilitate AR nuclear translocation and enhance downstream AR transcriptional activity in prostate cancer cells. Microtubule targeting chemotherapy blocks this pathway and suppresses AR signaling, through a negative feedback mechanism; AR signaling inhibits tubulin expression thus impairing the cytoskeleton structure and organization.

Despite the efficacy of taxanes in CRPC, the real benefit can vary according to the clinical setting and host factors. Clinical resistance often occurs and can be explained by various mechanisms. Some of these are the presence of p-glycoprotein or other drug transporters that impair the uptake of the drug, the presence of tubulin mutations or the overexpression of the βIII tubulin isotype that impairs the binding to β-tubulin and the presence of AR mutations or splice variants that do not require microtubule-based transport (51-54).

Hormonal treatments in CRPC

Novel approaches that target the AR signaling axis in CRPC patients are hormonal agents. Some of the most important hormonal agents that have demonstrated improved overall survival in CRPC are abiraterone and enzalutamide (55-57).

Abiraterone is a potent and specific steroidogenic inhibitor that irreversibly inhibits the enzyme CYP17A1, the most important enzyme that catalyzes two essential steroidogenic reactions, the 17α-hydroxylase and 17,20-lyase responsible for converting pregnenolone to 17-OH-pregnenolone and subsequently 17-OH-pregnenolone to DHEA and androstenedione (58).

Abiraterone at 1,000 mg daily with prednisone 5 mg twice daily has demonstrated an overall survival benefit in patients with metastatic CRPC who have progressed before and after docetaxel treatment.

The phase III COU-AA-301 trial evaluated abiraterone and prednisone (2:1) *vs.* placebo and prednisone in 1,195 patients with metastatic CRPC pretreated with docetaxel

and up to two lines of chemotherapy. Abiraterone was the first novel hormonal therapy to demonstrate a significant improvement in overall survival, with a 26% reduction in the risk of death (HR =0.74, 95% CI, 0.638-0.859; P<0.0001), and significant improvements in radiographic progression-free survival, time to PSA progression and PSA responses (54-56).

The phase III COU-AA-302 trial compared (1:1) abiraterone plus prednisone with placebo plus prednisone in asymptomatic or mildly symptomatic chemo-naive and ketoconazole-naive metastatic CRPC patients without visceral disease and with only bone or lymph node metastases. This trial showed that abiraterone was associated with delays in disease progression and a significant improvement in overall survival at 49 months of follow-up, despite the fact that 44% of patients in the placebo arm crossed over to active abiraterone (59).

Enzalutamide is a novel AR inhibitor that binds to the AR with eight times more affinity than bicalutamide. This hormonal agent possesses three mechanisms of action in blocking the AR. It blocks testosterone binding to the AR, impairs nuclear translocation of the AR and inhibits association of the AR with DNA (57,60). This drug is administered without corticosteroids and has shown an improvement in overall survival in both the pre and post chemotherapy settings.

The phase III AFFIRM trial was a randomized (2:1) study in 1,190 patients that investigated enzalutamide 160 mg/d or placebo in patients with metastatic CRPC who had previously received docetaxel. This study demonstrated a median benefit in overall survival of 4.8 months and a 37% reduction in the risk of death (HR =0.631, 95% CI, 0.529-0.752; P<0.0001) with enzalutamide *vs.* placebo in patients with progressive CRPC (61).

The randomized PREVAIL study evaluated (1:1) enzalutamide *vs.* placebo in 1,717 chemo-naive patients affected by metastatic CRPC. This trial showed that enzalutamide decreased the risk of death by 29% (HR =0.19, 95% CI, 0.15-0.23; P<0.0001), the risk of radiographic progression by 81% and delayed the initiation of chemotherapy in patients with metastatic CRPC (62).

Despite these excellent results, many patients treated with abiraterone and enzalutamide develop resistance to these therapies and our knowledge of the pathogenesis of resistance to these agents is improving but extremely limited.

It has been recently demonstrated that the presence of AR splice variants is correlated with resistance to abiraterone and enzalutamide. Antonarakis *et al.* utilized a quantitative reverse-transcriptase-polymerase-chain-reaction assay to assess AR-V7 splice variants in circulating tumor cells (CTCs) from 62 prospectively enrolled patients with metastatic CRPC who were initiating treatment with either enzalutamide or abiraterone. This study showed that the presence of the AR-V7 splice variant derived from the RNA in the CTCs of these patients was associated with an absolute absence of response to abiraterone or enzalutamide and poor survival. The AR-V7 splice variant may be one of the first biomarkers to individualize patients who respond to these hormonal agents, but these data must to be validated (63,64).

Conclusions

The treatment paradigm of prostate cancer is continuously evolving and increasing knowledge about the pathogenesis and heterogeneity of this disease is leading to new approaches that include both old and new agents. Both hormonal therapy and chemotherapy target AR signaling have been shown to corroborate the importance of the AR axis in the treatment of prostate cancer.

Despite the improved knowledge of prostate cancer molecular biology, the absence of adequate biomarkers hinders our abilities in selecting patients who may derive the most benefit from hormonal or chemotherapy treatments. Apart from molecular classification, the correct timing, optimal sequencing and the association of these therapies are all subjects of ongoing and future study.

Acknowledgements

Footnote

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Mechanisms of resistance in castration-resistant prostate cancer (CRPC)

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Abstract: Despite advances in prostate cancer diagnosis and management, morbidity from prostate cancer remains high. Approximately 20% of men present with advanced or metastatic disease, while 29,000 men continue to die of prostate cancer each year. Androgen deprivation therapy (ADT) has been the standard of care for initial management of advanced or metastatic prostate cancer since Huggins and Hodges first introduced the concept of androgen-dependence in 1972, but progression to castration-resistant prostate cancer (CRPC) occurs within 2-3 years of initiation of ADT. CRPC, previously defined as hormone-refractory prostate cancer, is now understood to still be androgen dependent. Multiple mechanisms of resistance help contribute to the progression to castration resistant disease, and the androgen receptor (AR) remains an important driver in this progression. These mechanisms include AR amplification and hypersensitivity, AR mutations leading to promiscuity, mutations in coactivators/corepressors, androgen-independent AR activation, and intratumoral and alternative androgen production. More recently, identification of AR variants (ARVs) has been established as another mechanism of progression to CRPC. Docetaxel chemotherapy has historically been the first-line treatment for CRPC, but in recent years, newer agents have been introduced that target some of these mechanisms of resistance, thereby providing additional survival benefit. These include AR signaling inhibitors such as enzalutamide (Xtandi, ENZA, MDV-3100) and CYP17A1 inhibitors such as abiraterone acetate (Zytiga). Ultimately, these agents will also fail to suppress CRPC. While some of the mechanisms by which these agents fail are unique, many share similarities to the mechanisms contributing to CRPC progression. Understanding these mechanisms of resistance to ADT and currently approved CRPC treatments will help guide future research into targeted therapies.

Keywords: Castration-resistant; disease progression; drug resistance; prostatic neoplasms

Introduction

Prostate cancer is the most commonly diagnosed non-cutaneous malignancy in men and remains the second-leading cause of cancer-related death in men (1,2). Despite advances in screening for and early detection of prostate cancer, a large portion of men continue to present with advanced or metastatic disease-approximately 20% of men in recent reports (3). Indeed, the morbidity from this disease remains high, with more than 29,000 prostate cancer related deaths in 2013 alone (1).

Androgen deprivation therapy (ADT), the standard of care for patients with biochemical recurrence after definitive primary therapy, locally advanced disease or metastatic disease, has been demonstrated to provide an initial benefit, but the majority of patients will progress to castration-resistant disease within 2-3 years (4).

Castration-resistant prostate cancer (CRPC), previously called hormone-refractory prostate cancer, is now understood to be a progression of disease despite medical or surgical castration. The paradigm shift is due to the understanding that CRPC is not hormone-refractory—

in fact, the androgen axis continues to play an important role in the function and growth of CRPC. Indeed, while other pathways can contribute to castration-resistance, the androgen receptor (AR) remains the most important driver in the continuum of CRPC.

Understanding the mechanisms of resistance that cause hormone-naive prostate cancer to progress to castration-resistance is the key to developing future therapy. In this review, we will review the current knowledge regarding the mechanisms leading to castration resistance, the agents currently available for treatment of CRPC, and the mechanisms of resistance against these agents.

Background

Understanding the androgen axis is a key component to understanding the mechanisms by which castration-resistance develops.

Androgen receptor (AR)

The *AR* gene on Xq11-12 encodes for a 110 kDa nuclear receptor with four distinct functional motifs—the amino-terminal domain (NTD), DNA-binding domain, hinge region, and ligand-binding domain (LBD) (5-7). The cytoplasmic receptor is bound by heat-shock proteins (specifically HSP90 chaperone complex) in the inactive state (8). Androgen binding, specifically dihydrotestosterone (DHT) or testosterone, to the LBD causes a conformational change that leads to dissociation of the HSP90 complex, homo-dimerization of the receptor, translocation to the nucleus, and binding to androgen-response elements (AREs) in the promoter region of androgen-regulated genes (6,9). This interaction with the promoter region is under the influence of many transcriptional coregulators. Over 150 proteins have been identified (10), and many are enzymes (histone acetyltransferases, methyltransferases, kinases) that act to open the chromatin structure to promote transcription.

Androgens

Prostate cancer growth and survival depends on androgens, the major ligands for the AR. Testosterone is the primary circulating androgen, with approximately 90% produced by Leydig cells in the testes and 10% produced by the adrenal cortex. Only a small portion (3%) of circulating testosterone is unbound and functionally active—the remainder is bound and sequestered by sex-hormone binding globulin and albumin. However, testosterone is not the primary functionally active androgen in the prostate microenvironment. Following diffusion into the cytoplasm, testosterone is converted by the enzyme 5α-reductase to DHT, which has a five-fold higher affinity for the LBD of AR (11-13).

Physiologic levels of androgens are required to promote growth and prevent apoptotic death. Therefore, the pathways under AR influence are varied, but focus on the functions of the luminal epithelial cells, including production of seminal fluid proteins such as prostate-specific antigen (PSA) and multiple genes in the metabolic pathway leading to increased protein and lipid synthesis (14-16).

Steroidogenesis, which leads to androgen production, is an important pathway to understand, as it can be fundamentally altered in CRPC. Testosterone is produced by the testes and adrenal gland, and then converted in the cytoplasm to DHT via the activity of 5α-reductase (17). However, in the presence of ADT, studies have demonstrated persistent levels of intratumoral DHT (18-21), suggesting that altered steroidogenesis pathways have been activated (20). Adrenal testosterone sources, unaffected by ADT, and intratumoral *de novo* androgen synthesis may be sources of persistent ligand-dependent AR activity in CRPC (22).

Androgen deprivation therapy (ADT)

Since Huggins and Hodges (23) first demonstrated the dependence of prostate cancer on androgen signaling, ADT through either medical or surgical castration has been the standard of care for metastatic and locally advanced disease. Surgical castration, or bilateral orchiectomy, removes testicular androgens from circulation by removal of the source. Medical castration is achieved through the use of different classes of agents. LHRH agonists and antagonists deplete the pituitary production of luteinizing hormone (LH) through negative feedback or competitive inhibition, respectively, which in turn prevents testicular testosterone production (24). Anti-androgens work as competitive inhibitors at the LBD of AR, thereby preventing androgen stimulation of AR. These agents, in conjunction, provide complete androgen blockade (25,26).

Castration resistance

Despite the initial response to androgen blockade, all patients will eventually progress to castration resistance.

Castration resistance is progression of disease, either clinical (development of metastatic disease, progression of pre-existing disease) or biochemical (three consecutive rises in PSA levels above nadir) in the presence of castrate levels of circulating testosterone (<50 ng/dL) (27,28).

Indeed, the biochemical recurrence of PSA, an AR regulated gene measured by serum levels, is evidence that CRPC is not hormone-insensitive. When adding first generation anti-androgens, such as flutamide or bicalutamide, to the treatment regimen of patients with advanced or metastatic disease, a decrease in serum PSA is often initially noted, indicating a response to direct AR blockage (29,30). However, serum PSA levels will again rise despite anti-androgen therapy, suggesting that the agent has begun functioning as an AR agonist; this is validated by the PSA decrease noted with anti-androgen withdrawal (31,32).

Further evidence for the critical role of the androgen axis in the development of CRPC lies in the finding that, despite castrate levels of serum testosterone, there remains a higher level of intra-tumoral androgens in CRPC compared to hormone-naïve prostate cancer (18-21). Recent studies have demonstrated that intra-tumoral androgen levels in CRPC are similar to those of eugonadal men, and in some cases even increased (22,33).

The AR persists in CRPC cells, and the re-activation of this axis by the following mechanisms appears to drive progression to castration-resistance.

AR dependent mechanisms of resistance leading to CRPC

The majority of mechanisms identified leading to castration-resistant are mediated by AR or the androgen axis. As seen in *Figure 1*, they can be categorized into five main subsets.

AR amplification and mutations/hypersensitivity pathway/ promiscuous pathway

Low levels of androgen persist despite androgen blockade with ADT. Within this microenvironment, a subset of cells develop sensitivity to these low levels of androgens either through amplification of the AR (hypersensitivity pathway) (34) or development of AR mutations that lead to activation by molecules other than androgens (promiscuous pathway) (35,36).

Amplification of the AR has been identified in a significant portion of CRPC cell lines, ranging from 30-80% (37,38). This finding is uncommon in hormone naïve prostate cancer and may be due to selective outgrowth of CRPC cells (36). This amplification enables CRPC to be hypersensitive to low level of androgens, which promotes progression of disease (35). As 20% of CRPC metastases have evidence of AR amplification, which is absent in hormone-naive metastatic disease, it may also contribute to metastases. In addition, recent studies have shown that exogenous overexpression of AR can lead to CRPC.

Related to this concept, a substitution of valine with leucine at codon 89 results in increased 5α-reductase levels in a subset of CRPC. This results in higher levels of DHT despite low circulating levels of testosterone. This mutation is more commonly observed in the African-American population, and has been associated with more aggressive, early onset prostate cancer (39,40).

There have been various point mutations identified in the *AR* gene itself that lead to increased AR activity in the presence of low levels of androgens as well as non-androgenic steroids, such as progesterone, hydrocortisone, estradiol, and certain AR antagonists. The substitution of threonine with alanine at codon 877 in LNCAP cells (41,42) and the substitution of histidine for tyrosine in CWR22 cells (43,44) are well described in the literature; other examples include L701H, V715M, W741C (45-47). While most of the mutations are predominantly in the LBD, mutations in the NTD and DNA-binding domain were also identified (48,49).

Co-activators and co-repressors

Over 150 different molecules have already been identified as co-activators and co-repressors for AR (10). The AR normally recruits a series of coregulator complexes, which can function to either enhance (co-activators) or repress (co-repressors) transcriptional activity. Many of these coregulators are enzymes that serve to modulate other proteins in the complex, either through phosphorylation, methylation, acetylation or ubiquitylation, but they have also been identified as molecular chaperones, recruiters of transcriptional machinery and RNA splicing regulators (50-52).

One coactivator, FKBP51, which is also an AR target gene, was found to be upregulated in relapsed LAPC-4 tumors grown in castrate mice (53). It promotes formation of a superchaperone complex by regulating the recruitment of p23, a co-chaperone, to ATP-bound Hsp90, which in turns keeps AR in a conformation with high-affinity for ligand binding. This promotes androgen-stimulated transcriptional activity and growth.

The steroid receptor coactivators (SRC) are a class of
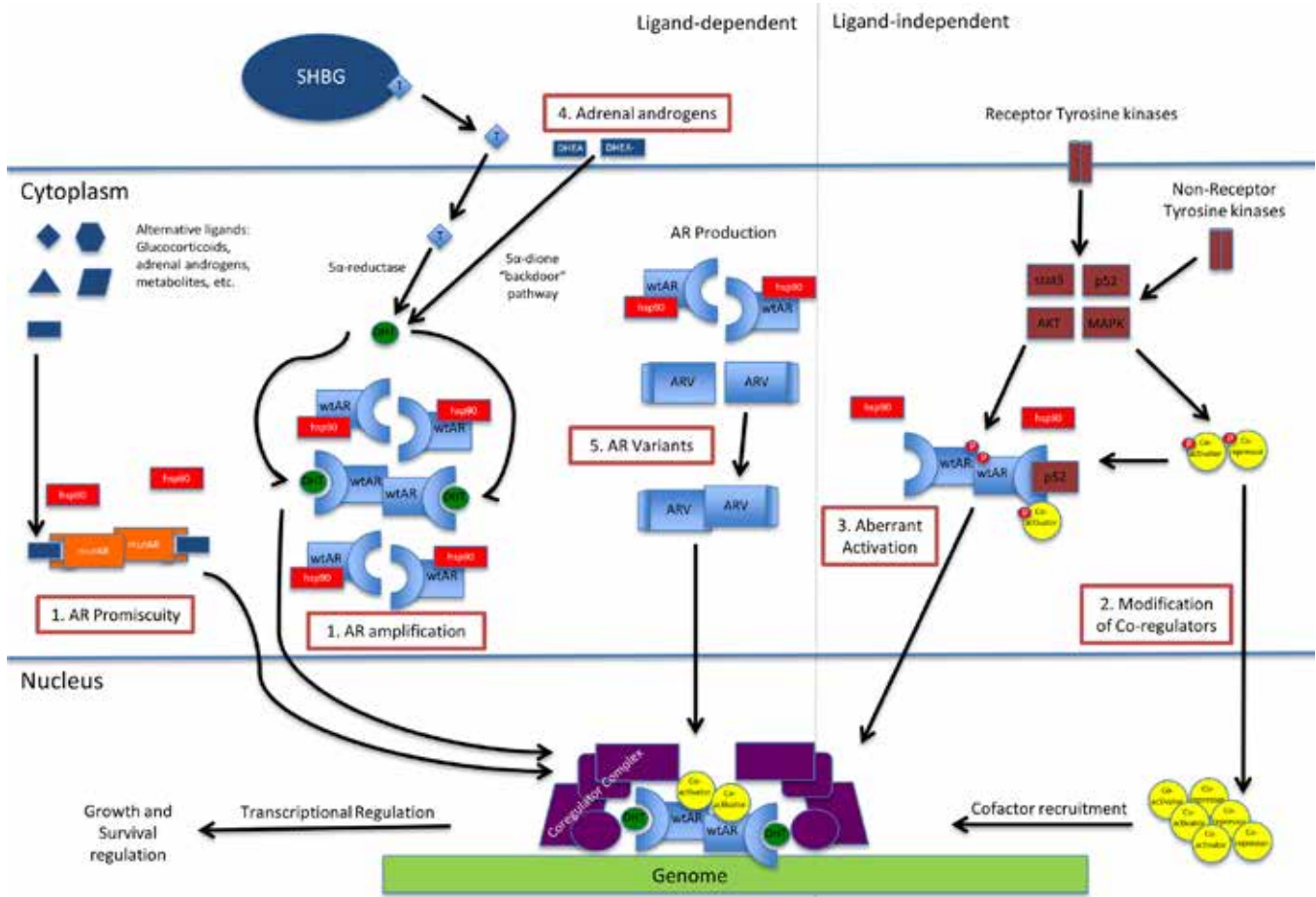
Figure 1 Androgen receptor-dependent mechanisms of resistance in hormone-naive prostate cancer leading to castration-resistance. wtAR, wild-type androgen receptor; ARV, androgen receptor variant; mutAR, mutated androgen receptor; T, testosterone; DHT, dihydrotestosterone; SHGB, sex hormone binding globulin.

AR coactivators capable of acetyltransferase activity, which in turn enhances AR-induced transcription by promoting formation of complexes between AR-associated enhancers/promoters and the transcription start site of AR target genes (54). The SRC class includes SRC-1, SRC-2 (TIF-2, GRIP-1, NcoA2), and SRC-3 (AIB). Xu *et al.* demonstrated that all three have been associated with prostate cancer progression (55). Ueda *et al.* identified SRC-1, when phosphorylated by MAPK under the influence of IL-6, was capable of both ligand-dependent and ligand-independent AR activation (56). SRC-3, in particular, tends to be over amplified in human cancers. Chung *et al.* demonstrated that SRC-3 is not overexpressed in androgen-dependent prostate cancer, but is overexpressed in poorly differentiated and more advanced prostate cancer, and is directly associated with prostate cancer progression—SRC-3 knockout mice were

effectively arrested at the well-differentiated stage and unable to progress to poorer pathology (57).

Other important pathways include p300/CBP, which promotes androgen-independent IL-6 mediated AR activation in the presence of STAT3 (58), and LSD1 and JMJD2c, lysine demethylases that demethylate the histone H3 proteins and lead to increased AR induced transcription (59). Many of these molecules have demonstrated AR-dependent and AR-independent effects, since their interaction is not limited to AR. Co-repressor proteins, on the other hand, have been found at reduced levels in CRPC.

Aberrant activation (post-translational modification)/outlaw pathway

While all the prior mechanisms mediate increased AR activity

in the presence of ligand, ligand-independent AR activation is also an important mechanism of progression to castration-resistance. Various *in vitro* studies have suggested that multiple growth factors, cytokines, and kinase pathways increase AR signaling, thereby promoting progression to castration resistance in a ligand-independent manner (60). Identification and characterization of those ligand-independent pathways can lead to additional targeted therapies.

The NF-κB family of proteins has been established as an important component of the oncogenic pathway in multiple human malignancies. There are five distinct NF-κB proteins, including the well-studied p65/p50 heterodimer, which has been shown to be constitutively active in prostate cancer. Another of the NF-κB pathways, the p100/p52 pathway has been of recent interest. The processing of p100 to p52 via molecules such as lymphotoxin β, B-cell activating factor, CD40 ligand, and stat3 (61) in prostate cancer, leads to significant hyperplasia and induced castration-resistant growth. This was accomplished by limiting ADT mediated apoptosis and cell cycle inhibition, but was done so in the presence of continued AR expression and activation, which suggested that p52 may activate AR during the progression of CRPC. p52 mediated its effects in an AR dependent manner by interacting directly with the NTD of AR. Downregulation of p52 in C4-2 cells led to the loss of constitutive activation of AR which suggested an androgen independent activation of AR (62).

The PI3K pathway is another important player in this process. The loss of the tumor suppressor PTEN protein, which is a negative inhibitor of the PI3K/AKT pathway, is identified in nearly all metastatic prostate cancers. Its activation has been associated with development of CPRC in various preclinical models (63-65). PI3K, specifically the p110β isoform, has been strongly associated with prostate cancer growth and progression, through basal activation of AKT in prostate cancer models. The PI3K/AKT pathway is downstream of key receptor tyrosine kinases (RTKs) such as EGFR, IGFR, c-met, but some studies suggest independent activation of this pathway (66). While it is also upstream of some critical signaling proteins, such as mTOR, it has also been found that AKT directly phosphorylates AR at two locations, Ser-217 and Ser-791, particularly in a castrate-state, though the clinical significance is not yet certain (67).

Src kinase, the key member in the family of non-RTK called Src family of kinases (SFK), has been a focus of our lab and our collaborators. Src, in the 25 years since its discovery as the first proto-oncogene identified, has been targeted in the treatment of multiple other malignancies (68,69). Our research into the role of Src in prostate cancer identified Src as a key molecule in multiple pathways that allow for progression of prostate cancer (70,71). Src is expressed in commonly used CaP cell lines CWR22Rv1, DU145, LAPC-4, LNCaP, and PC-3. As Src is not constitutively active, it has been difficult correlating Src protein expression levels with cell proliferation or aggressiveness *in vitro*. However, Src kinase is downstream of many important prostate-cancer influences—as it is activated by growth factors, cytokines, chemokines, and gastrin-releasing peptide, it has a pleiotropic effect on prostate cancer (68,70,71). Our laboratory group demonstrated that higher relative Src activation was associated with worse prostate cancer phenotypes, specifically DU145 and PC3, and the use of a novel SRC inhibitor AZD0530 helped elucidate a few of the pathways mediated by Src in prostate cancer cell lines (70). Activation of Src kinase has been linked to androgen-independent cell growth (72-74), inhibition of anti-apoptotic pathways (75-77), cell migration and adhesion (73), and tumor invasion (78), among other aspects of prostate cancer cell biology. Based on this preclinical data, AZD0530 (saracatinib) was taken to phase II clinical trial, but it was demonstrated to have minimal clinical efficacy as monotherapy (79). Lack of clinical efficacy was also noted with dasatinib; in the phase III clinical trial of docetaxel with dasatinib or placebo in chemotherapy-naïve CRPC patients, there was no improvement in overall survival (80). Other non-tyrosine kinases, such as Btk and Etk within the Tek-family of non-tyrosine kinases, are being targeted as well; recent work by Guo and colleagues demonstrated that CTN06, a novel dual inhibitor of Btk and Etk, induced apoptosis and autophagy, and also re-sensitized cell lines to docetaxel (81).

Growth factor pathways, such as IGF and KGF, bind and activate AR in a castrate state. Growth factor receptors, such as IGF-1R, IL-6R, and EGFR, control critical downstream growth and survival pathways such as MAPK, PI3K/AKT, and STAT signaling. Various RTKs, such as Her-2/neu, EGFR, and IGR-1R, enhance AR stability and activity, and in some cases, promote androgen independence. Her2/neu, for example, was found to promote xenograft cell growth via Ack1-kinase, which phosphorylates AR at tyrosine-267 and activates it (82). Targeting these pathways has shown some promise—cabozantinib (XL-184) inhibits tyrosine kinases of c-Met and VEGF, and in phase II clinical trial, demonstrated significant benefit specifically for CRPC patients with bone metastases; However, it did not reach its primary endpoint (bone pain alleviation) in phase III trial, with no significant

difference in bone pain alleviation between the treatment and control (mitoxantrone/prednisone) arms.

Altered steroidogenesis

CRPC develops in the presence of castrate-levels of circulating androgens. However, intra-tumoral levels of androgens in CRPC models have been established to be the same as or even higher than in eugonadal men, suggesting that there is alternative androgen production (18-22,33). This is likely due to adrenal production, specifically of androgen precursors of adrenal origin such as dehydroepiandrosterone (DHEA) and its sulfated form (DHEA-S), which can be converted to the highly active DHT via a "backdoor" pathway (83,84).

DHEA and DHEA-S, produced by the adrenal gland, are not affected by ADT and are still found in circulation. The molecules are converted to androstenedione (AD) either in the prostate or adrenal gland by 3βHSD, encoded by HSD3B. There are 2 isoforms, 3βHSD1 in the prostate and peripheral tissues, 3βHSD2 in the adrenal gland (85). The subsequent conversion from AD to DHT, in the absence of ADT, typically goes through testosterone as an intermediary, and requires 17βHSD3 and AKR1C3 (encoded by HSD17B3 and AKR1C3 respectively) and steroid-5α-reductase (two isoforms, encoded by SRD5A1 and SRD5A2). However, in the presence of ADT, the sequence can be reversed, leading to 5α-AD (5α-dione) serving as the intermediary between AD and DHT, bypassing testosterone completely. This alternative pathway, referred to as the "5α-dione" pathway, has been demonstrated to predominate in CRPC (86,87).

In addition to utilization of weak adrenal androgens in the 5α-dione pathway, recent assessment of CRPC cells has identified increased expression of steroidogenic enzymes such HSD3B1, HSD3B2, HSD17B3, AKR1C3, and SRD5A1 (20,87-89), which may contribute to *de novo* production of steroids and androgens. Up-regulation of SRD5A1 and concurrent down-regulation of SRD5A2 leads to higher levels of 5α-reductase-1, for which AD is a better substrate than testosterone (90-92). What drives the changes in transcription of these steroidogenic enzymes? Many single-nucleotide polymorphisms have been identified within the above enzymes, especially HSD3B1 and HSD3B2 (93), but the clinical significance of these is not yet clear.

AR variants

A more recent development has been the identification of splice variants of the AR (AR-Vs), which are constitutively active, typically due to the loss of the C-terminal LBD (94-97). Indeed, the amplification of AR seen in CRPC may contribute to the development of the splice variants. Most CRPC cell lines demonstrate low levels of AR-V, but 22RV1 express levels similar to full-length AR (44).

The functional implication of these variants is not yet fully understood. Direct measurement of splice variants has been limited by the lack of variant-specific antibodies, leaving only secondary assessment via RNA levels. ARV7 is the only variant that has a suitable antibody for staining, and immunohistochemistry staining has established increased expression in CRPC (95,96). However, transcribed RNA levels may not be completely reflective of protein levels. This suggests some post-translational control that has not yet been fully elucidated.

However, Hörnberg *et al.* reported high levels of splice variant expression in bone metastases compared to hormone-naïve prostate cancer, and that it led to CRPC and poorer prognosis (98). This study also demonstrated a discrepancy between RNA levels and protein levels, contributing to the difficulty in determining splice variant significance in CRPC development and progression.

The predominant variants are ARV1, ARV7 and ARV 567. Of the variants, ARV7 has been studied most extensively (95,96). As described above, it lacks the LBD, is located in the nucleus, and is constitutively active. It has been show to regulate both AR-regulated genes and a unique set of AR-independent genes (96), suggesting it has an overlapping but distinct role compared to full-length AR in prostate cancer cells (94).

A recently discovered variant, ARV8, actually lacks a DNA-binding domain. Therefore, it remains in the plasma membrane and its constitutive activity is limited to activation of cell signaling pathways (99). For example, Yang *et al.* demonstrated increased AR phosphorylation via an EGF-mediated SRC activation in the presence of this variant; its subsequent knockdown was associated with loss of this phosphorylation.

Mechanisms of resistance to current CRPC treatments

Based on this understanding of the development of CRPC, there are now approved medications for the management of patients who are castration-resistant. However, despite these new agents, all patients will eventually progress in their disease. Understanding the means by which prostate
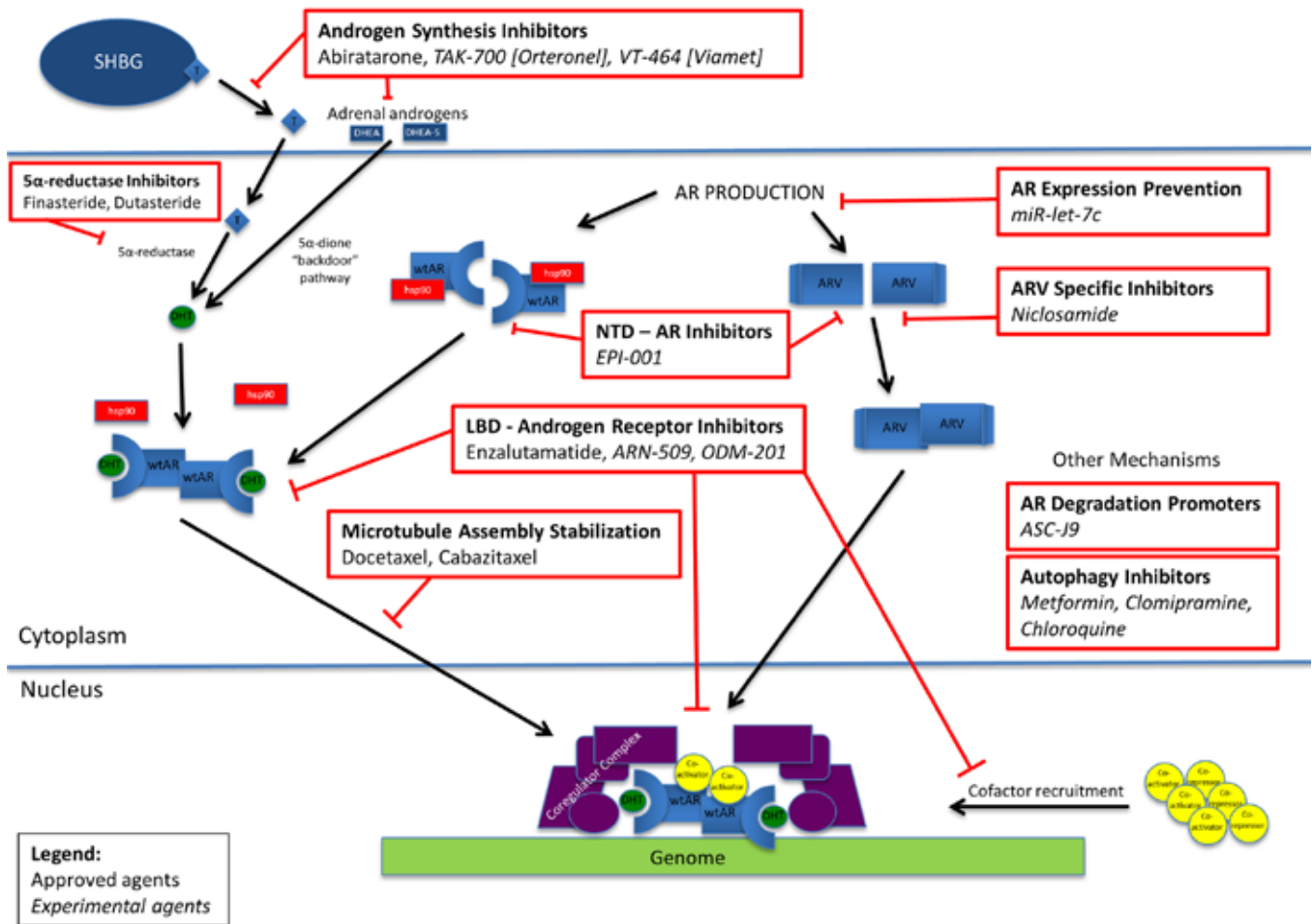
Figure 2 Current and experimental agents targeting the androgen axis. wtAR, wild-type androgen receptor; ARV, androgen receptor variant; LBD, ligand-binding domain; NTD, n-terminal domain; T, testosterone; DHT, dihydrotestosterone; SHGB, sex hormone binding globulin.

cancer overcomes these treatment modalities will help identify new treatment options.

Below, we will address the primary agents currently available, focusing on their mechanism of action and current knowledge about the resistance to their function. *Figure 2* provides an overview of the current and experimental agents affecting the androgen axis. As can be expected, there is crossover in many of these mechanisms, with these shared pathways being potentially significant future targets.

Docetaxel

Docetaxel is the current standard of care for patients who have progressed to castrate-resistant prostate cancer. SWOG 9916 and TAX327 demonstrated a 3-month survival advantage with docetaxel over mitoxantrone in CRPC patients (100-102), and until recently, it was the only approved primary therapy for CRPC. The recent CHAARTED trial, however, may have demonstrated a role for docetaxel as an initial treatment option for hormone-naïve prostate cancer in conjunction with ADT, as the combination was found to have a 17-month survival advantage (103).

Docetaxel is a well-known and studied chemotherapeutic agent used in the treatment of a variety of malignancies. It is an anti-mitotic chemotherapeutic agent that works by binding the β subunits of tubulin in microtubules, thereby stabilizing them and preventing the depolymerization required for mitosis (104-106), which induces apoptosis. In CRPC specifically, docetaxel leads to phosphorylation of

bcl-2 (B-cell lymphoma 2), which causes caspase activation and apoptosis *in vivo* and *in vitro* (107,108). Additionally, AR expression is reduced in docetaxel-treated CRPC cells and is thought to be due to AR nuclear localization and inhibition of signaling (109).

Drug-efflux in CRPC enables resistance to docetaxel. Multi-drug resistance proteins (MDRP) are well described in the literature, and include P-glycoprotein (P-gp), multidrug resistance protein 1 (MRP1), and breast cancer resistance protein (BRCP). These molecules cause active efflux of multiple therapeutic agents. DU145 and 22RV1 cell lines, when made docetaxel-resistant, have been found to over-express P-gp (110), while CRPC lines exposed to docetaxel have been found to have MDR1 genetic variations that are more docetaxel-resistant (111). Docetaxel-resistant CRPC lines also upregulate the class III β-tubulin isoform, which allows less taxane binding. Inhibiting class III β-tubulin restores docetaxel sensitivity in those same cells (112,113). In addition, LNCAP derived docetaxel-resistant cells demonstrated an F270I mutation in the class I β-tubulin, which had stronger taxane binding at baseline (114).

While the above mechanisms are docetaxel-specific, other mechanisms of resistance have been identified. Docetaxel resistance has been linked to apoptosis pathways, specifically upregulation of p53 and activation of PAR1. p53 is an important cell cycle regulator, often found over-expressed in prostate cancer. LNCAP cells over-expressing wild type p53 are more resistant to docetaxel activity than DU145 and PC3 cell lines, which have reduced or no p53 activity (115). Zhu *et al*. demonstrated this in docetaxel-resistant C42B cells *in vitro*—cells treated with docetaxel had p53 phosphorylation and activation, but taxane-resistant C42B demonstrated no phosphorylation (116). PAR1, through NF-κB activation, has been shown to reduce docetaxel-induced apoptosis (117).

In addition to blocking docetaxel-induced apoptosis, docetaxel's anti-mitotic activity itself directly initiates survival pathways in prostate cancer cell lines. Binding to the microtubules initiates pathways such as c-Jun N-terminal kinase (JNK), which in turns leads to activation of various transcription factors such as STAT-1, STAT-3, and NF-κB. Knockdown models of these transcription factors have been shown to be more sensitive to docetaxel-cytotoxicity (115,118).

Over-expression of cytokines and chemokines, such as IL-6, IL-8, and CCL-2, and chaperone molecules, such as HSP27 and HSP90, have been associated with docetaxel resistance, but no clinically significant inhibitors of these pathways have yet been identified. OGX-011, a second-generation antisense drug that inhibits the secretion of clusterin, a chaperone protein, was administered in conjunction with docetaxel in phase III trials, but did not meet its primary endpoint. Its activity focuses on CLU, a key protein that exists in two forms: nuclear CLU (nCLU) and secreted CLU (sCLU)—nCLU promotes docetaxel-mediated cell death while sCLU prevents it (119,120). Upon initiation of chemotherapy, especially docetaxel in prostate cancer cells, there is a shift in the balance towards sCLU, thought to be attributed to STAT-1 activation (110,121). However, inhibition of sCLU using antisense oligonucleotide re-sensitizes the cells to docetaxel (121,122).

Our lab group identified >1,600 genes that had altered expression in taxane-resistant C42B cells, with approximately 52% being upregulated. From this subset, we recently identified ABCB1, which belongs to the ATP-binding cassette (ABC) transporter family, among the top upregulated genes in the taxane-resistant cells. ABCB1 was highly expressed in taxane-resistant C42B cells, but virtually undetectable in taxane-sensitive C42B cells. Inhibition of ABCB1 expression resensitized C42B cells to docetaxel, and this was then confirmed in the DU-145 cell line (116). Apigenen, a natural molecule in the flavone family identified by Shukla and Gupta (123), was demonstrated to help resensitize cells to docetaxel therapy.

Abiraterone and androgen synthesis inhibitors

Abiraterone acetate (Zytiga) is a molecule structurally similar to pregnenolone that acts as an irreversible inhibitor of cytochrome p450, family 17, subfamily A, polypeptide 1 (CYP17A1). CYP17A1 is a member of the cytochrome p450 class of enzymes that serve as a catalyst for the oxidation of a variety of molecules. It has two consecutive enzymatic functions in the steroidogenesis pathway that contribute to the conversion of pregnenolone to DHT. Loss of CYP17A1 activity causes significant loss of androgen production in the peripheral organs, particularly adrenal androgens. It has been found to be 10-30 times more potent than ketoconazole, which is a non-specific inhibitor of p450 enzymes and previously has been used to generate rapid androgen ablation (106). The phase III trial COU-AA-301 demonstrated a 3.9-month survival benefit of abiraterone/prednisone over placebo/prednisone in patients who had progressed on docetaxel therapy (124). The subsequent COU-AA-302 trial demonstrated benefit in the pre-chemotherapy space, with improved radiographic

progression free survival, time to initiation of chemotherapy, and a trend towards improved overall survival (125).

Altered steroidogenesis was discussed as one mechanism by which CRPC develops. While abiraterone-naive CRPC cell lines utilize the "5α-dione" pathway to generate intra-tumoral DHT by bypassing testosterone, they are still dependent on adrenal androgens. By irreversibly inhibiting this critical upstream enzyme in the steroidogenesis pathway, abiraterone effectively causes a significant decrease in intra-tumoral androgen levels by preventing production of adrenal androgens.

However, despite its effectiveness in inhibiting the steroidogenesis pathway (126), abiraterone's effect is incomplete. Attard *et al*. demonstrated that while most urinary androgen metabolites and serum androgens were suppressed, the inhibition of CYP17 led to higher levels of urinary metabolite 3α5α-17HP, which correlated with the excretion of androsterone—which is the primary metabolite of 5α-reduced androgens such as DHT (127). This suggests that the use of abiraterone may push 17-hydroxyprogesterone towards the "5α-dione" pathway.

As can be expected, over-expression or mutations of CYP17A1 may also contribute to abiraterone resistance (128). Chang *et al*. demonstrated that the HSD3B1 (1245C) mutation previously mentioned as contributing to progression to CRPC has also been found in abiraterone-resistant xenograft models, though the clinical significance of this still needs to be elucidated (93). Mostaghel *et al*. demonstrated that abiraterone-treated cell lines responded with increased expression of CYP17A1, as well as increased expression of enzymes in the steroidogenesis pathway, including AKR1C3 and HSD17B3 (129).

Other androgen synthesis inhibitors are in development at this time, including TAK-700 (Orteronel) and VT-464 (Viamet), both of which are more selective for the 17, 20-lyase inhibition (130). TAK-700 is further in development, currently accruing for another phase III clinical trial, this time assessing efficacy in chemotherapy-naive CRPC patients; the initial phase III study in patients who had been treated with docetaxel demonstrated an improvement in radiographic progression-free survival (HR 0.755), but it did not meet the primary endpoint of improvement in overall survival (HR 0.894) (130).

Enzalutamide and androgen receptor (AR) inhibitors

In response to the many AR mediated mechanisms of resistance found leading to development of CRPC, there has been development of a new generation of androgen-receptor signaling inhibitors. The main agent in this class is enzalutamide (MDV-3100, ENZA, Xtandi), which has been demonstrated to have a multi-pronged approach—preventing testosterone binding to AR, AR nuclear translocation, AR binding to DNA, and co-activator recruitment (106). While the AFFIRM III trial demonstrated a 4.8-month survival benefit over placebo in CRPC patients who had failed docetaxel and the PREVAIL trial demonstrated an overall survival and radiographic progression-free survival over placebo in chemotherapy-naïve CRPC patients (131,132), not all the patients benefited from treatment—a subset of patients continued to progress, indicating that there are significant resistance mechanisms that need to be identified and addressed.

One mechanism by which CRPC develops resistance to enzalutamide, and potentially other treatment modalities, is the process of autophagy. Autophagy is a catabolic process that, besides being constitutively active at a low basal rate, is activated in response to stressors, allowing cells to use lysosomal-mediated degradation of cellular proteins and organelles to regenerate energy (133-135). Autophagy can be used by cancer cells to prolong their survival under harsh conditions of metabolic stress in the tumor microenvironment induced by various treatment modalities, but excessive or deregulated autophagy can push the cells toward autophagic cell death or type-II programmed cell death (136,137). Indeed, androgen deprivation has been shown to induce autophagy, and while the exact mechanism is unknown, suppression of mTOR appears to play a critical role (135,138). Prior studies, by our group and others, have established that administration of autophagy inhibitors, either as monotherapy or in conjunction with established therapies, has had effective cytotoxic result. We demonstrated that the use of clomipramine and metformin, both clinical autophagy inhibitors, significantly increased the cytotoxicity associated with enzalutamide *in vitro* and in mouse models—the enzalutamide/clomipramine combination decreased tumour size by 91%, compared with a 78% decrease with enzalutamide/metformin (135). There are currently many ongoing clinical trials assessing the role of autophagy inhibitors as concomitant therapy (139), including a study at our institution that has recently been approved to assess metformin and enzalutamide combination therapy.

AR point mutations are also important mechanisms of resistance to enzalutamide, just as in the development of CRPC. The Phe876Leu mutation in the LBD of AR has been reported to make enzalutamide into an agonist of AR,

though the clinical relevance of this change has not been documented (140,141). Similar effects were noted for the first generation anti-androgens bicalutamide and flutamide.

Another proposed mechanism is the "glucocorticoid receptor take-over" pathway. Glucocorticoid receptors are nuclear receptors similar in structure to the AR. Glucocorticoids initially have a suppressive effect on prostate cancer, and indeed, are often given in conjunction with early treatments of CRPC, including chemotherapy and abiraterone. However, the DNA binding domain of the glucocorticoid receptor is very similar to the DBD of the AR (142,143), and the glucocorticoid receptor has been shown to bind to many AR regulated genes, suggesting its upregulation in patients treated with chemotherapy or ADT may contribute to enzalutamide resistance (144).

Many of these mechanisms may also affect upcoming androgen-receptor inhibitors in a similar fashion. For example, ARN-509, another novel AR antagonist which is currently in the accrual phase of a multi-center phase III clinical trial, has been shown to be susceptible to the same AR F876L mutation that converts it to an agonist (145). Other agents currently being developed include ODM-201.

Targeting the androgen receptor (AR): the next step in prostate cancer therapy

As recently published in the *New England Journal of Medicine*, Antonarakis and collaborators demonstrated that 20-40% of circulating tumor cells in CRPC patients treated with abiraterone and enzalutamide have ARV7 constitutively active (146). More importantly, however, they demonstrated in this prospective trial that the subset of men with ARV7 in circulating tumor cells had a significantly lower PSA response rate, shorter progression-free survival and overall survival compared to men without ARV7 expression. This study, our own research (62), and studies by other groups (94,145-147) demonstrate that ARVs are an important mechanism of resistance to newer CRPC agents. Liu *et al.* demonstrated that AR-V7 was present in a number of prostate cancer cell lines and that it was able to activate the PSA promoter in LNCaP and PC3 cells in the absence of androgen (148). With the loss of the LBD on the AR as seen in ARV-7, CRPC cell lines overcome the loss of circulating and intratumoral androgens mediated by abiraterone. Loss of the LBD, and concurrent ligand-independent binding of AR to ARE's, is thought to be the underlying mechanism of resistance to enzalutamide. Li *et al.* demonstrated that knockdown of AR-V limited

androgen-independent growth rate of CWR22Rv1 cells and restored responsiveness to anti-androgens (147).

With the growing body of evidence pointing to the important role of ARV's in the development of resistance and the concurrent finding that many of the current mechanisms of progression to CRPC involve alterations in the AR pathway, targeting the AR appears to be the next major step in prostate cancer therapy.

Our lab previously identified niclosamide, used clinically to treat helminth infections, as an inhibitor of ARV7, by promoting its degradation; co-treatment with enzalutamide demonstrated a synergistic response (148). Similarly, we also established that miR-let-7c, a microRNA of the let-7 family, antagonizes AR expression via c-myc degradation, leading to inhibition of prostate cancer proliferation (149). Others have also started to focus on the AR itself as a target for therapy—either reducing its expression or promoting its degradation. Lai *et al.* have identified ASC-J9, a novel AR degradation enhancer currently utilized clinically for other pathologies (150). Sadar and colleagues have been focusing on EPI-001, a small molecule that inhibits the N-terminal domain (NTD), which is present on both wild-type AR and AR variants (151).

Perhaps by targeting the AR and its variants, we may be able to overcome the deficiencies of current CRPC treatments.

Conclusions

Prostate cancer, especially locally advanced and metastatic disease, continues to be a burden on the healthcare system. While the prognosis is good for men diagnosed with localized disease, the prognosis remains poor for men with more advanced disease. All current therapies, from ADT to chemotherapy, merely slow the progression of disease, but all patients inevitably progress on therapy. Understanding the mechanisms by which these patients develop resistance to ADT, then subsequently to docetaxel, abiraterone, and enzalutamide, is important to identify future targets of therapy.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Surgery and hormonal treatment for prostate cancer and sexual function

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Abstract: Prostate cancer (PC) is one of the most common cancers effecting men today. With earlier detection and improvements in available treatment modalities, there still remains significant morbidity associated with the treatment of PC. Male sexual health and erectile function are greatly impacted by these therapies and remain a concern to PC survivors. This article reviews the current literature on male sexual health following radical prostatectomy (RP) or androgen ablation therapy for PC. Each treatment modality affects male sexual function to an appreciable level, although certain patients have better outcomes if they have preoperative potency, are younger, or have nerve-sparing surgery. There is a delayed recovery up to 2 years seen in erectile function following RP. With androgen deprivation therapy (ADT), attempts can be made at different administration strategies and exercise may possibly play a role in maintaining erectile function. Penile rehabilitation protocols attempt to protect erectile function immediately following therapy through different modalities, although no one approach has been agreed upon.

Keywords: Androgen deprivation; prostatectomy; prostate cancer (PC); erectile dysfunction (ED)

Introduction

Prostate cancer (PC) is the most common cancer in the aging male population and the second leading cause of cancer death (1). The American Cancer Society estimates 1 in 7 men will be diagnosed with PC in their lifetime, with the average age at 66 years old (1). The risk of PC increases with age. However, because of the slow progression of the cancer, the majority of men diagnosed do not die from the disease. In fact, the ACS quotes the relative 10-year survival rate as 99% (1).

Due to the chronic nature of this cancer, and the extended time from premalignant lesion to "clinically relevant" cancer, treatment should focus not only on survival but also on quality of life and sexual health (2). The goal of treatment should be to minimize the risk-benefit ratio. However, this goal is limited by the lack of complete understanding of the pathophysiology, as well as the

heterogeneity, of the disease. Despite this fact, it is widely known that, as whole, androgens promote the growth and progression of PC. Even with a diversity of androgenic interplay among diseased individuals, there appears to be a common side effect among all treatment modalities, as each has a degree of negative impact on male sexual health and function (2). Common alterations to male sexual health include erectile dysfunction (ED), changes in penile length and girth, pain with sexual activity, and dysfunctions of ejaculation and orgasm. Among these, ED is oftentimes cited as the major concern of men following treatment for PC (3). Primary treatment modalities for PC consist of active surveillance, surgical removal of the prostate, radiation, and androgen deprivation therapy (ADT). In this review we will focus on prostatectomy and androgen deprivation, and their effects on male sexual function.

In order to understand the sexual dysfunction resulting from PC treatment, it is necessary to first understand the

normal physiology. Normal male sexual function requires the involvement and coordination of multiple regulatory systems and is thus subject to the influence of psychological, hormonal, neurological, vascular, and cavernosal factors. The initial obligatory event required for male sexual activity, the acquisition and maintenance of penile erection, is primarily a vascular phenomenon. The arterial dilatation and venous compression required for erection is triggered by neurologic signals and facilitated only in the presence of an appropriate hormonal milieu and psychological mindset. An alteration in any of these factors may be sufficient to cause sexual dysfunction (4,5).

Radical prostatectomy (RP)

RP is primarily a treatment option for patients with localized PC, and is not indicated for patients with clinical evidence of regional lymph node involvement or distant metastases or when there is tumor fixation to adjacent structures. Moreover, in the recently published Scandinavian Prostate Cancer Group 4 trial it was found that prostatectomy, for localized disease, has significantly lower incidence of death from all causes, death from prostate, distant metastases, and use of ADT compared with those who undergo watchful waiting. The benefits were most pronounced in those less than 65 years of age at diagnosis and in those with intermediate risk disease (6).

However, as with all treatment options, prostatectomy is associated with a number of risks. The most commonly reported postoperative complications include ED and urinary incontinence (2). ED following RP has been reported in 60-70% of men, although definition of ED varies in reported sources (3). The etiology of ED following RP is most likely multifactorial-mechanical or thermal injury intraoperatively or postsurgical inflammation can lead to neuropraxia or permanent damage of the cavernosal neurovascular bundle. Ligation of the accessory internal pudendal arteries also plays a role in postoperative ED, as it decreases arterial inflow leading to subsequent hypoxia and apoptosis (4). Chronic loss of erections in itself contributes to these ischemic changes through decreased blood flow, cavernosal smooth muscle fibrosis, apoptosis, and collagen deposition (3,7). Corporal veno-occlusive dysfunction also may be seen causing clinically evident venogenic impotence (3,7). The nerve-sparing technique for RP, first described by Walsh in 1982, has reported rates of 40-86% positive erectile function following surgery; however still 90% of men will experience some initial decline in sexual function (8).

An alteration of the hypothalamic pituitary axis may also explain the initial ED and urinary incontinence seen following RP, as it has been noted that in the immediate post-operative period there is greater sexual dysfunction and incontinence than there is 15 years out. This can be explained by one prospective study, which enrolled 100 men with clinically localized PC to evaluate the serum levels of testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) following RP. Immediately following surgery, a drastic decline in serum testosterone was observed, along with compensatory rise in LH and FSH. At three months out from surgery, testosterone levels were seen to normalize, however LH and FSH remained elevated. It was postulated by the authors that this could explain the delayed recovery of erectile function and urinary control that is seen following RP (9).

Sivarajan et al. prospectively examined sexual function and erectile function in men undergoing open RP over a 10-year period. Men in the study completed a sexual function survey at baseline and at increasing intervals over this time period. The expected initial decline in both measured outcomes was seen, followed by a time-dependent improvement through 2 years post RP. Sexual function appeared to remain stable in the 2-10 year postoperative period. However, younger men and those with pretreatment potency were more likely to actually continue to see improvements in erectile function past 2 years. Despite this, all treatment groups, including RP, radiation treatment and active surveillance, are noted to be subject to time dependent changes of erectile function (10).

RP has also been shown to decrease emission, incontinence during sexual activity, and decrease pleasure with orgasm (10). Loss of penile length and girth is also reported. One study saw up to a 3 cm decrease in stretched penile length at 12 months out from treatment. It has been postulated that parasympathetic damage secondary to cavernosal nerve injury leads to overcompensation of the sympathetic nervous system and release of factors responsible for penile shortening (3). Other changes in penile appearance are seen, including curvature and onset of Peyronie's disease, all of which negatively impact male sexual health and thus decrease post treatment quality of life.

In a post-operative analysis of men who underwent RP, Dubbleman et al. found that orgasmic function was preserved in 73.4% after a bilateral nerve sparing procedure, in 70.9% after a unilateral nerve-sparing procedure and in 54.0% after non-nerve sparing technique. Indicating that orgasmic dysfunction plays a relatively minor role in post

prostatectomy sexual dysfunction (11). However, it has been noted that PC survivors may experience lack of ejaculation at the point of orgasm or urinary incontinence associated with orgasm (climacturia), and the impact of these changes may be challenging for patients and their partners. It has been noted however, that climacturia does not significantly impact sexual satisfaction (12).

Another treatment modality frequently used in localized disease burden is radiotherapy (RT). RT, like RP, is also associated with multiple risks. Commonly observed risks include bowel dysfunction, sexual dysfunction and urinary incontinence. The prevalence of stated risks among patients with localized disease undergoing RP versus those undergoing RT was evaluated in the Prostate Cancer Outcomes Study (PCOS), a cohort comprised of 1,655 men with localized PC. In this study the functional status of patients' bowel, sexual and bladder functions were assessed at baseline, and at 2, 5, and 15 years following diagnosis. It was found that, at 2 and 5 years post treatment, ED and urinary incontinence were more likely to occur in patients who underwent RP. While bowel urgency was more likely to occur following RT at 2 and 5 years post treatment. However, despite the declines in all functional domains, seen in both treatment groups, during 15 years of follow-up no significant differences in disease-specific functional outcomes were observed. This suggests that both treatment options decrease quality of life in patients with localized disease to an equal extent (13).

Since the median life expectancy following treatment of clinically localized PC is 13.8 years, it is imperative to uncover and address the long-term quality of life outcomes when discussing treatment options with patients. In order to provide the patient with the ability to make the best decision, evidence based prediction models may be used. One such model has been developed for ED by Alemozaffar *et al*. In their study they examined men within the Prostate Cancer Outcomes and Satisfaction with Treatment Quality Assessment (PROSTQA) cohort with early stage PC who opted to undergo prostatectomy, external RT, or brachytherapy. Pretreatment characteristics of individual patients, quality of life regarding sexual function, and treatment paradigms were included in the prediction model. The primary outcome was erections defined as "firm enough" for intercourse based on the EPIC-26 (Expanded Prostate Cancer Index Composite). Using this data, models predicting erectile function 2 years out were developed. These were validated using a similar community based-cohort. In this cohort, satisfactory erectile function was

reported in 177 of 511 [35% (95% CI, 30-39%)] men status post prostatectomy. Younger age, less comorbidities, lower prostate specific antigen (PSA), lower risk PC, erectile function prior to treatment, better sexual quality of life questionnaire scores, and plan for nerve-sparing surgery were associated with greater probability of erectile function 2 years out using univariable analysis. Multivariable analysis, however, only showed younger age, better pretreatment sexual functioning score, and nerve-sparing surgery as factors leading to potency 2 years following surgery (14). By sharing this evidence with patients, and enabling them greater insight into their personal treatment risks, post treatment quality of life may be increased.

Androgen ablation

The role of androgens in the pathophysiology of PC has been well documented, as it is known that androgen receptor signaling is critical for PC growth and survival (15,16). The first mention of this role was made in 1941 by Huggins and Hodges, with their observation that castration levels of testosterone led to PC regression. This provided the nidus from which ADT developed (17,18). However, a precise understanding of androgenic stimulation and the mechanism by which it effects the initiation and progression of PC has yet to be elucidated, and is likely multimodal and subject to patient specific genetic aberrations (19). Despite this, recent evidence supporting a favorable risk-benefit ratio for ADT in PC is currently limited to men with high-risk or metastatic disease. This is in part because ADT has been associated with a number of constitutional and somatic side effects. Similarly, ADT use has been limited in localized PC due to its association with lower PC-specific survival and no increase in overall survival compared with conservative management (15).

There are still mixed opinions as to whether or not ADT should be used as monotherapy in intermediate and high risk patients. The EUA recommends primary ADT if there is symptomatic locally advanced PC or positive nodal involvement (20). However, the National Institute for Health and Care Excellence (NICE) does not recommend ADT as monotherapy for men with intermediate and high risk localized PC. On the other hand, it has been shown, that for patients undergoing RT, adjunct ADT improves overall patient survival relative to RT alone (21-24).

ADT plays a major role in the treatment of metastatic prostate disease, and is considered mandatory treatment in symptomatic patients, as immediate hormonal therapy may

improve cancer-specific survival for men with advanced PC and maximal androgen blockade might increase overall survival at 5 years (21,23,25). The corollary, however, is that ADT has also been associated with increased adverse events and reduced quality of life.

The three most common adverse effects experienced with ADT are hot flashes, bone fractures and impotence with eventual ED. ED is a particularly distressing side effect and may develop in 10-30% men after ADT therapy (26,27). Similarly, in a cohort analysis from a randomized trial, 75% of men reported ED at 5 years following neoadjuvant androgen deprivation plus external beam radiation therapy for localized PC (26).

ADT, accomplished either by surgical or medical means, induces ED via decreased testosterone. The loss of testosterone causes decreased libido and decreased arterial dilatation and flow leading to sexual dysfunction (28). In order to alleviate sexual dysfunction, many modifications of ADT therapy have been looked at. One such alteration is intermittent ADT, which may be used in men with rising PSA after local therapy but no evidence of metastases. This strategy was tested by Cook *et al.*, who randomized patients with rising PSA after either primary or salvage RT to continuous ADT versus intermittent ADT and found no difference in overall survival but significantly better sexual desire in the intermittent group (P<0.001). Unfortunately in men with metastatic disease, intermittent ADT cannot be recommended as a strategy to reduce sexual dysfunction because of the inadequate therapeutic response (21,24).

Another ADT treatment option for patients, which has been shown to decrease sexual side effects, is antiandrogen monotherapy instead of a gonadotropin-releasing hormone (GnRH) agonist. A randomized trial of leuprolide versus bicalutamide 150 mg found less of a decline in sexual interest in the bicalutamide group. However, a decrease in the efficacy of this substitution must be considered (24,29). Nonetheless, exercise is likely the safest means by which sexual side effects may be minimized on ADT. Cormie *et al.* investigated the effect of a 12-week exercise program on sexual activity in 57 PC patients undergoing ADT and found a significant (P=0.045) adjusted group difference in sexual activity following the 12-week intervention. Following the intervention, the exercise group had a significantly higher percentage of participants reporting a major interest in sex (30).

In conjunction to a direct physiological decrease in libido and erectile function, ADT is also associated with decreased penile length and testicular size which is associated with

great regret and likely contributes to the sexual side effects of ADT (21). As such, continued research and verification of current evidence minimizing sexual dysfunction in ADT treated men should continue.

Penile rehabilitation

For PC survivors, sexual dysfunction following PC diagnosis and treatment is common and greatly impacts quality of life. The etiology of sexual dysfunction is multifactorial, but has been tied to orgasmic dysfunction, penile changes, climacturia and a variety of psychological causes.

Yet another contributing factor to sexual dysfunction is decreased penile length. Loss of penile length is a source of patient bother and distress and is seen after RP and ADT. Recent exploration into treatment options has found that decreased flaccid penile length may be reduced and or prevented with treatment with phosphodiesterase-5 (PDE-5) inhibitors after RP. On the other hand, in patients with non-metastatic disease, who undergo short term ADT, it has been shown that men recover supra-castrate testosterone levels. As such these patients should return to pretreatment penile length. The recovery of potency and sexual desire in these patients is dependent upon age and ADT duration (31).

Animal models exhibiting bilateral cavernosal nerve damage have demonstrated corresponding corporal smooth muscle apoptosis and fibrosis. PDE-5 inhibitors in rat models have shown reversal of these changes, with preservation of smooth muscle content and decrease in previous fibrotic deterioration. Despite this evidence seen in animal models, there remains no consensus regarding the clinical effectiveness of PDE-5 inhibitors in preserving the penile morphological changes seen (32). Loss of penile length has been observed in men following bilateral nerve sparing RP (33). The different studies examining this phenomenon had various designs and reported findings in an inconsistent manner (32,33). In addition to this, no studies have shown a "connection between corporal fibrosis and penile size changes" (33).

ED remains the most common cause of sexual dysfunction after RP. The treatment of ED is aimed at penile rehabilitation and attempts to address loss of erections by preventing the post-treatment changes. Such changes lead to ischemia, apoptosis and fibrosis. It is thought that this pathway can be interrupted with therapy to improve erections, with a goal of "natural spontaneous erections" (34). Options available for treatment include

PDE-5 inhibitors, intraurethral alprostadil, intracavernosal injections, and vacuum erection devices (VED). Despite evidence available for each, no consensus has been reached on a single protocol for penile rehabilitation following PC treatment.

The REACTT trial (effects of tadalafil treatment on erectile function recovery following bilateral nerve-sparing RP) was a randomized controlled trial with the primary outcome of erectile function in men treated with PDE-5 inhibitors following bilateral nerve-sparing RP. The study specifically examined tadalafil daily and on demand versus placebo in men with preoperative normal erectile function and clinically localized PC. At nine months follow-up, recovery of erectile function was significantly higher in the once daily group than in the control group; this was not seen in the on demand group. However, following a drug-free washout period, there were no significant differences in either group compared to placebo. Penile length, evaluated as a secondary study outcome, was significantly less affected in the once daily group compared to placebo; this effect was not observed with on demand dosing. Although the primary objective of the authors was not met, there appears to be a potential role for once daily tadalafil for penile length protection. In a previous study examining vardenafil, on demand and daily dosing resulted in significant improvement in erectile function compared to placebo. Although this seemed to conflict, the authors attributed this to the differing pharmokinetic profiles of each drug (35).

Technological and medicinal advancements have widened ED treatment options. A variety of external penile support devices exist (36). One such external device is the handheld penile vibrator, which is thought to be a good option for penile rehabilitation as it increases the neurotransmitters from the cavernous nerve terminals that are involved in penile erection. Another option is low intensity extracorporeal shockwave (LI-ESW), which is thought to improve erectile function through recruitment of endogenous stem cells (37). A VED is another option for patients; in fact studies show it is the second most commonly used method for penile rehabilitation after RP. VED uses negative pressure to distend the corporal sinusoids and to increase blood inflow to the penis (38).

Despite the wide array of therapeutic options, a study done by Megas *et al.*, which compared penile prosthesis surgery to oral PDE-5 inhibitor administration, in men with ED after nerve-sparing RP, found that currently penile prosthesis provides the most satisfactory option for patients with severe ED (39). However, the study did note that the efficacy and satisfaction results of both treatment types are considered acceptable. As such when notifying patients of the risks of therapeutic options for PC, one should also consider the availability of corrective treatments.

Proposed management algorithm

There is no consensus regarding the best rehabilitation program, but based on our clinical practice early initiation of treatment is warranted. After having a discussion with the patient regarding available treatment modalities and the evidence, or lack thereof, supporting the role of each in penile rehabilitation, take an individualized approach to addressing ED in this select patient population. If the patient is willing, it may be beneficial to take an aggressive approach in management. Despite differing evidence, there does appear to be some role of early initiation of rehabilitation in the recovery of erectile function following PC treatment. PDE-5 inhibitors have been shown effective, at least in the short-term, for recovery of erectile function and even in maintaining penile length. Since the psychosocial factor of ED should not be ignored, even a short, if not sustained, benefit could assist in a man's recovery of sexual function. VED, which are inexpensive and have minimal side effects, should be employed for possible reversal of post-treatment tissue changes. At 6 weeks post-treatment, if PDE-5 inhibitors have failed, offer the patient intracavernosal injections, but no later than 3 months out. If the patient's function remains suboptimal at a predetermined timeframe, it would be reasonable to offer penile implant, which has excellent satisfaction, or alternate therapies, such as intracavernosal injections, intraurethral therapy, or vibratory stimulation based on patient preference and motivation. Throughout management, erectile function should be assessed with a validated questionnaire at frequent intervals and with the same survey so as to follow response objectively (see *Figure 1*).

Conclusions

With the advent of the PSA era and the ability to diagnose and treat PC earlier, quality of life is a major consideration when choosing treatment (40). Male sexual health is hampered by the therapies currently available for PC. It is imperative to discuss the risks associated with each respective treatment option with men prior to PC treatment. With the development of clinical prediction models and recent studies examining the expected course of erectile function and overall male sexual health, an
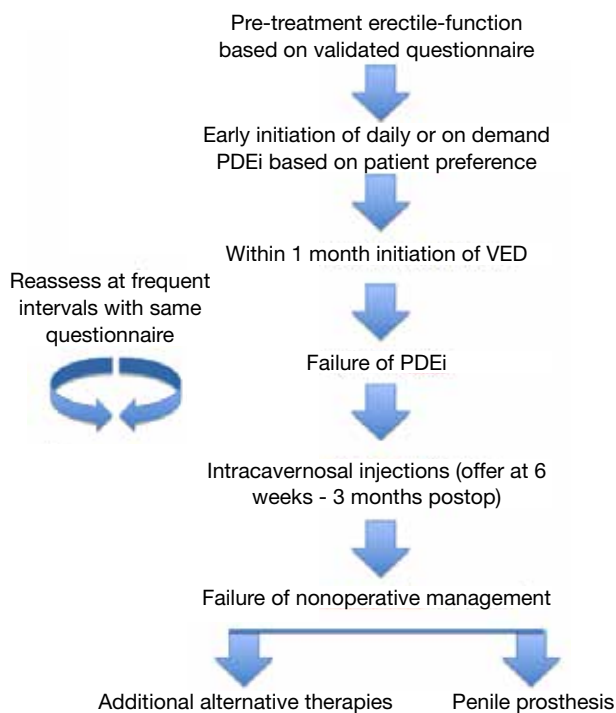
Figure 1 Proposed penile rehabilitation.

individualized discussion with each patient can hopefully be achieved. Options for penile rehabilitation are available; however, no consensus on duration or modality of choice has been defined. The future of clinical research should focus on prospective randomized controlled trials examining optimum treatment aimed at achieving spontaneous erections sooner following treatment.

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Footnote

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Challenges to treat hypogonadism in prostate cancer patients: implications for endocrinologists, urologists and radiotherapists

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Abstract: The literature suggests that the serum testosterone level required for maximum androgen receptor (AR) binding may be in the range of nanomolar and above this range of concentrations; this sexual hormone may not significantly affect tumour biology. This assumption is supported by clinical studies showing that cell proliferation markers did not change when serum T levels increased after exogenous T treatment in comparison to subjects treated with placebo. However, a considerable part of the global scientific community remains sceptical regarding the use of testosterone replacement therapy (TRT) in men suffering from hypogonadism and prostate cancer (Pca). The negative attitudes with respect to testosterone supplementation in men with hypogonadism and Pca may be justified by the relatively low number of clinical and preclinical studies that specifically dealt with how androgens affect Pca biology. More controversial still is the use of TRT in men in active surveillance or at intermediate or high risk of recurrence and treated by curative radiotherapy. In these clinical scenarios, clinicians should be aware that safety data regarding TRT are scanty limiting our ability to draw definitive conclusions on this important topic. In this review we critically discuss the newest scientific evidence concerning the new challenges in the treatment of men with hypogonadal condition and Pca providing new insights in the pharmacological and psychological approaches.

Keywords: Hypogonadism; prostate cancer (Pca); psychotherapy; radiotherapy; testosterone; treatment

Introduction

Up to 25% of older men experience hypogonadism, and the prevalence is higher in men with comorbid disease (1-5). Hypogonadal men have low serum testosterone levels and symptoms of androgen deficiency, including a decrease in energy and libido, muscle mass and bone density, as well as impairment in cognition and sexual function, and depressive symptoms (6). During the past decade, there has been increasing awareness of the health benefits conferred by testosterone replacement therapy (TRT). TRT for hypogonadism increases muscle mass and

bone mineral density, decreases fat mass, and improves mood, libido, and sexual performance (7). However, TRT may not be appropriate for all men. The most important and controversial implications are with regard to the use of testosterone therapy in men with symptomatic testosterone deficiency and a history of prostate cancer (Pca). There is an historical fear that administration of exogenous testosterone may increase risk of developing Pca or an aggressive form of the disease. Pca was explained by what was called the androgen hypothesis: (I) androgens play a key role in the etiology of Pca, (II) high testosterone is a risk factor for Pca, (III) low levels of testosterone are

protective, and (V) administering testosterone to men with existing Pca universally causes rapid growth—something every trainee at that time learned was like "pouring gasoline on a fire", or "feeding a hungry tumor". Although the dramatic effects of androgen deprivation therapy in Pca are indisputable, current evidence fails to support the concept that increasingly high serum testosterone leads to ever-greater growth of benign or malignant prostate tissue (8).

Testosterone levels as a risk determinant for Pca

It is a common belief that that higher testosterone levels are associated with increased probability of developing Pca whereas lower levels of this sexual hormone should be associated with a lower risk of developing Pca. A growing body of evidence suggests that this is a false myth since lower testosterone levels have been associated with a greater risk of developing PCa. Additionally lower testosterone levels are a negative pathologic predictor of poor outcomes in men suffering from Pca. A recent study on men with Pca, García-Cruz and co-workers found that testosterone level was inversely related the percentage of tumor in the biopsy and that lower testosterone levels were related to a higher risk of Pca progression (9). In patients treated by radical prostatectomy (RP) and low testosterone levels a significantly higher incidence of extra-prostatic invasion and biochemical recurrence were observed (10).

A logical question would be whether it is the low testosterone that increases the risk of Pca or if it is the Pca that increases the risk of having low testosterone. In this regard literature seems to support the latter. Significant increases in serum testosterone levels and gonadotropins have been reported after RP even in the absence of androgen deprivation (ADT). Authors found that one year after RP, a significant increase in luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels was observed with a significant increase in the serum testosterone levels. Interestingly, men with higher Gleason score (7 to 10) had lower serum testosterone levels at baseline with respect to men with Gleason score 2 to 6. These data seem to suggest that may be a significant impact of high grade Pca on the hypothalamic-pituitary axis. Other authors also found that Pca exerts an inhibitory effect on testosterone synthesis, with a significant increase in testosterone, LH, and FSH one yr. ehen tumor is removed by RP (11). Finally, a cross-sectional study of 55 men with localized Pca (12) showed an increase in the serum levels of LH, FSH and dihydrotestosterone (DHT) of 53%, 21% and 13%,

respectively with no significant changes in any other serum hormone investigated.

TRT in men treated by RP

A study population of 3,886 men with Pca and 6,438 age-matched controls, found no relationship between Pca risk and serum concentrations of testosterone, DHT, or free testosterone (13).

Muller *et al.* reported on 3,255 men in the placebo arm of the reduction by Dutasteride of Pca Events (REDUCE) trial who underwent planned prostate biopsies at 2 and 4 years. and found that baseline serum testosterone and DHT levels were unrelated to Pca detection or grade (14).

No prospective, controlled studies have yet been performed with adequate population sizes and duration to definitively assess Pca risk with testosterone therapy, but evidence to date fails to suggest increased risk. A meta-analysis of 19 placebo-controlled testosterone therapy cases found no significant increase in Pca or development of prostate specific antigen (PSA) >4.0 ng/mL in men treated with testosterone therapy versus placebo (15). Shabsigh *et al.* conducted a systematic review of 11 placebo-controlled studies and found that men who received testosterone therapy had neither increased Pca risk nor greater Gleason grade among those who developed Pca (16).

Several investigators have reported the use of testosterone therapy in men after curative treatment for Pca.

Kaufman reported no biochemical or clinical evidence of cancer recurrence in seven men who received testosterone therapy after prostatectomy, with the longest follow-up 12 years (17).

Agarwal *et al.* reported , no cancer recurrences in ten hypogonadal patients with organ confined Pca treated with prostatectomy and testosterone therapy (18). Khera *et al.* found that TRT is effective in improving testosterone levels, without increasing PSA values, in 57 hypogonadal men who have undergone prostatectomy (19).

Pastuszak *et al.* performed a review of 103 hypogonadal men with Pca treated with testosterone after prostatectomy (treatment group) and 49 non hypogonadal men with cancer treated with prostatectomy (reference group). There were 77 men with low/intermediate (non-high) risk cancer and 26 with high risk cancer included in the analysis. All men were treated with transdermal testosterone, and evaluated for more than 36 months. Median follow up was 27.5 months, at which time a significant increase in testosterone was observed in the treatment group. A significant increase in

prostate specific antigen was observed in the high risk and non-high risk treatment groups with no increase in the reference group. Overall 4 and 8 cases of cancer recurrence were observed in treatment and reference groups, respectively. Although this preliminary data may suggest that testosterone therapy may not harm or eventually protect against Pca development or recurrence (20), the limited number of studied patients and the absence of a randomization design do not allow us to generalize these conclusions to all patients.

These studies confirm the new concept that testosterone therapy may actually protect against Pca development or recurrence. Also, emerging data demonstrating that androgens promote less aggressive phenotypes and inhibit dedifferentiation in some Pca cell lines (21,22).

Indeed, in a recent publication, San Francisco *et al.* reported that low levels of free testosterone represented a significant risk factor for Pca disease reclassification (progression) in men undergoing active surveillance (23).

However, it should be recognized that the number of reported cases is still small and heterogeneous. There is clearly the need to design and conduct randomized trials for assessing the impact of TRT on Pca since the current recommendations, which suggests to limit TRT to symptomatic hypogonadal men successfully treated for Pca after a prudent interval, derived from retrospective analyses. The timing of T therapy initiation remains undefined. For men who underwent RP the "prudent interval" is achieved once the PSA is no longer detectable. The situation is less simple for men who received radiotherapy since undetectable levels might not ever be achieved (7).

TRR in men treated by curative radiation therapy (RT)

Treating hypogonadal men by TRT after RT can pose specific and potentially serious problems. A well-known phenomenon happening in subjects treated by RT is that PSA levels, after this treatment, do not become undetectable and a transient increase in the PSA values after nadir achievement can cause confusion regarding recurrence versus other benign causes. Additionally, short- and long-term ADT by LH-RH analogues is considered a standard of care in association with RT in intermediate and high-risk Pca improving Pca specific mortality and overall survival (24,25). The Baylor group retrospectively reviewed their data on hypogonadal men (13 patients) receiving TRT after treatment with either brachytherapy or external beam

radiotherapy (EBRT) (20). Four patients fall into a very low or low risk for recurrence, seven in the intermediate risk, and two in high risk, respectively. Four patients received ADT with RT, and three of them also received TRT, which was held during treatment. No significant changes in PSA were noted during 67.3 months (median 2.5 yrs) of follow-up. Although the small sample size and the short follow-up, the authors suggested the use of TRT in hypogonadal men treated with curative RT. Sarosdy analyzed 31 patients treated with brachytherapy and followed for a mean of 60 months (26). Three patients received combined EBRT and brachytherapy with 14 patients also receiving concomitant ADT. Interestingly, one patient experienced a rise in PSA after TRT, which steadily declined thereafter, and only one patient with 5-year post-brachytherapy has a PSA greater than 0.5 ng/dL. In no patients documented recurrence, or progression of disease and for this reason, no men stopped TRT. Morales treated five hypogonadal men with TRT once the PSA nadir was reached after EBRT, and none had evidence of recurrence based on PSA or digital rectal exam (DRE) (27). Davila and co-workers reviewed six men treated with EBRT for Pca and treated with TRT. None of whom developed biochemical recurrence although the follow-up was of only 9 months after radiation course (28).

While we have yet to see the randomized trial that will answer the question of risk associated with TRT in men Pca and treated by RT, there are interesting data that suggest as men with very low or low risk Pca according to National Comprehensive Cancer Network (NCCN) guidelines may be considered eligible for a treatment with TRT when clinically indicate. For patients with intermediate or high risk for recurrence who reach castration range during treatment with short- and long-term ADT, particular caution must be used. In fact, according with the saturation model, Pca is sensitive to T levels only in the castrate range, above which androgen receptors (ARs) are saturated and more T does not produce more growth (29). The picture is more clear in patients treated with RP, as any PSA elevation thereafter is concerning for disease recurrence. Some authors suggest that TRT may be optimal for men with "no residual disease," which may be defined for patient treated with RP as a PSA <0.2 and <2 ng/mL for the post-radiation population (30).

Morgentaler and co-workers studied a cohort of men with untreated Pca and treated with RTR for hypogonadal condition (31). Thirteen with these clinical characteristics received TRT for a median of 2.5 yrs. Gleason score at biopsy ranged from 6 to 7 in the 13 studied subjects. During

TRT mean serum testosterone concentration increased from 238 to 664 ng/dL and mean PSA as well as prostate volume did not significantly change. On re-biopsy, in 54% of subjects no evidence of Pca was observed, while two subjects experienced an upgrading of pathologic stage with no change in the oncological parameters during follow-up. No local progression or distant disease was observed in this study. Morales studied seven hypogonadal patients with untreated and treated Pca (32). Clinical criteria to stop TRT were (I) an increase in PSA level >1 ng/mL quarterly or (II) a PSA doubling time less than 12 months. After discontinuing TRT, a return to pretreatment PSA levels was an indication to reinitiate TRT. Four patients demonstrated unpredictable increases in PSA level, occurring immediately or as late as after 36 months after starting TRT. One patient with a rising PSA later underwent RP. In one patient, intermittent TRT resulted in synchronous changes in PSA levels.

Clinical experience with testosterone in men with castration resistant Pca

A body of biological and clinical evidence suggests that pulsed T treatment may have a positive impact on the biology of Pca. In this regard, an anti-proliferative effect from immediate T boosts within the physiological range was observed in androgen-sensitive Pca cells by other authors (33-35). Brendler at the Brady Urological Institute (36) used parenteral testosterone in a number of men with CRPC. He found a clinically significant improvement in several measurable parameters which included decreased pain, decreased prostate size and decreases in acid and alkaline phosphatase. Similarly, Prout and Brewer (37) administered parenteral testosterone in men who were either hormonal naive or recently or long-term castrates. In the long-term castrate group four of five men treated with testosterone for at least one month experienced clinical benefit. Of note, one man in this group with severe back pain, weakness and anorexia had a 10-month response with complete cessation of pain and decrease in acid phosphatase. On the contrary the five remaining patients received testosterone for less than 19 days and experienced tumor progression. More recently, Mathew (38) reported a case report on the use of testosterone gel replacement therapy in a man with castration-resistant prostate cancer (CRPC) and observed a sustained decrease in PSA that lasted for approximately one yr. Recently, two Phase I studies reported the results of the use of testosterone gel in men with CRPC. In the first study, Szmulewitz selected 15 men

with rising PSA and minimal bone disease. He evaluated the effect of increasing doses of transdermal testosterone in this cohort of men with early CRPC (39). Groups of five men were treated with 2.5, 5.0, or 7.5 mg/day of transdermal testosterone reaching a serum concentrations T of 305, 308, and 297 ng/dL, respectively. In this study only one patient had symptomatic progression whereas three patients had a decrease in PSA and men treated at the highest dose had a prolonged time to progression. No significant toxicity was observed in the studied cohort except one man who experienced grade 4 cardiac toxicity 53 weeks after T therapy. In the second study, Morris evaluated the effect of transdermal testosterone at a dose of 7.5 mg/day administered for 1 week, 1 month or until disease progression in small cohort of 12 patients with CRPC (40). They observed no grade 3 or 4 toxicities and no pain flares. Average serum testosterone levels were within normal limits and no objective responses were observed. Four patients had declines of PSA of at least 20% and 1 patient out of 12 achieved a >50% decline in PSA. These clinical results from a limited case series or case reports seem to suggest that systemic testosterone can be administered to men with CRPC and minimal disease burden. However no evidence exists that more advanced CRPC disease may respond to testosterone treatment as hormone sensitive Pca in early stage. Additionally no practical indications may be derived from this limited number of clinical evidence. A very comprehensive explanation of molecular events leading to tumour cell growth inhibition under rapid cycling of physiologic or supra-physiologic T boost may be explained by studies defining bipolar androgen therapy (BAT) (33-35). AR may function as a licensing factor for DNA replication, which may be important for the proliferation properties of Pca tumour cells (34). In this regard, without a timely and complete AR degradation during mitosis, the origin of DNA replication remains AR-bound stalling, in this way, the re-licensing for the subsequent cell cycle. Therefore, it seems clear that T level is a critical event in determining AR degradation. Rapid concentration changes obtained under both physiological and supra-physiological T supplementation may prevent AR degradation during mitosis, thus stabilising this receptor and resulting in G1/S-phase growth arrest (34,41).

Testosterone as protective biological determinant against the development or recurrent Pca

Although a low number of studies have investigated

men receiving TRT after RP, there is a lower rate of Pca recurrence and progression in this cohort of patients compared to subjects not receiving TRT after RP. Although most patients with Pca treated with surgical and non-surgical treatment with curative intent may be considered oncologically cured, around 15% to 40% will experience a biochemical-recurrence (42). Interestingly the recurrence rates of subjects being treated with TRT after RP seems to be even lower than found in men a low risk of recurrence and not treated with TRT (21). The explanation of this paradox was explained by Sonnenschein who evaluated the biological response of LNCaP tumor cells to different concentrations of androgens (43). The evidence derived from this study seems to suggest that the proliferative response in this cellular model may be not directly mediated by intracellular ARs and those androgens were able to trigger an inhibition of cell proliferation at higher concentrations. Other authors suggested that testosterone may have a beneficial effect on Pca by promoting the insurgence of a less aggressive phenotype (44).

New frontiers of Psychotherapy in men with hypogonadism and Pca

Most of the men live the experience of diagnosis and treatment of Pca with high levels of stress in all aspects of life.

Specifically, the diagnosis may prefigure itself not only as a threat to survival, but also to prospects for future well-being of the patient in several ways: from the physical to the social, from the familiar to the sexual.

In fact, although many men often deal with great courage this experience, some have high levels of psychological and sexological stress (45,46).

On an equal age, men with Pca have a higher probability of having erectile dysfunction (ED) of 10-15 times (47). Other distressing effects associated with the treatment include shortening of the penis (68%), loss of sexual desire (60-80%), orgasms unsatisfactory (64-87%), sexual dissatisfaction overall (61-91%) (48,49). These effects may lead to altered sexual performance; changes in relationships with partners; progressive reduction in sexual fantasies and in self-esteem (49,50).

In addition, many men are hesitant to seek help from a sexual health expert (49). This is particularly problematic for men who have undergone RP, where a rapid return to sexual activity (3 months after the surgery), associated with couple therapy, may increase the recovery rate of spontaneous erections and improve responses to treatment

for ED (51).

However, existing medical and support services are geared exclusively towards the patient, not paying enough attention to the couple's relationship and ignoring the needs of the partner, who are less likely to consider their sexual needs and more focused on the physical and psychological recovery of their man (52,53).

The psychological distress of female partners may increase if they have a limited knowledge about the post-operative course and treatment modalities.

In addition, female partners may be reluctant to share their discomfort with their partners, avoiding to adding new stress to the couple; This is even more likely when the discomfort regards the sexuality (52). This lack of communication leads the partner to live on their own and with limited tools anguish and anxiety derived from the male oncological pathology (54). The discomfort experienced by women is also aggravated by the demands of emotional support from their partners, which leads to having to manage not only their own anxiety, but also the anguish of their husbands (52,55).

Patients and partner capacity to deal with Pca and subsequent treatment side effects are correlated (56) and may have a negative impact on the marital relationship (57). The reactions of the partner to sexual dysfunction and the support they provide seem to influence the level of acceptance of sexual changes experienced by humans (58). In addition, the ability of the female partner's pleasure during sexual activity (in the absence of overt dysfunction) is a strong predictor of improved sexual satisfaction in the male partner (49).

This leads to the need to provide targeted support to couples that promotes communication and adaptation to sexuality post-cancer. This strategy, for example, has led to excellent results in couples with women suffering from breast or gynaecological cancer and will promote communication strategies that included the topic of cancer (59).

In contrast to women, men are less likely to seek psychological help and are reluctant to use sexual aids effective after treatment of Pca, despite the high levels of sexual dissatisfaction with the results of treatment. The lack of engagement with psychosocial support programs after Pca has been described in connection to a conflict with the values that underpin masculine identities (60).

Effective support interventions need to use delivery methods and sources that are acceptable for this group of patients. Men prefer individual consultations to support sexuality after Pca (61). Specifically, the interventions most

accepted are Internet-based (62,63) and self-help groups, where discussions among peers provide emotional support and information, reducing the feeling of social isolation (64). A feasibility study of a program of support among patients with Pca reported a decrease of depressive symptoms and a better self-efficacy in the short term, with people who speak more frequently about incontinence, ED and prostate-specific antigen test (65). In addition, a randomized controlled trial of a training program group focused on treatments for Pca (66), found that only with the addition of Peer discussion to the provision of information by an expert allow a better understanding and internalization of information, compared to a control group. An advantage of peer supportprovided by the patients veterans is that it is inexpensive compared to approaches provided by professional nurses.

Although this approach is very promising, currently there are no randomized controlled trials to evaluate the effectiveness of peer support in the reduction of psychological distress and sexual. However, the reduction of the costs of peer support than professional approaches, although not yet quantified, makes this a potentially lucrative source of support. As well, research to date has not identified an effective way to improve sexual and psychosocial adjustment for both men with Pca and their partners.

One of the latest interventions to improve sexual and psychosocial adjustment for both men with Pca and their partners is "Proscan for Couples" (67), a randomised control trial of a couples-based intervention that targets the specific challenges couples experience at diagnosis of localised Pca and after RP. Intervention components include psycho-education; cognitive behavioural strategies; couple relationship education focused on relationship enhancement and helping the couple to conjointly manage the stresses of cancer diagnosis and treatment; and specific psychosexual education and sexual communication. The protocol consisted of sexuality intervention DVD plus eight sessions of telephone support by peer support volunteers or professional nurse, planned from post-surgery to six months later.

This study has identified when distress in couple is highest during diagnosis period, and consequently realizing more specific and individual interventions trough a cost-effective and easily translatable approach.

The impact of Pca and hypogonadism on the psychological well-being

It is well-known how Pca bears on psychological well-being.

In fact, both the diagnosis and the relative treatment may lead to dramatic consequences, damaging the sexual and, in some cases, the general self-esteem. However, the negative relation between Pca and psychological well-being may be also reinforced by the hypogonadal condition, which, in some cases, may coexist with the cancer.

Some studies have highlighted the impact of testosterone decrease on mood. In particular, a review (68) has evidenced the role of low testosterone levels in the development of anxiety and depression. In fact, it is known that hypogonadal men may suffer with more probability of anxiety disorders and major depressive disorder, compared to men with physiological androgens levels. *Vice versa*, several researches suggest that testosterone-replacement therapy in hypogonadal men may improve mood, alleviates anxiety, and mitigates symptoms of depression. This data remains, however, not replicable in all studies.

The suggestion of a worsening by the side of a low testosterone levels is given by another study (69) investigating the prevalence of sexual dysfunctions, anxiety, depression, and a poorer quality of life in young patients with congenital hypogonadotropic hypogonadism (CHH). As supposed by researchers, low endogenous levels of testosterone might be related to the increased incidence of psychological symptoms.

Hence, since the man with both a diagnosis of a Pca and of a hypogonadism may be of a major risk to develop anxiety disorders or major depressive disorder, this patient should be supported by a psychoterapic intervention, in order to re-establish the global well-being.

Conclusions

Clinical and biological data suggest no clear relationship between testosterone levels and growth of Pca above the range of castration. While no randomized controlled trials have been specifically conducted to answer to this clinical issue retrospective clinical data indicate that TRT may be used safely in highly selected men with Pca. The negative attitudes with respect to testosterone supplementation in men with hypogonadism and Pca may be justified by the relatively low number of clinical and preclinical studies that specifically dealt with how androgens affect Pca biology. More controversial still is the use of TRT in men in active surveillance or at intermediate or high risk of recurrence and treated by curative radiotherapy. In these clinical scenarios, clinicians should be aware that safety data regarding TRT are scanty limiting our ability to

draw definitive conclusions on this important topic. Long-term data and more prospective or randomized studies are needed to conclusively change the current paradigm regarding TRT and Pca growth.

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Footnote

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Role of hormonal therapy for prostate cancer: perspective from Japanese experiences

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Abstract: Hormonal therapy has been playing an important role in the treatment of prostate cancer. However, it has recently been the subject of criticism that it shows minimal effectiveness, it may reduce patients' quality of life, and induce adverse effects. On the other hand, next-generation hormonal drugs have provided new strategies for hormonal therapy to overcome advanced prostate cancer. Therefore, it is necessary to accumulate further clinical evidence concerning the efficacy and adverse effects of hormonal therapy. And, what is important for the treatment of prostate cancer is how we use hormonal therapy most effectively. This article presents a review of the possible roles of hormonal therapy for prostate cancer based upon experience in Japan.

Keywords: Adverse effects; hormonal therapy; prostate cancer

Introduction

Hormonal therapy for the treatment of prostate cancer was first introduced about 70 years ago by Huggins and Hodges (1). At that time, methods of hormonal therapy consisted of surgical castration and/or estrogen therapy. Such treatment was only used in cases of advanced prostate cancer, because surgical castration results in permanent androgen deprivation. However, the development of luteinizing hormone releasing hormone (LH-RH) analog allowed us to compensate for androgen deprivation in such cases, and the indications for hormonal therapy have therefore changed. Hormonal therapy is frequently used as neoadjuvant and/or adjuvant therapy in patients undergoing radical prostatectomy or radiotherapy. Furthermore, hormonal therapy is sometimes used as the primary treatment for localized prostate cancer, especially in aged patients. Thus, hormonal therapy has been widely used. However, it has recently been the subject of criticism that it shows minimal effectiveness (2), it may reduce patients' quality of life (QOL), and induce adverse effects (3,4). On the other hand, next-generation hormonal drugs have

provided new strategies for hormonal therapy to overcome advanced prostate cancer.

This article presents a review of the possible roles of hormonal therapy for prostate cancer based upon experience in Japan.

Theoretical background of hormonal therapy

Most prostate cancer cells express androgen receptor (AR). In prostate cancer cells, dihydrotestosterone (DHT) is converted from testosterone produced in the testis. DHT, which binds with androgen receptor (AR) in the nuclei of prostate cancer cells, activates androgen-responsive genes, and finally plays a major role in the proliferation of prostate cancer cells. AR is a member of the steroid hormone receptor superfamily, and is activated by androgens resulting in androgenic effects on androgen-target organs. Therefore, androgen deprivation by surgical or medical castration could theoretically suppress growth of most prostate cancer cells, because serum testosterone concentrations fall to less than 50 ng/mg after castration. However, testosterone and

DHT are also converted from dehydroepiandrosterone (DHEA) and androstenedione secreted from the adrenal gland, and it has been reported that approximately 40% of androgen in prostate tissue is derived from the adrenal gland (5) (intracrine hormone synthesis). We also showed that approximately 25% of testosterone in prostate cancer tissue remained after castration (6). These results suggested that ADT for prostate cancer requires not only surgical or medical castration using LH-RH analog but also antiandrogen agents. Based upon these findings, combined androgen blockade (CAB) using castration and antiandrogen agents was advocated. On the other hand, antiandrogen agents block the activities of androgens by various mechanisms, i.e., suppression of LH secretion in the pituitary gland, inhibition of androgen binding with AR, and suppression of androgen-AR complex translocation to the nucleus. Therefore, it is possible that the different clinical outcomes of CAB treatment are due to the various types of antiandrogen agent used (7).

Role of hormonal therapy in reatment of advanced prostate cancer

Hormonal therapy is still the first choice for treatment of advanced prostate cancer, because it is useful in more than 90% of cases of advanced prostate cancer. There has been some controversy whether CAB is superior to castration alone. Recently, the results of a phase 3 randomized controlled trial of CAB in advanced prostate cancer showed that LH-RH analog +80 mg of bicalutamide was more effective than LH-RH analog alone, with favorable safety profiles and cost-effectiveness and without deterioration of QOL (8). Although the effectiveness of CAB treatment has been confirmed, most patients with advanced prostate cancer unfortunately experience relapse, a condition known as hormone refractory prostate cancer (HRPC). Chemotherapy using docetaxel is the standard treatment in such cases of relapsed prostate cancer after primary hormonal therapy failure. Other modalities of hormonal therapy using other antiandrogen agents (9), glucocorticoids, estrogens, or ketoconazole can be used as the second or third line of hormonal therapy, and have frequently been effective in so-called HRPC. Therefore, HRPC was shown to not be necessarily hormone-independent, and therefore it has been renamed castration-resistant prostate cancer (CRPC).

Mechanisms of relapse after first line hormonal therapy

Relapsed prostate cancers can be divided into 3 types after first line hormonal therapy, as shown in *Figure 1*. The first is AR signal-independent cancer, which can survive without the AR signal. This is the real HRPC, which is indicated for chemotherapy. The second is AR signal-dependent but ligand-independent. The third group still has the ligand-dependent AR signal. This type is CRPC, because it shows no more response to conventional hormonal therapy using CAB. The mechanisms of CRPC are thought to be as follows. First, these lesions are thought to have greater sensitivity of AR to androgen. AR signaling can be amplified by AR overexpression, AR mutations, or changes in AR-interacting factors, such as cofactors. With such increased sensitivity of AR, even low levels of androgen can induce AR activation. The second mechanism of CRPC is intraprostatic formation of androgens. As mentioned above (5,6), approximately 25-40% of DHT remain in castrated prostate tissue in which enzymes that convert progesterone to androgen were shown to be overexpressed. This DHT is converted from precursor steroids, which are derived from the adrenal gland and peripheral tissues. This relatively low concentration of DHT may be sufficient to stimulate AR signaling *via* increased sensitivity of AR.

Treatment of CRPC

Given the several mechanisms of CRPC (10), many therapeutic agents have been developed. The first target in CRPC is inhibition of androgen biosynthesis in prostatic cancer tissues (*Figure 2*).

As ketoconazole completely inhibits androgen synthesis, it could artificial adrenal deficiency. However, it could be useful when used carefully supplemented with prednisone (11). As estrogen is known to induce adverse cardiovascular effects, its use has been limited. However, it has been reported to be very effective in Japanese CRPC patients (12). As flutamide sometimes induces hepatic dysfunction, its use as first line treatment has been decreasing. However, flutamide not only has antiandrogenic effects but also suppressed androgen biosynthesis (7). Therefore, with close attention, the above-mentioned drugs should be reconsidered for use as second line hormonal therapy for CRPC, at least until the next-generation hormonal drugs described below become available.

Inhibition of CYP17 is promising, because upregulation of CYP17 expression has been demonstrated in CRPC
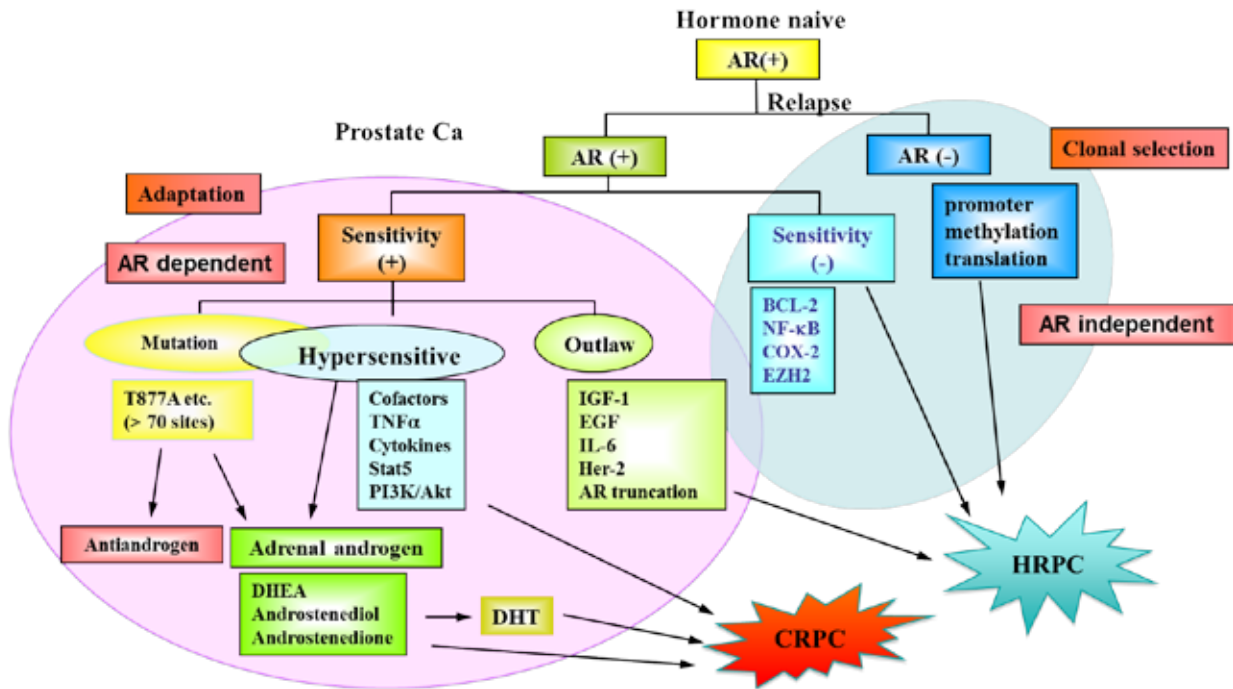
Figure 1 Mechanisms of relapse after first line hormonal therapy
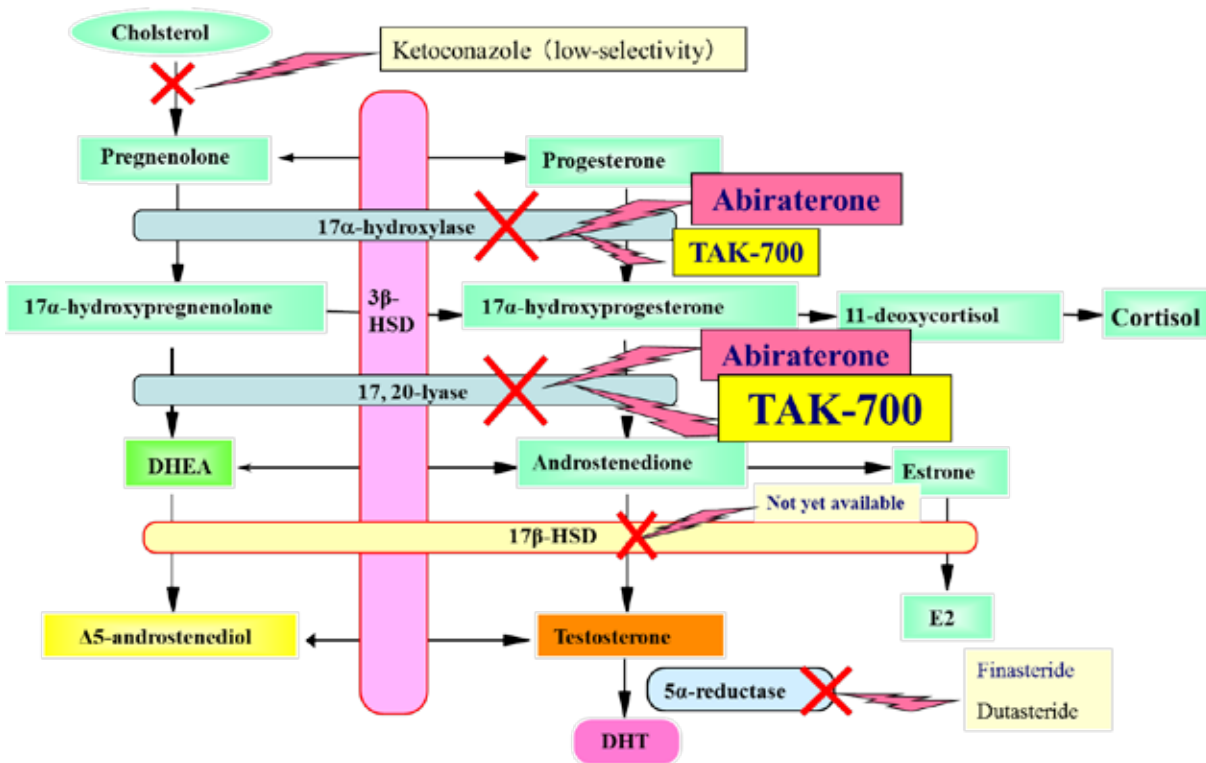
Figure 2 Therapeutic agents which could inhibit androgen biosynthesis in prostate cancer tissues

tissues (13). CYP17 catalyzes two essential reactions in androgen biosynthesis, 17-hydroxylase and C17,20 lyase (14-16). Three novel selective inhibitors of CYP17 are currently under development. Abiraterone acetate is a small-molecule CYP17A1 inhibitor. As abiraterone acetate inhibits both 17-hydroxylase and C17,20 lyase, glucocorticoid replacement is necessary. Recently, clinical trials to compare the effectiveness of abiraterone plus prednisone with those of prednisone plus placebo in CRPC patients previously treated with docetaxel showed significant improvement in overall survival of patients treated with abiraterone plus prednisone (17,18) and the US FDA has approved its use in treatment of advanced prostate cancer progressing after docetaxel treatment. TAK-700 (orteronel) is a more selective inhibitor of CYP17, because inhibition of C17,20 lyase is more potent than that of 17-hydroxylase (19). Thus, glucocorticoid replacement may be unnecessary or its requirement may be only minimal in comparison to patients treated with abiraterone. Phase 3 studies of TAK-700 are currently underway in both CRPC patients who have received chemotherapy and CRPC patients who are chemotherapy naive. TOK-001 is also a selective inhibitor of CYP17 (20). This compound also downregulates AR expression.

MDV3100 is a novel second generation antiandrogen. MDV3100 has greater binding affinity for AR to inhibit DNA binding of androgens to AR (21). MDV3100 also inhibits nuclear translocation of androgens. Furthermore, it can inhibit the association of AR and DNA within the cell nucleus. In a phase 1/2 multicenter study of 140 patients with CRPC, MDV3100 showed overall ≥50% PSA decrease in 56% of patients (22). The AFFIRM phase 3 study was conducted in men with CRPC who had progressed after treatment with docetaxel-based chemotherapy. The trial stopped in November 2011 because a planned interim analysis showed a 37% reduction in the risk of death with MDV3100 over placebo (median, 18.4 *vs*. 13.6 months; HR, 0.631).

Treatment for AR signal-dependent but ligand-independent CRPC

Although intraprostatic androgen concentration can be reduced by next-generation hormonal drugs, AR can sometimes autonomously maintain transcription ability without ligand. Molecular targeted therapy may be indicated in such cases in which AR-interacting proteins upregulate transcriptional activity of AR by cross-talk.

On the other hand, an AR splice variant has autonomous transcription ability (23). However, variant AR is thought to bind to AR-binding domain as a heterodimer with intact AR. Therefore, MDV3100, which inhibits ligand binding to AR, may be effective for such variant AR-induced CRPC (24).

Ideally, direct AR-targeted therapy may be most effective; such a drug has recently been reported (25), and the results of clinical trials are awaited.

How should we select the second line treatment after relapse of the first line hormonal therapy?

Many next-generation hormonal drugs are now available. On the other hand, new chemotherapeutic and immunotherapeutic drugs have also been developed. Therefore, it is very important to establish strategies for the sequential use of such drugs after relapse of the first line hormonal therapy. Many factors, including the duration of effectiveness of the first line hormonal therapy, immunohistochemical findings of re-biopsied prostate tissue, and serum & intraprostatic concentrations of androgens, may be helpful to decide on the next drug(s) to be administered. Of course, clinical trials for such purposes are required.

Role of hormonal therapy for high-risk or locally advanced localized prostate cancer

Patients with high-risk or locally advanced prostate cancer with high Gleason score, elevated PSA level, and advanced clinical stage have a high probability of treatment failure after initial management by single-treatment modalities, such as hormonal therapy (26), radical prostatectomy, external beam radiation therapy (EBRT), or brachytherapy (27,28). Therefore, it is important to establish the most effective treatment strategy for patients with high-risk prostate cancer. As high-risk patients may have locally advanced disease with direct extension and/or micrometastases, various combinations of treatments have been developed to augment cancer-specific survival. Neoadjuvant and/or adjuvant hormonal therapy offer synergistic enhancement of radiation therapy or radical prostatectomy due to induction of apoptosis. Moreover, hormonal therapy may play a role in elimination of occult systemic disease (29,30). Whereas many studies have demonstrated benefits of hormonal therapy used in conjunction with EBRT to treat locally advanced prostate cancer (31-35), questions remain, including the details of

426

Namiki et al. Hormonal therapy for prostate cancer

the duration, timing, and contents of hormonal therapy. The results of the Radiation Oncology Group trial (RTOG)-9202 regarding the effectiveness and adverse effects of hormone therapy are very informative (36). These results suggest that cause-specific benefits of hormone therapy may have been offset by deaths from other causes induced by hormone therapy. As the prolonged use of hormonal therapy results in increased incidence rates of adverse events, investigation of the optimal duration of hormonal therapy with maximization of clinical outcome and minimization of toxicity is a logical step in the management of localized high-risk prostate cancer. Further, we should determine which patients with high risk prostate cancer will actually benefit from hormonal therapy even if there is some compromise in QOL associated with the adverse event profile of this treatment method. Clinical trials have demonstrated the superiority of longer periods of adjuvant hormonal therapy (34). Therefore, with sufficient care to prevent adverse effects due to hormonal therapy, better outcomes may be achieved with longer periods of neoadjuvant and/or adjuvant hormonal therapy. Tri-modality treatment (EBRT + brachytherapy + hormonal therapy) has attracted attention as another method to produce better outcomes in cases of high-risk prostate cancer (37). According to the American Brachytherapy Society (ABS), brachytherapy alone is not recommended for high-risk prostate cancer but can be used as a boost in conjunction with EBRT (38). In this multimodal approach, a combination of brachytherapy and EBRT theoretically delivers a possible escalated dose to the prostate and at the same time to extracapsular cancer extension. Although the ABS provides no clear indications for neoadjuvant and/or adjuvant hormonal therapy with combination of brachytherapy and EBRT in high-risk prostate cancer, the duration of hormonal therapy could be reduced with such multi-modality radiotherapy. A new trial has just begun to investigate the optimal duration of hormonal therapy in combination with brachytherapy and EBRT (39).

In contrast to the many efforts to develop better treatment regimens for radiotherapy with hormonal therapy, there have been few clinical trials investigating the effectiveness of neoadjuvant and/or adjuvant hormonal therapy with radical prostatectomy (40). One reason for this is that early studies of neoadjuvant hormonal therapy did not confirm the improvement of overall survival despite improvements in the pathological findings. In addition, surgeons may have less interest in medical treatments, such as hormonal therapy. However, surgeons should consider the best methods for improving the results in cases of high-risk prostate cancer, because recent reports have indicated the superiority of radiotherapy for high-risk prostate cancer compared with radical prostatectomy (41). Recently Dorff *et al.* reported that 2 years of adjuvant androgen deprivation therapy (ADT) after radical prostatectomy resulted in an extremely low rate of disease recurrence and prostate cancer-specific death for high-risk patients in the SWOG S9921 Study (42).

Finally, it should be stressed that it may be possible to eradicate prostate cancer death even in the high-risk or locally advanced prostate cancer with appropriate use of hormonal therapy in combination with radiotherapy or radical prostatectomy. Therefore, further well-designed clinical trials are required.

Efficacy of primary hormonal therapy for localized or low-risk prostate cancer

Hormonal therapy is not recommended as the primary treatment for localized prostate cancer according to representative guidelines, such as the National Comprehensive Cancer Network (NCCN) guidelines. However, according to the Japanese cancer registration statistics, many patients with localized prostate cancer have actually been treated with primary hormonal therapy (43). Despite urologist's explanation regarding the various treatments for localized prostate cancer, many patients select primary hormonal therapy in Japan (44). It is likely that many patients with localized prostate cancer select primary hormonal therapy because such medical treatment is more acceptable than more invasive treatments, such as surgery, at least for many Japanese patients. In addition, urologists themselves may also influence patients' decisions because they have experience regarding the effectiveness of primary hormonal therapy.

The ethnic background of patients may play an important role in the effectiveness of hormonal therapy and in susceptibility to adverse effects. The efficacy of hormonal therapy has been compared between Japanese-Americans and Caucasians living in Hawaii (45). Both groups had similar backgrounds, but both overall and cause-specific survival rates of Japanese-Americans were better than those of Caucasian subjects (*Figure 3*). The overall survival rate was also compared among Caucasian, Chinese, and Filipino patients living in Hawaii. The Chinese subjects showed similar trends to Japanese patients. Therefore, sensitivity of prostate cancer to hormonal therapy and susceptibility to
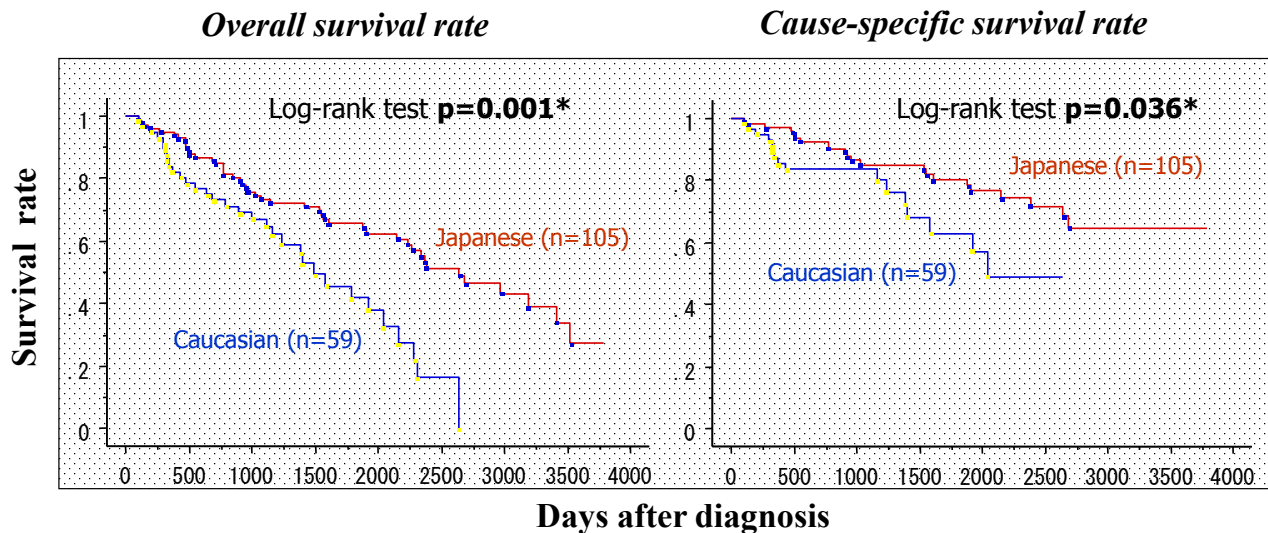
Overall survival rate

Cause-specific survival rate

Figure 3 Comparison of the efficacy of hormonal therapy between Japanese-Americans and Caucasians living in Hawaii (modified from reference 45)

adverse effects may differ among ethnic groups.

Akaza *et al*. reported that overall survival of patients with localized or locally advanced prostate cancer treated with primary hormonal therapy was equivalent to life expectancy of age-matched subjects in the healthy population (46). Before Akaza's report Egawa *et al*. had already reported that primary hormonal therapy was as effective as radical prostatectomy with regard to disease-specific survival rate in localized prostate cancer (47). In their report, disease-specific survival rate at 10 years of 56 patients with well-differentiated prostate cancer treated with primary hormonal therapy was 100%. Why is the outcome of primary hormonal therapy so excellent, especially in well-differentiated prostate cancer? Kitagawa *et al*. analyzed the histological effects of hormonal therapy in specimens from patients treated with radical prostatectomy after neoadjuvant hormonal therapy (48). They reported that histologically cured or nearly cured patients accounted for more than 40% of the total number. In addition, the recurrence-free survival rate of patients with histologically complete apoptosis was 100%. These results suggest that some cases of localized prostate cancer could be cured by primary hormonal therapy alone. Schulman *et al*. also performed neoadjuvant hormonal treatment for 3 months before radical prostatectomy in patients with localized prostate cancer, and reported good histological effects (49). Labrie also reported that long-term control of about 80%

of Stage B prostate cancers could be achieved with primary hormonal therapy (50).

These reports raise questions about which groups of patients would be good candidates for primary hormonal therapy. We performed a retrospective review of the efficacy of primary hormonal therapy in 628 patients with localized or locally advanced prostate cancer treated with primary hormonal therapy at 7 institutions in Japan, and attempted to predict patients in whom the disease could be controlled for long periods by primary hormonal therapy (51). Disease-specific and overall survival rates at 8 years in all patients were 89.1% and 75.0%, respectively. In addition, disease-specific survival rate at 8 years of patients given combined androgen blockade (CAB) treatment was 95.3%, which was significantly higher than that of patients treated with castration alone. We classified the patients into three risk groups based on pretreatment PSA level and Gleason score according to a modification of the D'Amico risk grouping (52). Disease-specific survival rates at 8 years of low-, intermediate-, and high-risk groups were 97.6%, 95.4%, and 78.3%, respectively. Next, we divided low- and intermediate-risk patients into two groups with PSA level <0.2 ng/mL after hormonal therapy. The time to PSA level <0.2 was within 6 months in 192 patients (good response group, Group G). These patients accounted for 30.6% of the total patient population. We classified the 139 patients in whom the PSA level did not fall below 0.2 within 6 months
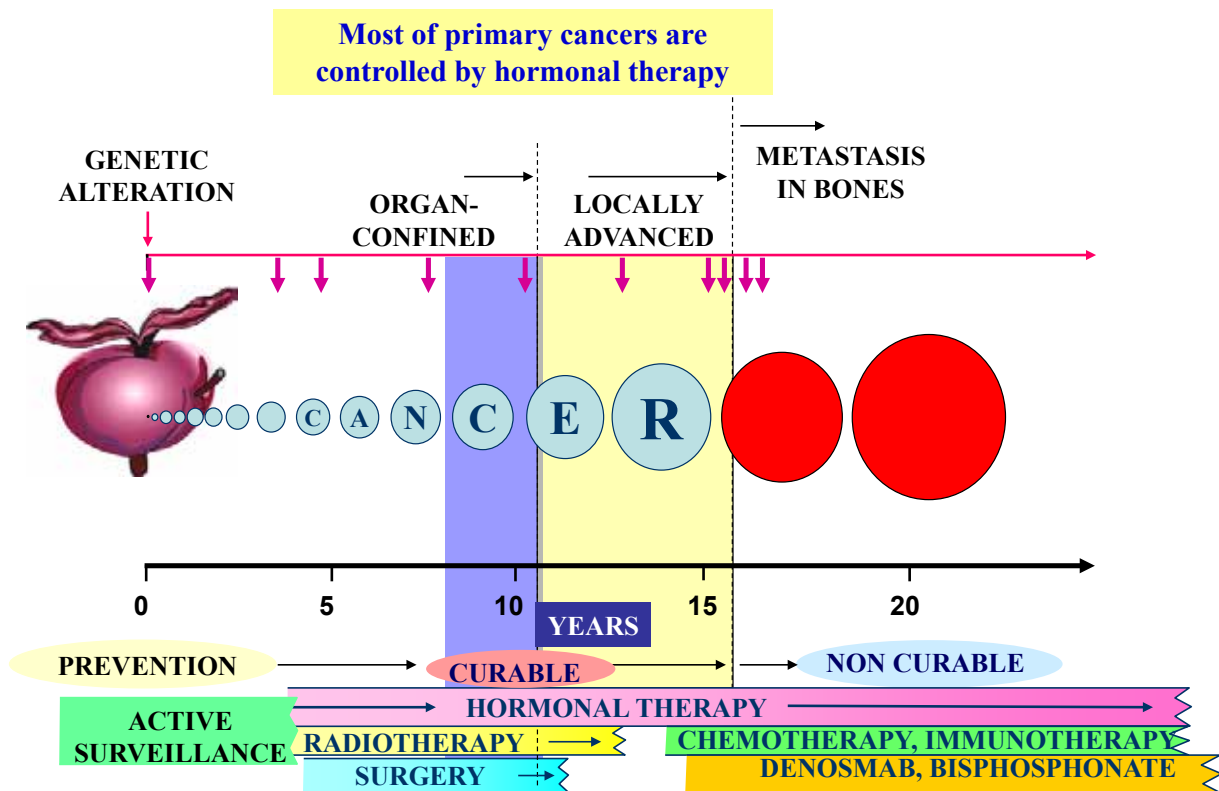
Figure 4 Evolution of prostate cancer (modified from reference 53)

as the poor response group (Group P). The disease-specific survival rates at 8 years of Groups G and P were 98.9% and 94.0%, respectively. Notably, there were no cancer-related deaths during the observation period among the 133 patients in Group G receiving CAB treatment in this study. Although a randomized controlled trial may be necessary for utilization of primary hormonal therapy in patients in whom such treatment is considered more effective, based on the results of our study T1c-T3 patients with PSA level ≤20 ng/mL and Gleason score ≤7 may be good candidates for the initial hormonal therapy. These patients accounted for 52.7% of the total number of T1c-T3 patients in our study. Hormonal therapy may be suitable as the initial treatment in such patients, but changing to another curative regimen or combination therapy with radiotherapy or radical prostatectomy should be considered if the PSA value does not decrease to <0.2 ng/mL after 6 months of hormonal therapy. However, in patients in whom the PSA value drops to <0.2 ng/mL within 6 months of the commencement of hormonal therapy, continuation of the same regimen may

be reasonable with careful observation.

Another preference for early-stage prostate cancer patients involves active surveillance. No study in PSA screened low-risk cancer has ever shown that treatment is better than no treatment. Therefore, further investigations are necessary to compare the disease-specific or progression-free survival rates of a low-risk group, such as Group G, with those of an active surveillance group. The PIVOT trial has recently shown that radical prostatectomy did not reduce mortality to a greater extent than observation in men with low PSA or low-risk prostate cancer. However, even cancer cells for which observation alone without treatment was at first thought to be sufficient are not always inactive after long periods. These cancer cells may become impossible to control due to malignant transformation by gene mutation during follow-up (*Figure 4*) (53). In addition, most patients are anxious about the status of their disease, and few are willing to rely solely on active surveillance (54). Another possible problem is the period over which hormonal therapy should be continued. Labrie

et al. performed long-term hormonal therapy in stage B and C patients and discontinued the treatment in patients who did not show PSA recurrence. An increase in PSA occurred in only 2 of 33 patients with stage B and C prostate cancer who stopped treatment after continuous CAB for more than 6.5 years. In addition, seven of eight patients with localized prostate cancer who received CAB treatment continuously for 6.5-9.0 years before stopping treatment showed no PSA failure at least 5 years after cessation of CAB. CAB treatment was restarted in patients showing PSA recurrence after cessation of the initial hormonal treatment, and control was achieved again in most cases. Thus, it was concluded that CAB treatment for 7 years may be suitable in such cases. Recently, Tanaka *et al.* also investigated when hormonal therapy could be discontinued based on nadir PSA levels after commencement of treatment. They concluded that a relatively short period, *e.g.*, 3 years, may be sufficient in cases in which the nadir PSA dropped to <0.01 ng/mL (55). Although intermittent hormonal therapy was reported to be useful for the treatment of advanced prostate cancer to maintain sensitivity to androgens (56), care is required in application of this treatment for localized prostate cancer as cancer that could be controlled over the long-term or may be cured by appropriate hormonal therapy (50) may progress to develop more malignant potential by incomplete androgen ablation.

According to the modified D'Amico classification reported previously (51), disease-specific and progression-free survival rates of the high-risk group treated with primary hormonal therapy at 5 years were 87.8% and 58.8%, respectively. From these results long-term control by primary hormonal therapy seems difficult in the high-risk group. However, Mizokami *et al.* (57) reanalyzed the previous data and showed that the results for the high-risk group are not necessarily pessimistic in patients in whom the PSA value drops below 0.2 ng/mL. They proposed that high-risk prostate cancer patients should be first treated with neoadjuvant CAB. Then, once a PSA value of <0.2 has been reached, patients with favorable parameters (Gleason score ≤6, pretreatment PSA ≤20, time to PSA <0.2 ng/mL within 6 months after commencement of hormonal therapy) are likely to have reduced likelihood (<25%) of relapse at 10 years after commencement of CAB. Therefore, such patients could select any treatment option, *e.g.*, surgery, radiotherapy, or primary hormonal therapy. However, they recommend that poor responders to neoadjuvant CAB should be treated with more intensive therapy using CAB combined with high dose rate

(HDR)-brachytherapy, intensity-moderated radiotherapy (I-MRT), or some forms of chemotherapy.

QOL and medical cost

Long-term hormonal therapy is sometimes criticized for reducing patients' QOL. In our institution, the QOL of prostate cancer patients treated with primary hormonal therapy was investigated using the Androgen Deficiency in Aging Male (ADAM) questionnaire to allow comparison with healthy aged men who visited our institution for medical examinations. Surprisingly, the QOL of men receiving primary hormonal therapy was rather better than that of the healthy controls, except for sexual function in men aged 50-59 years (57). Indeed, most prostate cancer patients reported no anxiety regarding their primary disease or side effects of the treatment. Kato *et al.* evaluated health-related QOL (HRQOL) in Japanese men receiving hormonal therapy for prostate cancer using SF-36 and USLA-PCI59). They concluded that general HRQOL was mostly unaffected by hormonal therapy and that most patients did not report sexual anxiety despite deterioration of sexual function. These reports suggest that QOL of prostatic cancer patients receiving hormonal therapy is rather better than previously thought, at least in Japan (58).

Medical costs can also be a significant issue. The medical costs of hormonal therapy are higher than those of other treatments, but there are costs that are calculated directly, such as medical costs or transportation for hospital visits, and costs that cannot be calculated, such as loss of employment for disease treatment or psychological burden. Therefore, estimation of cost is very difficult, and further studies are required to compare costs with other types of treatment.

Adverse effects

Several recent studies indicated that ADT increases the incidences of cardiovascular disease and bone fractures. Keating *et al.* demonstrated that GnRH agonist increased the risk of diabetes mellitus (DM), coronary heart disease (CHD), myocardial infarction, and sudden cardiac death compared with the risks in patients without hormonal therapy (59). However, their study had some limitations. First, this was not a randomized study. Therefore, patients receiving GnRH agonist may have been associated with higher levels of background factors contributing to DM

430

Namiki et al. Hormonal therapy for prostate cancer

Table 1 Management strategies for ADT-associated morbidities

Complication	Management strategy
Cardiovascular risk factors and disease	1) Non-smoking
	2) Consultation for diet and exercise
	3) Regular monitoring of serum lipid profiles
Osteoporosis and fractures	1) Regular monitoring of BMD
	2) Consultation for exercise, diet with adequate calcium and Vit D intake
	3) Bisphosphonates
Endocrine and metabolic dysfunction	1) Consultation for nutrition, exercise, and weight control prior to ADT
	2) Regular monitoring of HbA1c and fasting blood sugar
Hot flash	1) Chlormadinone acetate
	2) SSRI
Sexual dysfunction	1) PDE-5 inhibitors
	2) Cavernous injection of PGE-1
	3) Vacuum erection devices

or heart disease. For example, older men who are more likely to receive hormonal therapy are also more likely to develop DM or CHD. Second, we cannot exclude the possibility that men receiving regular injections were more likely to be diagnosed with DM or CHD because of the greater frequency of medical consultations. D'Amico *et al.* showed that a subset of men age 65 years or older who received 6 months of ADT demonstrated shorter intervals to fatal myocardial infarction compared with men in this age group who did not receive ADT (60). However, this study was criticized by the authors of another paper recently published in the same journal (61). One major criticism was that D'Amico *et al.* did not show any difference in total number of fatal myocardial infarctions between groups. Their study was also criticized for its short treatment duration, shorter follow-up, and the lack of information on cardiovascular disease (CVD) risk factors. Efstatiou *et al.* described the first analysis using data from a large prospective study to directly address the potential relationship between GnRH agonists and cardiovascular mortality (61). In this study, patients with locally advanced prostate cancer who selected radiotherapy were randomly assigned to one of two arms. Patients in arm 1 received radiotherapy plus adjuvant hormonal therapy for 4.2 years on average. Those in arm 2 initially received only radiotherapy, and thereafter 64% of patients received salvage hormonal therapy after recurrence. Pretreatment characteristics, including CVD risk factors, were similar between the two arms. Surprisingly, at 9 years,

cardiovascular mortality rate for men treated with adjuvant hormonal therapy was 8.4%, which was less than the rate of 11.4% for men without adjuvant hormonal therapy. However, patients with established CVD risk factors were significantly associated with greater cardiovascular mortality. Therefore, criticism of hormonal therapy should not be simplistic, but rather should focus on decreasing cardiovascular risk factors and managing CVD.

With regard to the adverse effects of hormonal therapy data for the general population show that the incidence of ischemic heart disease is much lower in Japanese than in Westerner subjects. The incidence of bone fractures is much lower in Japanese than in Western populations. Based on these data, we expect that the adverse effects of hormonal therapy will be less in Japanese populations. Akaza *et al.* conducted the J CaP study as a surveillance study of hormonal therapy in Japan (62). The data showed that the cardiovascular mortality rate in Japanese patients undergoing ADT was almost the same as the rate in the general population, as expected. Nevertheless, androgen deprivation could induce a variety of adverse effects even in Japan, because the adoption of a more Western lifestyle may increase the susceptibility to adverse effects of hormonal therapy. Therefore, efforts should be made to prevent or minimize such adverse effects. Management strategies for ADT-associated morbidities are shown in *Table 1*. It is well known that bone mineral density is decreased during long-term ADT. Therefore, the fracture rate after ADT is not low. We performed

a nonrandomized prospective study to confirm the usefulness of bisphosphonate for improvement of bone mineral density in patients receiving hormonal therapy (63). Whereas bone mineral densities of patients not receiving risedronate continued to decrease, those of patients receiving risedronate increased. Management of endocrine and metabolic dysfunctions, such as DM, is very important, although most urologists do not pay adequate attention to such nonsurgical issues. Androgen deficiency is now attracting attention as one of the causes of metabolic syndrome. Basaria *et al.* reported that hormonal therapy induces metabolic syndrome, which they detected in more than 50% of men receiving long-term ADT (64). Therefore, we should carefully manage patients receiving hormonal therapy, and this is not as difficult as performing complicated surgery. The Endocrine Society Clinical Practice Guidelines will be helpful in preventing cardiovascular disease and DM. Furthermore, we should make our own clinical guidelines for urologists managing prostate cancer patients with hormonal therapy.

Patient satisfaction

The Prostate Cancer Outcome Study yielded interesting results (65). In this study, patient satisfaction was compared after each treatment, i.e., watchful waiting, primary androgen deprivation, radiotherapy, and radical prostatectomy. Satisfaction was higher in men receiving primary ADT than in those managed by watchful waiting or radical prostatectomy. In addition, most patients indicated that they would make the same choice if they had to select the treatment again. Thus, in patients requiring hormonal therapy, criticism of primary ADT should not be simplistic, but rather efforts should focus on decreasing its adverse effects.

Conclusions

Since its introduction about 70 years ago by Huggins and Hodges (1), hormonal therapy has played as important role in the treatment of prostate cancer. Recently, however, hormonal therapy has been the subject of frequent criticism. Some authors reported that it showed minimal effectiveness, while others suggested that it may reduce patients' QOL and induce adverse effects. Such reports should be evaluated very carefully (66). From Japanese experiences using hormonal therapy, we suspect that there may be ethnic differences in efficacy and adverse

effects of hormonal therapy. Therefore, it is necessary to accumulate further clinical evidence concerning the efficacy and adverse effects of hormonal therapy. We should also strive to decrease its adverse effects, because changes in lifestyle may increase susceptibility to the adverse effects of hormonal therapy even in Japan. It is expected that new hormonal compounds, such as selective androgen receptor modulators [SARM (67)] capable of specifically targeting prostate cancer, will be developed in the near future.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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How to evaluate sexual health in cancer patients: development of the EORTC sexual health questionnaire for cancer patients

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Background: The aim of the study is to describe the development of a comprehensive European Organisation for Research and Treatment of Cancer (EORTC) questionnaire to assess sexual health of female and male cancer patients and for cancer survivors.
Methods: According to the EORTC guidelines, the development of an EORTC sexual health questionnaire is typically organised in four phases. The first phases comprise a literature search following interviews with patient and health care professionals (HCPs) (phase 1) and the operationalization into items (phase 2). The translation process is formally conducted according to the EORTC QLG Translation guidelines with a rigorous forward-backward procedure supported by native speakers.
Results: Studies on sexuality in oncology patients which were identified by a literature search predominantly focused on issues of activity, experiences of sexual dysfunction, and satisfaction with sexual functioning. The literature review identified themes beyond these aspects. In total 53 potentially relevant issues were presented to 107 patients and 83 HCPs, different evaluations were found.
Conclusions: A questionnaire that includes physical, psychological, and social aspects of sexuality of cancer survivors will be needed. Pre-testing and validation of the questionnaire will be done in future (phases 3 and 4). Divergent ratings of patients and professionals should be further investigated.

Keywords: Cancer; sexual health; European Organisation for Research and Treatment of Cancer (EORTC) sexual health questionnaire

Introduction

An individual's sexual response can be affected in a number of ways that involves the physical, psychological, interpersonal, and behavioural aspects of a person. The most common sexual problems for people who have cancer are loss of desire for sexual activity in both men and women, problems achieving and maintaining an erection in men, and pain with intercourse in women (1). Unlike many other physiological side effects of cancer treatment, sexual problems do not tend to resolve within the first year or two of disease-free survival (2-5) rather, they may remain constant and fairly

severe or even continue to increase. Long-term effects of different treatment on sexual functioning have been studied in cervical cancer survivors (6,7). Existing research has mainly focused on women who have breast or gynecologic cancer and men who have prostate cancer (8). Less is known about how other types of cancers affect sexual health. Although it is unclear how much sexual problems influence a survivor's rating of overall health-related quality of life, these problems are clearly bothersome to many patients and interfere with a return to normal post-treatment life. In 2014, the National Cancer Institute at the National Institutes of Health published data on some form of sexual

dysfunction occurring in 40-100% of persons diagnosed with cancer (1,9-13). Sexual dysfunction is less broadly defined than sexuality and is characterized by dysfunction of one of the four phases of the sexual response cycle, or pain during intercourse (14,15). The World Health Organization (WHO) defines sexuality as a central aspect of being human throughout life and encompasses sex, gender identities and roles, sexual orientation, eroticism, pleasure, intimacy and reproduction. Comparatively, sexual health is defined as a state of physical, emotional, mental and social well-being relating to sexuality, and is not merely the absence of disease, dysfunction or infirmity (16). Cancer and cancer therapies are frequently associated with changes in sexual health. However, there is a lack of consensus regarding valid outcome measures for assessing sexual functioning in cancer patients (12,17). There is no single self-report measure that can be recommended for cancer clinical trials (18,19) on the basis of a broader definition of sexual health. Several modules for various cancer sites (e.g., breast, gynecologic, prostate) were developed by the European Organisation for Research and Treatment of Cancer Quality of Life Group (EORTC-QLG) which include a limited number of sexual functioning items. The U.S. National Institutes of Health PROMIS® Network has already developed a self-report measure of sexual function and satisfaction for cancer populations (20). However, the existing questionnaires do not cover the whole range of sexual health. Currently, there is no comprehensive instrument that assesses aspects of sexual health in a broader sense.

The aim of the study is to develop a comprehensive EORTC questionnaire to assess sexual health of male and female cancer patients and for cancer survivors. We describe the development of the sexual health questionnaire according to the EORTC guidelines (21).

Materials and methods

According to the EORTC guidelines (21) the development of an EORTC questionnaire is typically organised in four phases. The first phases comprise a literature search following interviews with patients and health care professionals (HCPs) (phase 1) and the operationalization into items (phase 2).

Literature search and interviews

The literature search is focused on sexual health in cancer patients with a search strategy which was kept broad. The

following combinations of keywords were used to identify papers in PubMed covering the period January 1993 to January 2012: 'neoplasms[mesh]' or 'neoplas*[tw]' or 'tumor[tw]' or 'tumors[tw]' or 'tumou*[tw]' or 'cancer*[tw]' or 'carcinom*[tw]' or 'oncolog*[tw]' and 'sexuality[mesh]' or 'sexual function or sexual function[all fields]' or 'sexual function[tiab]' or 'sexual dysfunction' or 'sexual dysfunction [all fields]' or 'sexual dysfunction[tiab]'. Articles focusing on sexuality that conceptually exceeds issues relating to sexual function, sexual activity or sexual response cycle in cancer patients and/or cancer survivors were selected through title and abstract screening. For inclusion, original research articles had to be full reports in English and published in peer-reviewed journals. Qualitative and quantitative studies are included in the review. Additionally, an evaluation of measures related to sexuality in cancer patients was conducted (Den Oudsten *et al.*, unpublished data). Issues which resulted from the literature search were summarized in an issue list. The same list was then presented to patients and HCPs recruited by collaborators within the EORTC Quality of Life Group for feedback on appropriateness of content and breadth of coverage on a four-point Likert scale. The following demographic data from the HCPs were asked: professional background, cancer sites they were most familiar with, duration of being involved in the care of patients with cancer, sex and country. A case report form including the most important clinical data related to disease and therapy was given to patients. The list of issues was translated into several languages. Members and collaborators of the EORTC-QLG spread out the questionnaire to other HCP specialists in oncology such as psycho-oncologists, radiotherapist or gynaecologists in more than 20 institutions in different countries (*Table 1*).

For the final decision which issues on sexual health are definitely important for cancer patients, mean scores for relevance and priority ratings were defined according to the EORTC-QLG guidelines (21) using the following criteria: (I) mean score for HCPs <2 versus ≥2; and (II) mean score for patients <2 versus ≥2; (III) priority should have been rated by more than 30% of the HCPs; and (IV) >30% of patients as highly relevant to be included. Issues that met three or four of these criteria were kept in the list. Issues that met less than three criteria were deleted, unless the interviews provided strong arguments for retaining them. Additional new issues were included if they were mentioned by a considerable number of patients or HCPs. Based on the remaining issues the items for the provisional questionnaire were developed.

Table 1 Issue list

Sexual activity

  Frequency of sexual activity

  Reasons for being sexually inactive

  Satisfaction with the frequency of sexual activity

  Importance of having an active sexual life

  Level of hesitation to initiate sexual activities

Sexual desire

  Frequency of sexual desire

  Level of desire

  Distress caused by decreased libido

  Satisfaction with frequency of sexual desire

  Satisfaction with level of desire

Sexual arousal

  Frequency of sexual arousal

  Level of sexual arousal

  Satisfaction with level of sexual arousal

Orgasm

  Ability to achieve an orgasm

  Difficulty to reach an orgasm

  Satisfaction with the ability to orgasm

  Satisfaction with the frequency of orgasm

Side-effects influencing sexual activity

  Incontinence (urine/fecal) during foreplay or intercourse

  Hair loss (indirectly) affecting sexual response

  Fatigue/lack of energy affecting sex life

  Scarring/organ loss (indirectly) affecting sexual response/ satisfaction

Pain

  Frequency of pain during/after sexual activity

  Level of pain during/after sexual activity

Intimacy

  Change in amount of affection expressed

  The level of emotional intimacy

  Satisfaction with level of affection or intimacy

Fear

  Fear that sex will be painful

  Fear of injury during intercourse

  Fear harming the incision during intercourse

Table 1 (*continued*)

Table 1 (*continued*)

Communication/Relationship issues

  Satisfaction communication partner

  Partner is afraid to touch, afraid to cause pain

  Experience of emotional distance from spouse

  Insecurity regarding ability to satisfy the partner

  Partner response to changes in sexual functioning: accepting/rejecting

Distress/bothered/satisfaction (general)

  Level of comfort with one's sexuality

  Change in presence sexual fantasies

  Level of sexual enjoyment

  Sexual satisfaction

  Reduced sexual enjoyment

  To what extent are sexual dysfunctions distressing

Health care/aids

  Need for care because of sexual difficulties

  Communication about sexual issues with health professionals

Male sexual health

  Ejaculation

    Dry orgasm

    Retrograde ejaculation

  Erectile function

    Ability to get an erection

    Ability to maintain an erection (firm enough for sex)

    Satisfaction with ability to maintain erection or level of firmness

    Level of confidence in getting an erection and keeping one

  Masculinity

    Change in masculinity/feeling less masculine

Female sexual health

  Lubrication

    Insufficient/decreased lubrication

    Frequency of spotting/bleeding after sexual intercourse

  Feminity

    Change in femininity/feeling less feminine

    Masturbation (included after discussion during an EORTC QLG meeting)

Operationalization into items

The process of translation is formally conducted according to the EORTC QLG Translation guidelines with a rigorous forward-backward procedure (18) supported by native speakers. The underlying English issues will be operationalised into items first. If items already have been used in another validated EORTC questionnaire they normally are included in their original wording. Questions are phrased in a way that they fit into the 4-point answering format of the EORTC ranging from "not at all" to "very much". A time frame of 4 weeks is chosen as suggested in the EORTC guidelines (21). A standardized introduction was modified for the issue of sexual health.

Phase 3 is the pilot-testing of the final item list and is actually under construction. A validation of the questionnaire will be done in phase 4.

Results

Results of the literature search

Many generic and cancer-specific sexual health issues have been identified in the literature review. A total of 4,518 articles were screened of which 3,461 articles were excluded because they did not focus on cancer patients and/or did not report sexuality as an outcome measure. Another 924 articles were excluded because they were literature reviews or presented only domain scores or were on sexual activity and sexual response cycle only. Finally, 65 articles were included in the systematic review. Apart from the frequency of sexual activity, sexual dysfunction and overall levels of satisfaction with sexual functioning, a number of issues were identified: sex-related guilt, anxiety, embarrassment, future prospects, quality of the relationship with the partner, changed feelings of sexual attractiveness, partner's response to the changed situation, and the effectiveness and side effects of (medical) sex aids. These topics were used as underlying issues for the provisional sexual health questionnaire.

Issues concerning sexual attractiveness were restricted to quantitative studies only. Many questionnaires for measuring different aspects of sexual health were found in the reviewed literature, but cancer-specific instruments were underrepresented. Sexual functioning was often assessed as part of (health-related) quality of life. Frequencies and sexual satisfaction were commonly assessed with a single item only. Qualitative designs to assess sexual functioning were used in 20 studies. The detailed results of the literature review are described elsewhere (Den Oudsten *et al.*, unpublished data). Issues identified by the literature search were first checked for clinical relevance. A shortened list of 53 issues was then reviewed by a great number of patients (n=107) and HCPs (n=83).

Characteristics of patients and HCPs

Most of the patients had breast cancer (n=43; 40%), followed by colorectal (n=17; 16%), and head and neck cancer (n=14; 13%). All over the group cancer sites were well-balanced. The majority of patients were treated with surgery followed by radiation therapy and chemotherapy, and were under active treatment when they were interviewed. Half of the patients had no evidence of disease (n=53; 50%); a smaller number was newly diagnosed (n=17; 16%) or had a recurrent disease (n=11; 10%). A progression was stated for 10 patients. About a half had completed their treatment within the last 5 years, 8% more than 5 years ago and the others have not completed yet or did not provide any information on that. There were more female than male patients interviewed with a mean of 55 years (±11). The majority was living with a partner or family and had a sexual partner and was well-educated (*Tables 2,3*).

Various experts from different disciplines, mainly employed as medical doctors (n=53; 64%), psycho-oncologists (n=16; 21%) or nurses (n=3), reviewed the issue list. Gender was equally distributed and their clinical experiences mostly ranged from 10 to 20 years. Most of the HCPs were familiar with gynecologic cancer (n=29; 26%), followed by colorectal/gastrointestinal (n=21; 19%), breast (n=18; 16%), prostate/testicular (n=9; 8%) and other cancer sites (e.g., head and neck or lung cancer) (n=33; 31%).

Interview results

Most of the issues on satisfaction as well as the importance of having an active sexual life were rated as highly important in the sexual health questionnaire by both patients and HCPs. There were several issues referring to satisfaction (e.g., satisfaction with the level of affection or intimacy, satisfaction with the frequency of sexual activity, satisfaction with the communication with the partner and sexual satisfaction). No specific issues for female patients were rated as highly important. Male patients additionally rated their level of confidence in getting an erection and keeping one as very important. These issues met all four criteria on relevance and priority. Issues on sexual arousal, orgasm-

Table 2 Sociodemographic patient characteristics (N=107)

Characteristics	N	%
Gender		
Female	66	62
Male	41	38
Age (years)		
20-35	3	3
36-50	31	29
51-65	54	50
66-85	17	16
Missing	2	2
Living situation		
Living with partner or family	90	84
Living alone	14	13
Living with others	2	2
Missing	1	1
Sexual partner		
Yes	90	84
No	15	14
Missing	2	2
Education level		
Post compulsory school education	47	44
University level	30	28
Compulsory school education or less	24	22
Missing	6	6

Table 3 Clinical patient characteristics (N=107)

Characteristics	N	%
Treatment status		
Active treatment	72	67
No active treatment	34	32
Missing	1	1
Treatment		
Surgery	76	71
Radiation therapy	66	62
Chemotherapy	62	58
Anti-hormonal therapy	28	26
Others	5	5
Status of disease		
No evidence of disease	53	50
Newly diagnosed	17	16
Recurrence	11	10
Progression	10	9
Missing	16	15
Years since diagnosis		
Less than 5 years ago	80	75
5 to 10 years ago	20	19
More than 10 years ago	3	3
Missing	4	4
Time of completion		
During the last 5 years	45	42
More than 5 years ago	9	8
Not completed yet	4	4
Missing	49	46
Menopausal status (N=66)		
Post-menopausal	30	45
Pre-menopausal	19	29
Treatment-related menopause	13	20
Missing	4	6

related issues and general issues on sexual enjoyment were also rather important for female and male patients and HCPs.

A very low relevance and priority had the issues on hair loss, masturbation and sexual desire (frequency, level). Also issues about fear harming the incision during intercourse, retrograde ejaculation and changes in sexual fantasies did not fulfill the criteria to be further included.

When considering the mean relevance only, all presented issues except five were more often rated as highly relevant for cancer patients by HCPs than by patients. There seems to be a similar relevance for sexual desire (frequency, level), satisfaction (with level of affection or intimacy and with the communication with the partner) and the level of emotional intimacy. All other issues were rated more relevant to be included in the questionnaire for HCPs, not for patients.

The greatest differences in the estimated relevance

(>1.10) were found for issues on incontinence, masturbation, pain and fear, always rated higher by the interviewed HCPs (>1.0). Comparable results were found for issues for male sexual health (ejaculation) and issues on masculinity/femininity (*Tables 4,5*).

Priority for inclusion was assessed in the same way for most of the 53 issues from the list. Only level of emotional intimacy and change in amount of affection expressed was

Table 4 Mean relevance for issues rated similar by patients and HCPs

Issues	Mean relevance	
	Patients	HCPs
Frequency of sexual desire	1.76	1.76
Level of desire	1.70	1.79
Satisfaction with level of affection or intimacy	2.24	2.31
The level of emotional intimacy	2.10	2.13
Satisfaction communication partner	2.35	2.43

HCPs, health care professionals.

Table 5 Mean relevance for issues rated different by patients and HCPs

Issues	Mean relevance	
	Patients	HCPs
Incontinence	0.87	2.39
Masturbation	0.88	2.02
Frequency of pain during/after sexual activity	1.39	2.54
Level of pain during/after sexual activity	1.26	2.57
Fear that sex will be painful	1.06	2.46
Fear of injury during intercourse	0.87	2.08

HCPs, health care professionals.

much more higher prioritised by patients than by HCPs. Although many issues on body-related changes were less relevant for patients on average, they thought that these topics should be included in such a questionnaire of sexual health for cancer patients anyway (e.g., incontinence).

Highest differences in the evaluated issues, identified for relevance and priority, were found for frequency of pain (during/after sexual activity), incontinence, fear that sex will be painful, and for communication about sexual issues with HCPs. While the interviewed HCPs found those issues more important to integrate in the sexual health questionnaire, patients found them less relevant.

Item development

On the basis of the results a provisional item list was established ("EORTC SHQ-Cxx"), supported by the translation coordinator of the EORTC Quality of Life Department. Issues fulfilling all four criteria on relevance and priority were automatically included. Issues within the categories of sexual desire, sexual arousal and pain were combined each. Issues implying norms, such as frequencies or content-related redundancy were not included. Negative

wording or ambiguity was additional reasons for exclusion. The reduced list of 22 items with an additional question on reasons for not being sexually active was reviewed by two independent native English speakers before starting the translations process. At least three languages representing an English-speaking country, a country from Northern and Southern Europe were invited for preparation of pilot-testing, which is the next step in the EORTC procedure to develop a validated EORTC questionnaire.

Discussion

The results of the literature search were considered for the development of the issue list which was presented to patients and HCPs. Interview results were then analysed and item development process was done before continuing with pretesting.

Studies on sexuality in oncology patients which were found in the literature search predominantly focused on issues of activity, experiences of sexual dysfunction, and satisfaction with sexual functioning. The literature review identified themes beyond these aspects and provided interesting results on current instruments used for measuring sexual health. No questionnaire currently exists that focuses on physical, psychological, and social aspects of sexuality for cancer survivors. Based on the available interview results most important issues to fill this gap were identified: satisfaction and the importance of having an active sexual life such as the level of confidence in getting an erection and keeping one for male patients. Different evaluations by HCPs and patients were found for most of the issues. Pain, incontinence, fear and communication about sexual issues with HCPs were issues which were highly relevant for HCPs but not for patients. Less research exists, in which possible reasons have been identified. Body image can change during different phases of cancer treatment. As shown in a review article by Cororve Gingeret *et al.* 2014 (22) patients are found to be most concerned about body image in the immediate postoperative period and soon after completing other forms of treatment (23). In a study with 225 Canadian cancer patients, 44% used the Internet to learn more about their condition and 14% wished their medical teams had provided them with links, but did not independently search for medically relevant information about their condition. While education level was correlated with web based information seeking, age was not (24). Why fear as an issue was less important for our interviewed patients than for professionals would be

interesting to further investigate.

Strength of the study is the fact that the development of the sexual health questionnaire is based on current literature, patient and HCP interviews from many different countries according to the EORTC guidelines (21). The literature review systematically examined sexual health issues in cancer beyond general ratings of sexual activity, symptoms, and sexual dysfunction. As other literature reviews (25-28) have described patients' sexual activities, types of sexual dysfunction and generic levels of sexual satisfaction, this review uniquely took as its focus sexual health issues for all cancer patients and survivors. Including interviews of more than 100 patients with different cancer sites and many professionals from different disciplines also increases the quality of the development process. The provisional module will then be pre-tested in various languages to identify and solve potential problems with wording. For pilot-testing patients from many different countries and cultures are foreseen (phase 3) after the translation process was finished and ethical approval was done. Also the translations into several languages and the cross cultural distributions for developing the questions follow a standardized procedure (18). The questions are designed for male and female patients with different cancer sites during all stages of their disease.

It is suggested that future studies broaden their focus to encompass sexual health related topics (body image, self-esteem, relationship functioning and communication) rated as important in cancer survivors which is not evident yet. But these issues should be addressed in clinical trials to get information on a possible impact for clinical practice. The instrument will be designed as a 'stand-alone' sexual health measure that can be used in clinical trials as well as in clinical studies. The results of trials which use the sexual health questionnaire adequately can improve sexual health care.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The study was approved by the ethical committee (ID: EK 25-273 ex 12/13) and patient consent was obtained.

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Pharmacologic and surgical therapies for sexual dysfunction in male cancer survivors

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Abstract: The recent recognition that many men experience sexual dysfunction following their diagnosis and treatment of genitourinary cancers, has led to the development multiple varied strategies that attempt to restore or preserve that function. In this manuscript we review the understanding of why it happens, highlight novel management strategies and discuss the concept of penile rehabilitation (PR) following prostate cancer (PCa) treatment, glans preserving strategies among men diagnosed with penile cancer and address the controversial issue of testosterone therapy in men with PCa.

Keywords: Sexual dysfunction; treatment modalities; cancer survivors

Introduction and classification

Despite rapid advances in diagnosis and therapeutics over the past decade, cancer remains a major public health concern worldwide. The negative quality of life (QoL) impact of cancer and its treatment on sexual function has become more important as greater survival rates and recognition of its impact have been reported (1). Importantly for the clinician, even cancers that do not directly involve sexual organs can result in sexual dysfunction as a consequence of the adverse effects of multi-modal treatment. Cancer related sexual dysfunction in this population of male cancer patients includes erectile dysfunction (ED), structural changes within the penis, ejaculatory dysfunction and hypogonadism among many others (2). While we recognize that additional and important sexual dysfunctions such as climaturia, penile deformities, ejaculatory dysfunction and other concerns develop, given the wide scope of these issues we have limited our discussion to those areas where there exists adequate literature to support therapeutic conclusions.

Materials and methods

A literature search for original and review articles published in the English language was performed using a PubMed database ending October 2014. Search keywords were

prostate cancer, bladder cancer, penile cancer, testicular cancer, male cancer survivors, male genital cancer, sexual dysfunction, treatment male cancer survivors, prostate cancer treatment, radical prostatectomy (RP), erectile dysfunction (ED), penile deformities, hypogonadism, ejaculation, orgasmic. The selected articles were reviewed by the authors and their findings/conclusions incorporated into the manuscript.

Prostate cancer (PCa)

PCa remains the most common solid organ nondermatologic cancer in men in the USA. It is estimated that there are nearly 2.8 million men living with a history of PCa in the USA, and more than 90% of all PCas are discovered in the local or regional stages, for which the 5-year relative survival rate approaches 100% (3). Therefore, preservation of continence and erectile function (EF) two factors contributing to long term QoL in this population, are among the most significant interests of patients with local PCa.

Men with PCa often struggle with sexual dysfunction, both before and after treatment. The rates of sexual dysfunction after treatment are quite high despite the development of minimally invasive technology, evolving surgical techniques and a greater understanding of the

anatomy of the pelvis. Following PCa treatment, 70% of men complain of worsening sexual function, which is frequently attributed to RP or radiation therapy (XRT) (4).

Male sexual dysfunction after PCa treatment is truly a diffuse clinical symptom complex, but for simplicity is often divided into three groups: (I) ED; (II) penile deformities; and (III) ejaculatory and orgasmic dysfunctions.

Erectile dysfunction (ED)

The true incidence of ED after PCa therapy is unknown. The contemporary literature reports rates of ED after RP to be around 60-70%, with some robotic and laparoscopic reports citing rates as low as 5-10%. Consensus has been reached on risk factors which include: patients age, preoperative EF status, comorbidities (diabetes, hypertension, hypercholesterolemia), surgical technique (nerves sparing) (5-7). ED after RP is most often attributed to neuronal and/or vascular injury to the cavernous neurovascular bundle at the time of surgery. Subsequent neuropraxia, inflammation and ischemia result in failure of spontaneous erections, which can lead to persistent hypoxia, cavernous smooth muscle apoptosis and ultimately corporal fibrosis. Controversial data exists which support early intervention and penile rehabilitation (PR) strategies attempting to prevent these pathophysiologic changes from becoming established and irreversible (8-10). Age and normal preoperative sexual function parameters emerged as independent determinants of patients' desire to preserve postoperative sexual functioning (11). Briganti et al. recently reported a preoperative risk stratification tool aimed to assess the probability of EF recovery after open bilateral nerve-sparing RP (BNSRP), using cardiac risk factors, age and EF as determinants. The resulting tool was able to stratify patients into three groups according to the relative preoperative risk of post-RP ED: low (age ≤65 years, IIEF-EF ≥26, CCI ≤1), intermediate (age 66-69 years or IIEF-EF 11-25, CCI ≤1), and high risk (age ≥70 years or IIEF-EF ≤10 or CCI ≥2). According to the risk-group stratification at 12 months post-operative, EF recovery rate was 82%, 57% and 29% in the low-risk, in the intermediate-risk and in the high-risk group, respectively (P<0.001) (12).

Interestingly, controversy exists concerning another parameter that has been widely reported to affect the rate of post-prostatectomy ED. Given the rapid recent acceptance of minimally invasive approaches to RP, the surgical approach such as open RP (ORP), laparoscopic RP (LRP) or robotic RP (RALP) has been studied in terms of EF

outcomes. Tal et al. reported that the rate of EF recovery found in open, laparoscopic and robotic RP at 57%, 58% and 73% respectively (13). A cumulative meta-analysis of studies reporting EF in preoperatively potent patients, demonstrated a range of potency rates after LRP vs. ORP vs. RALP at 48 months were 58-74% and 49-74% and 60-100% respectively (14). Moreover RALP also seems to promote a more rapid EF recovery as compared with ORP and LRP (15). However, conflicting data exists on this topic and no clear statement can be made at this time, clearly demonstrating that robotic compared to laparoscopic or open is truly superior with respect to erectile preservation at this time. There are need randomized controlled study for accurate results (16).

Potvedin et al. reported that the outcomes of intrafascial vs. interfascial BNSRP techniques for RARP. The recovery rates of EF at 3, 6, and 9 months in the intrafascial group were 24%, 82%, and 91%, respectively, whereas in the interfascial group, they were 17%, 44%, and 67%, respectively. However, the intrafascial technique was associated with higher positive surgical margins rates in patients with pT3 disease (17). Xylinas et al. showed that the robot-assisted intrafascial approach provided early satisfactory functional results with respect to postoperative potency (18).

Consequently, preoperative EF appears to be the best independent predictor of postoperative EF, with age, nerve sparing technique and cardiac risk factors also contributing to recovery prediction.

XRT-induced ED is thought to result from neurovascular bundle injury and is related to the amount of radiation given near the penile bulb (19). Two recent prospective trials showed an incidence of ED in 30-40% of the patients treated with external beam XRT (EBRT). Prospective studies have reported an increase of ED between 1 and 2 years after radiotherapy (RT), whereas ED rates did not seem to change after 3 years (20,21). The PCa outcomes study (PCOS) demonstrated that the actual rates of ED between RP and radiation groups are similar at 15 years post treatment. The results showed that 87.0% of patients in the prostatectomy group and 93.9% of patients in the RT group reported an inability to achieve an erection sufficient for intercourse (22,23).

Recent data on brachytherapy indicates that it may provide better preservation of EF compared with EBRT alone or in combination with hormone therapy (24). Vascular comorbidities may have a significant role in ED after XRT. Wang et al. recently reported data from 732 patients who treated for CaP with XRT. Patients with three

vascular comorbidities were almost twice as likely (75%) to develop ED at 4 years after XRT, compared with patients with no vascular comorbidities (44%) (25).

Penile deformities (penile length loss and curvature)

Penile shortening (PS) and Peyronie's disease (PD) has been reported after RP. Several studies have shown that penile length decreases after RP, however rates of PS varied between 0-55% depending on whether a subjective or objective method was used for evaluation and on what cutoff value is used to define PS (26-29).

The pathophysiology of PS is clearly unknown. A number of mechanisms have been proposed and include anatomic alterations, neural damage, sympathetic nervous system over activity and histological alterations such as apoptosis (30). PS can be divided into two groups; early phase PS which occurs immediately after RP related to neural damage and sympathetic hyperactivity and late phase PS associated with histological alterations as well as fibrotic accumulation (31). Non-nerve-sparing surgery and ED have repeatedly been shown to be associated with loss of penile length after RP. Contemporary theories place hypoxia and cavernosal smooth muscle apoptosis as the most culpable cause for this length and girth loss. Strategies to reduce length loss and preserve function attempt to mitigate the hypoxia largely through return of early erections with injection therapy or use of PDE-5 inhibitors (PDE5Is) (32-34).

The first long-term prospective study on PS was published by Gontero et al. in 2007. They reported that PS after RP peaks at the time of catheter removal and it continues for at least 1 year. Longer preoperative stretched penile length, NS surgery and recovery of EF appeared to be independent protective factors on penile length loss at 1 year (35). Frey et al. recently reported that 47% of patients had penile length loss in excess of 1 cm. Patients reported a subjective length loss between 1 and 3 cm, 3 and 5 cm and more than 5 cm was stated at 33%, 11% and 1% respectively. This study showed that a high BMI increased the risk of PS. This finding may be caused by the prepubic fat pad covering the proximal part of the penis, which can be misinterpreted as penile length loss (36). In another study, Briganti et al. found no changes in penile length 6 months after NSRP in patients with normal EF before surgery when precise measurements were performed. When the same patients were asked to subjectively estimate if their penis had shortened after the operation, 14% answered affirmatively (26). As a result NS and postoperative EF,

strongly correlate with preservation of penile length.

The prevalence of PD (penile curvature) in the normal population is not well established, but prevalence estimates of 3.7-7.1% are widely felt to be reasonable (37). The incidence of penile PD among men with PCa is higher than the normal population and Tal et al. found that incidence of PD after RP was 16.7% in 1,161 patients (38). In another study the patients who were referred for ED after RP were asked if they had noticed an altered penile curvature or narrowing. The rate of patients who had clinical fibrosis on their penises was 41% and 24% of those patients having a deformity that resembled a waistband, and 93% patients who had measurable curvatures. Of the patients with clinical fibrosis, 70% reported a subjective shortening of the penis with an average length loss of 24% (39). The pathogenesis of PD is still not clear but some authors have suggested that repetitive micro-trauma of the penile tissue during sexual intercourse can induce PD (31).

Ejaculatory and orgasmic dysfunctions

Although the exact cause of orgasmic dysfunction after RP is unknown, it is clear that removal of the prostate and the seminal vesicles may in itself impact orgasmic pleasure as ejaculation is no longer possible. In addition, the correlation between orgasmic function and postoperative potency, nerve sparing, and urinary control implies that nerve damage may play a role (40).

In a prospective study, Le et al. (n=620) showed that the percentage of patients with a "good" or "very good" ability to achieve orgasm was reduced from 65% at baseline to between 25% and 30% postoperatively. Age <65 years, higher levels of education, NS surgery, lack of comorbidities and good EF after surgery were positively correlated with the ability to reach orgasm (41). On the other hand, Tewari et al. found that 80% of patients who underwent RP patients had normal orgasmic function (42). This might be because of a high rate of nerve sparing and good postoperative EF in the study. Predictors of good orgasmic function were age <60 years and nerve-sparing surgery (P<0.001). The latest study on orgasmic dysfunction after RP was performed by Frey et al. In their study the rate of orgasmic alteration after RP was 5% anorgasmia, 60% reported decreased intensity of their orgasms and for unknown reasons, 6% of the patients in the sexually active group noted an increase in the intensity of their orgasms. The remaining 29% reported no change in their orgasm intensity (36).

Anejaculation after RT is infrequently reported, and the

446

Kadioglu et al. Therapies for sexual dysfunction in male cancer survivors

published data on this issue are scarce. Sullivan *et al*. recently evaluated the effect of RT on ejaculation in patients with PCA. In their study 16%, 69% and 89% of patients reported to have lost the ability to ejaculate in an antegrade fashion after prostate RT at 1, 3, and 5 years respectively. They have found that Age >65 yrs, androgen deprivation therapy (ADT), prostate <40 g, each year post-RT and dose >100 Gy were independent risk factor for anejaculation (43).

Penile rehabilitation (PR) after prostate cancer (PCa) therapy

The optimal treatment modality or rehabilitation strategy for ED after PCA therapy does not exist today. According to a survey by International Society for Sexual Medicine (ISSM), the practice of erectile rehabilitation is commonly performed in the clinical setting and up to 87% of these specialized sexual medicine physicians utilize some form of erectile rehabilitation. The survey showed that 95% used PDE5Is, 75% used intra corporeal injection (ICI), 30% used vacuum device, and 9.9% used intraurethral prostaglandin (44). Since the survey was conducted among ISSM members, it may not accurately reflect the tendencies of the whole urology community. The first clinical study in support of PR was reported by Montorsi *et al*., who showed that intracorporeal alprostadil injection positive effected EF after RP (45). Currently, there are several treatments modalities for ED after PCa therapy such as PDE5Is (sildenafil, tadalafil, vardenafil, udenafil, avanafil), ICIs, vacuum erection device (VED) and intraurethral alprostadil (IUA).

PDE-5 inhibitors (PDE5Is) (sildenafil, tadalafil, vardenafil, udenafil, avanafil)

PDE5Is facilitate an erection by locally increasing cGMP levels in the penile tissues through inhibition of metabolism when neural function exists. With adequate preservation of cavernous nerves, PDE5Is reduce cGMP metabolism and thereby increase its concentration, which promotes corporal smooth muscle relaxation and enhanced blood flow. Several animal studies suggests that neuropraxia after RP may lead to hypoxia, apoptosis, venous leak and fibrosis of the corpora cavernosa. The central theme of many rehabilitative strategies is early PDE5Is administration which may reduce hypoxia and the subsequent cascade of events outlined above (46,47). There is no standard regimen for PR, however PDE5Is are widely recommended as first-line treatment for ED after PCa therapy. Typically, response rates to PDE5Is improve as time passes after RP and rates of response range widely from 15% to 80% (48,49).

The first randomized and placebo-controlled trials that assessed the clinical effects of PDE5Is in PR were conducted by Padma-Nathan *et al*. Their study randomized 76 patients after NSRP to double-blind sildenafil (50 or 100 mg) or placebo nightly for 9 months and reported that nightly sildenafil markedly increased the return of normal spontaneous erections (27% and 4% in the placebo group) (50). The second study was a multi-center, placebo-controlled trial by Montorsi *et al*. This study enrolled over 600 patients for a double blind treatment period followed by a washout period and then an open label phase with vardenafil on demand. The patients in their post-prostatectomy period were randomly assigned to use daily vardenafil, on-demand vardenafil, or placebo control. IIEF scores greater than 22 were reported in 24.8%, 32%, and 48.2% of patients for placebo, vardenafil daily and on-demand dosing, at the end double blind phase respectively. This trial showed that the efficacy of on-demand or daily PDE5Is after prostatectomy were similar in terms of EF. The final evaluation of this trial demonstrated efficacy of PDE5Is when taken on-demand in this population of men post RRP, but not a true rehabilitative effect, given that no significant potency advantage was measured among the groups at the end of the open label phase (51).

To date, there is no consensus on the appropriate PDE5Is agent, dose or timing, for post-RP erectile rehabilitation. Pavlovich *et al*. investigated the effect of nightly or on demand 50 sildenafil after NSRP in a double-blind, randomized controlled trial that enrolled a total of 100 men who had IIEF-EF >26, aged <65 years. The patients were randomized to either nightly sildenafil group or the on-demand placebo (nightly sildenafil group), or on-demand sildenafil and nightly placebo (on-demand sildenafil group; maximum on-demand dose six tablets/month) for 12 months. The authors found no significant differences in IIEF-EF scores between nightly and on-demand treatment after RP (52).

Another well designed study was published by Montorsi *et al*. in 2014. This trial investigated the efficacy of tadalafil 5 mg once daily and tadalafil 20 mg on demand versus placebo after RP. The proportion of patients reaching the IIEF-EF >22 was significantly higher in the tadalafil once daily group than in the placebo group while the comparison between tadalafil on demand and placebo was not statistically significant at 9 months after surgery. At 10.5 months, the time point in which the efficacy of spontaneous EF without drug was assessed and was the primary endpoint showed no

statistical difference among the groups (53).

Interestingly however, an important secondary endpoint defining penile length preservation, an index of cavernous smooth muscle preservation, a goal of rehabilitation was found to be statistically superior at 13.5 months post-op in the daily tadalafil group compared to the on-demand group. Additionally at 13.5 months clinically and statistically greater responses were measured in the daily tadalafil arm for SEP question 3. These data indicate that early use of daily tadalafil may preserve cavernous smooth muscle during the critical early phase and results in enhanced PDE5I response at later time points (52). Mulhall *et al.* investigated the efficacy of avanafil 100 mg, and avanafil 200 mg for the treatment ED after BNSRP. Their study showed that patients randomized to 100 and 200 mg avanafil had an improvement in IIEF-EF domain score of 3.6 and 5.2, respectively, compared with 0.1 for placebo (54).

Timing of rehabilitation and choice of treatment remains a major clinical controversy. Several animal studies have clearly shown that early treatment is critical for endothelial and smooth muscle protection and reduction of corporal fibrosis. The current literature suggests that PR should be started as early as possible, indeed should be begin after the day of surgery if possible (55). This means that PDE5Is may be most effective if initiated as early as the diagnosis and surgery date confirmed. Moreover, Moskovic *et al.* instructed their patients to take sildenafil 25 mg nightly as well as to use alprostadil 250 μg urethral suppositories three times per week, beginning one week prior to surgery (56).

The effect of PDE5Is on orgasmic function after RP was investigated by Nehra. In this study, significant improvements in orgasmic function were found with doses of both 10 and 20 mg vardenafil compared with placebo. These improvements were accompanied by significant improvements in satisfaction with EF (P<0.0001 for both groups). In this context, it seems likely that the improvements seen in orgasmic function were caused by improvements in EF (57).

PDE5Is have proved effective in the treatment of ED after RT for PCa. There are numerous well designed randomized controlled trials that address treatment ED after RT. One prospective, placebo controlled study compared tadalafil with placebo taken on demand in patients with PCa after RT treatment. They found that 67% of the patients reported an improvement in EF with tadalafil compared with only 20% in the placebo group (P≤0.0001) (58). Pisansky *et al.* published the effect of tadalafil 5 mg compared to placebo

on PCA with ED after XRT. The results for the primary endpoint demonstrated retention of EF in 79% of patients in the tadalafil group *vs.* 74% in the placebo group (P=0.49). The study was not powered to detect a 20% difference between the groups (59). In contrast to these findings, Zelefsky *et al.* designed a similar study where patients received daily sildenafil (50 mg) or placebo. They found that 81.6% of patients on daily sildenafil and 56.0% of those on placebo achieved a functional erection with or without ED medication at 24 months (P<0.045) (60).

Intracavernosal injection (ICI)

ICI with alprostadil alone or in combination with papaverine or phentolamine is an effective option in men who respond poorly to PDE5Is (61). ICIs induced erections result in enhanced cavernosal oxygenation and penile stretch, both of which are known to be protective of erectile tissue structure and function (62). ICI post prostatectomy rehabilitation success rate was investigated by Prabhu *et al.* on 135 men through 8 years and only 44% of those men declared some level of satisfaction as well as pre-operative erectile status was independently associated with use of ICI (63).

Raina *et al.* reported that 68% of patients had sufficient erection for sexual intercourse after ICI therapy. Long-term (>3 years) data have revealed high dropout rates (50%), most often attributed to discomfort, fear, or inconvenience associated with injection (64). Nelson *et al.* investigated injection anxiety and pain in men who used ICI for 4 months after radical pelvic surgery. The study showed that the frequency of ICI use was 29% week, 26% 1/week, 40% 2/week, and 5% 3/week, whereas the IIEF-EF score increased 8 to 22 compared to baseline. They found that injection anxiety on average, was moderately high (score =5.7 on 0-10 point scale) at the first injection training and this significantly decreased at the 4-month follow-up (score =4.1). The result of their study showed that despite the passage of time the rate of anxiety score not changed (65). Moreover, Coombs *et al.* demonstrated RP as an independent risk factor of ICI therapy failure (66).

Vacuum erection device (VED)

The VED is the only non-pharmacologic strategy among the choices for men who do not respond to PDE5Is or for those who have a contraindication (67). Numerous publications have suggested that VED therapy improves EF in 84-95% of patients (68,69). VED therapy uses

negative pressure to distend the corporal sinusoids and to increase blood inflow to the penis. Lin *et al*. found that the mean O_2 saturation of corporeal blood immediately after VED-induced erection was 88.25%. Of the blood in a VED-induced erection, 62% was arterial, and 38% was venous in origin in rats (70). Welliver *et al*. investigated the effect of VED therapy on the penile oxygen concentration in 20 men in a pilot study. They measured penile oxygen saturation before and after VED therapy. They showed that use of VED significantly increased 20% and 55% in both glanular and corporal oximetry compared with baseline respectively (71). Köhler *et al*. designed a pilot study and randomized study to compare early (1 month after surgery) to late (6 months after surgery) use of VED. The results showed that the early use of VED for rehabilitation significantly improves the IIEF-EF scores and preserves penile length compared with control group (EF score: 12.4 *vs*. 3.0) after six months following surgery. However patients did not have adequate erection for spontaneous intercourse at the end of study in either group (72). In a similar study, by Raina *et al*., patients were randomized as either daily VED users or controls for a 9 month period. Although the reduction in penile length and girth were reported in 23% (14) of the VED users and 63% of controls, no statistical difference was found between the two groups (17-29%) in terms of erection adequate for successful intercourse (73).

Penile prostheses

The surgical placement of a penile prosthesis is widely used for ED, particularly in men unresponsive to medical management. Interestingly, according to the SEER-Medicare database only 0.8% of patients who chose a penile implant were after PCa therapy (74). In another study by Menard *et al*. investigating 400 post RP patients who underwent penile prosthesis, while complication rates were less than 5% for infection, revision, mechanical failure, the overall satisfaction rate was reported as 86.1%. In addition, these patients were compared with vasculogenic ED patients who underwent penile prosthesis placement. No significant difference was detected in complications (mechanical failure, infection) or surgical satisfaction rates (86% *vs*. 90%) (75). While penile prosthesis was shown to be superior to PDE5Is in terms of overall improvement at 12, 18, 24 months after the surgery by Megas *et al*., function and satisfaction scores were similar in both groups (76).

Recently an alternative reservoir placement has been suggested for patients with ED and radical pelvic surgery history such as prostatectomy, cystectomy or colon surgery. To date the conventional retropubic reservoir placement has been posterior of transversalis fascia (PTF). Despite the rarity of complications, very grave complications such as vascular or bladder injury may happen during this approach (77). Stember *et al*. investigated the complications of reservoir placement between posterior or anterior to the transversalis fascia (ATF) and demonstrated that no injuries to major blood vessels or bowel occurred in neither of these approaches (78). In a similar study published by Karpman *et al*., AMS 700 conceal or spherical reservoir was used in 747 patients in a prospective, multicenter study. The authors compared satisfaction and complications rates of PTF (n=572) and ATF (n=177) groups and showed that ATF placement approach was as safe and highly satisfactory as PTF (79).

Hypogonadism and testosterone replacement

Hypogonadism is present in more than 20% of men after RP and is often worsened by ADT. Due to prolonged absence of erections, ADT may lead to corporal fibrosis and decreased penile length (80). Testosterone replacement therapy (TRT) in hypogonadal CaP survivors is controversial because TRT may increase the risk of CaP recurrence. However, current evidence supports the safe use of TRT in hypogonadal CaP survivors. Landau *et al*. reported that PCa recurrence was insignificant in those who underwent TRT to treat hypogonadism that occurs before and after RP (81). Most notably, Pastuszak *et al*. recently reported on the use of TRT in 103 hypogonadal men following RP for CaP. Although TRT use did result in slight PSA elevation, there was no associated increase in cancer recurrence at a median of 27 months of follow-up (82).

Bladder cancer

Bladder cancer, the fifth most common cancer in men in the United States, typically presents as a superficial transitional cell carcinoma that is locally resectable and curable. However, in a small minority of men their cancer is muscle invasive requiring more aggressive treatment with a greater risk of sexual dysfunction. Therapeutic options for this population include surgery, radiation and chemotherapy, usually associated with radical cystectomy with urinary diversion (83). The long-term prognosis for those who undergo radical cystectomy continues to improve with advances in technique and earlier diagnosis, regrettably

rates of ED and sexual QoL loss remains high (84).

The etiology of ED after radical cystectomy (cystoprostatectomy) is strongly correlated with the peroperative injury to neurovascular bundle. A large number of animal models exit describing the type and extent of this injury which has been classified as traction, percussive, thermal, transection and devascularization injury. Thus, treatment is often associated with the loss of sexual function, the most impactful and frequent of which is ED (85). The high prevalence of post-surgical ED has driven researchers to consider whether NS cystectomy is a safe alternative in the treatment of bladder cancer. Several studies have reported accepted rates of potency after nerve sparing radical cystectomy that range from 42% to 71% (86-88). NS surgery is associated with greater rates of positive outcomes in EF. Most men experience a temporary decrease in function immediately following surgery, which is then followed by a steady return to function (89). Numerous studies showed that age is an important predictive factor of ED after nerve sparing radical cystectomy. Schoenberg et al. reported the potency rates of 101 patients after nerve sparing radical cystectomy were 62% in men 49 years and younger and 20% in men 70-79 years old (87). Asgari et al. recently investigated the effects of urinary diversion type on sexual function in 41 patients who underwent ileal conduit urinary diversion and 40 patients with orthotropic ileal neobladder substitution who underwent non-nerve sparing radical cystectomy. The baseline total EF scores of the patients were similar for both groups (26.74 vs. 26.70). At 12-month following surgery, the mean total EF scores were 5.52±1.24, and 15.60±1.61, in ileal conduit and ileal neobladder groups, respectively (P=0.001). At the post first year, 14 (35.0%) of the ileal neobladder patients were able to achieve vaginal penetration and maintain their erection for intercourse, whereas this rate decreased to 4 (9.8%) in patients with ileal conduit (P=0.006). This study demonstrated the superiority of patients with orthotopic ileal neobladder substitutes to the ones with ileal conduits in terms of EF (90).

Prostate preservation during radical cystectomy provides better postoperative EF. Basiri et al. randomized 24 radical cystectomy patients with initial high IIEF scores (>20) into a prostate sparing group (12 patients) and non-sparing group (12 patients). Group 1 [12] had prostate sparing and group 2 [12] had non-sparing cystectomy. After a follow-up time of 39 months, 2 and 10 of the patients lost their erections after the operations in groups 1 and 2 respectively. In addition mean IIEF scores were 19.8 and 5.7 in former and latter groups respectively. This study showed that the patients who underwent prostate sparing surgery had better EF when compared to non-sparing patients (91).

As a result, PR for ED after radical cystectomy is identical to the PCa survivor rehabilitation, PR should be started with PDE5Is as soon as possible after surgery. Early intervention is associated with better sexual functions and satisfaction rates in the light of current literature (92,93).

Penile cancer

Penile cancer is relatively rare (0.58/100,000 men) in the developed countries of the world. However, in some regions of Africa, South America and Asia the incidence of penile cancer can be up to five times higher (94). Penile cancer and its treatment can negative effect sexual function and intimacy, body image, urinary function mental health and QoL. Maddineni et al. reported that penile cancer treatment negatively affected well-being in up to 40% of patients with decreased sexual function in up to 60% (95).

Kieffer et al. have investigated the impact on QoL, after treatment for penile cancer in 90 patients, 54 with penile sparing surgery and 36 with partial penectomy. The authors found that men treated with penile sparing surgery scored significantly better than those who underwent partial penectomy on the orgasmic function scale. However, there was no statistically significant difference in EF, sexual desire, intercourse satisfaction or overall sexual satisfaction (96). Yang et al. recently published a similar study. The authors compared sexual performance between partial penectomy and glans preserving surgery in 135 patients. Patients treated with glans preserving surgery had better performance in all of the IIEF domains score. They also had significantly higher satisfaction (64.4% vs. 13.9%) and intercourse confidence (55.6% vs. 5.6%) compare to men who underwent partial penile amputation (97).

Recently, brachytherapy have been recommended for initial treatment of invasive T1, T2 and selected T3 penile cancers by consensus guideline (98). Delaunay et al. investigated the effect of brachytherapy on sexual function in 47 patients with penile cancer and cancer specific survival rates of 90.7% and 87.6%, were reported at 2 and 5 years respectively. They reported that 58.8% of patients had adequate sexuality after treatment and 47.3% stated that brachytherapy had not affected their sexuality and 15.8% of them had mild changes. Consequently, most patients stated that brachytherapy had little or no influenced on their sexual life (99).

Testicular cancer

Most testicular cancers are diagnosed early and approximately 70% of patients are diagnosed at a localized stage. Typically treatment of testicular cancer begins with inguinal orchiectomy. After inguinal orchiectomy, early stage seminomas are often treated with radiation (45%), however late-stage seminomas (65%) and non-seminomas germ cell tumors (NSGCT) are generally treated with chemotherapy especially at high stages of disease. The 5-year relative survival rates are 99.0% for tumors diagnosed at a localized disease (100).

ED has been reported in 12-40% of men treated for testicular cancer, regardless of cancer treatment method (101). The etiology of ED in these patients is multifactorial depends on how the patient were treated. Psychogenic ED may be attributable to changes in body image after orchiectomy, loss of sense of manhood after orchiectomy, reduced feelings of well-being and other psychosocial changes associated with cancer (102). Pühse *et al.* recently reported on the prevalence of sexual dysfunction in a group of 539 survivors of testicular cancer and found that 35% had reduced sexual desire, 42% had reduced sexual activity and ED was present in 32%, with three-quarters of the latter group having an impaired ability to maintain an erection during intercourse (103). Tal *et al.* investigated the pathogenesis of ED 12 months after the completion of therapy in 76 men with testicular cancer. The study population consisted of, 66% patients had seminoma and received XRT, 79% of had NSGCT and received chemotherapy, 18% underwent primary retroperitoneal lymph node dissection (RPLND) and 20% underwent post-chemotherapy RPLND. The authors found that a total of 26% of patients had total testosterone levels <300 ng/dL and 84% complained primarily of loss of erection-sustaining capability. None of patients had an abnormal Doppler ultrasonography (DUS) finding. Mean (SD) peak systolic and end-diastolic velocities were 48 [16] and 1.2 (2.2) cm/s, respectively. Moreover 88% of patients responded to PDE5Is use, with erections sufficient for penetration. This result suggests that ED in testicular cancer survivors is primarily non-vasculogenic (104).

Anejaculation may be observed in post-chemotherapy patients who underwent RPLND. The anejaculation rate of 7% was reported following nerve sparing RPLD (105). Hsiao *et al.* investigated the effects of pseudoephedrine therapy on patient anejaculation one year after post-chemotherapy RPLND. The anejaculation was a result of retrograde ejaculation or emission failure in 15% and

85% of cases respectively. None of patients with failure of emission responded pseudoephedrine therapy while 50% patients with retrograde ejaculation were responders for sperm retrieval with masturbation (106).

Conclusions

Sexual dysfunctions are common in male patients with cancer and have been shown to vary in intensity and frequency according to treatment modality, age, pre-existing sexual function and many other factors. Management of sexual dysfunction in PCa and bladder cancer survivors can be difficult, but various effective management options exist. The early intervention of rehabilitative strategies may prevent loss of penile length and increased EF score. Despite several preventive and therapeutic strategies being available, there is no evidenced-based specific recommendation on the optimal rehabilitation or treatment regimen at this time. PDE5Is, ICIs, using vacuum constriction devices, after bladder or PCa therapy, have been shown to be useful in achieving EF. The definitive strategy to restore natural erections in this population remains elusive but ongoing research continues to strive towards that goal. Finally penile prostheses should be suggested for the non-responders to medical therapy.

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Footnote

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452

Kadioglu et al. Therapies for sexual dysfunction in male cancer survivors

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Co-constructing sexual recovery after prostate cancer: a qualitative study with couples

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Background: Men are likely to experience deterioration in sexual functioning as a consequence of treatment for prostate cancer. Indeed, sexual difficulties are common across all treatment modalities.
Objective: To determine the impact of treatment for prostate cancer on intimacy and sexual expression/relationships from the perspective of couples.
Methods: An observational study was conducted including in-depth interviews with 18 people affected by prostate cancer; comprising eight couples and two individual men.
Results: Four categories were identified that illustrated the impact of prostate cancer on intimacy and sexual recovery. These related to social influences and language used to describe the loss or recovery of sexual activities; difficulties in discussing sexual activity with clinicians; the clash of individual impact of prostate cancer recovery versus the impact on the couple, and the re-integration of sexual activities into the relationship.
Conclusions: Though only one person in a partnership experiences cancer, these data indicated the extent to which prostate cancer treatment also impacts on partners. The study indicates that adjustment to erectile dysfunction (ED) takes time, but is a highly significant event in couples' lives and its importance should not be under-estimated. Consequently, we suggest that relational models of care should be considered, whereby side-effects are recognised as impacting on both members of the partnership (for example ED, or lack or ejaculate). Supportive care in this context, therefore, may best be based on a relational approach using language and interventions that are appropriate to the patient and their situation.

Keywords: Prostate cancer; sexuality; couples; survivorship

Introduction

Prostate cancer is the highest incidence cancer diagnosis for men worldwide, with approximately 35,000 men diagnosed with the condition in the UK each year (1). Despite rising detection rates via increased public awareness and prostate specific antigen testing, however, mortality has remained static (1). Consequently, increasing numbers of men are surviving long beyond the original diagnosis and experiencing the long-term effects of the disease and its treatment. Sexual dysfunction presents a particular challenge, with men and their partners experiencing deterioration in sexual

functioning as a consequence of all treatment options.

Surgery is known to result in erectile dysfunction (ED) (2), with recovery of function often occurring up to five years following treatment (2). Even nerve-sparing surgery impacts on sexual functioning (3), though less invasive surgical techniques are being championed to reduce impact on sexual and urinary function (4). ED is also a known treatment consequence of external beam radiotherapy and brachytherapy (5). Studies suggest that the overall impact on sexual functioning is likely to be underestimated in the quality of life (QoL) literature (in part due to the methodological limitations of fixed questionnaire scales) (6),

Table 1 Socio-demographic and medical data of participants

Sample characteristic	N [%]
Ethnicity (n=18)	
White British and White Irish	10
African Caribbean, Australian, Chinese, Greek, North African, Filipino, Taiwanese	8
Employment (n=18)	
Retired	4
Housewife	4
Professional	5
Semi-professional	1
Manual	4
Sexual orientation (n=18)	
Heterosexual	14 [78]
Homosexual	4 [22]
The men's treatment (n=10)	
Prostatectomy	4 [40]
Hormone therapy	1 [10]
Radiotherapy/HIFU	1 [10]
Combination of two therapies	3 [30]
Combination of three therapies	1 [10]
Stage of treatment	
On treatment (hormone therapy)	2
Follow-up	8

HIFU, high-intensity focused ultrasound.

and emerge as a concern as men enter follow-up (3). Despite these data, the impact of prostate disease on sexual functioning has been described as an outcome that has been neglected by urologists (3,7). Research focus and clinical practice developments have tended to concentrate on the use of assistive technologies: primarily pharmacological interventions, to help some men achieve more normative erectile function (8). However, such responses are limited as they may lack integration with couple-focused strategies which, if delivered effectively, could enhance the success of biomedical interventions. Further, prostate cancer is often portrayed as a disease of individual men (9), whereas research has increasingly pointed to the shared nature of the experience within a unique couple dynamic (10-13), with evidence demonstrating poorer sexual functioning when compared to the general population (11). Studies have also started to emphasise partners as 'unpaid/ unrecognised carers', indicating the need for a further

precedent to appreciate and address their needs in order to support and sustain their physical, emotional and caregiving role (11).

Despite the growth in prostate cancer research, the majority of studies in prostate cancer have focused on the impact of management of ED as an iatrogenic consequence of androgen therapy or radical prostatectomy (14). The impact on partners has been a more recent development but attention is being concentrated on this issue (15,16). In the latter study the issue of distress in female partners was examined and revealed the shared nature of the cancer event, and the need to reassess the situation once prostate cancer had been confirmed and to accept the challenges, threats and losses facing them, manage changes and create a meaningful intimate and social life (15). Furthermore there have been questionnaire studies exploring how prostate cancer couples rate each other's coping and distress. One study demonstrated that higher QoL, in both groups, was associated with higher education levels, lower avoidant coping, and higher relationship satisfaction (17).

The present study sought to document the intimate experiences of men and their partners post-treatment, focusing particularly on qualitative accounts of the impact in relation to sexual functioning and how these concerns were managed between themselves, and discussed in the clinic.

Materials and methods

In-depth interviews were conducted with participants recruited from two inner-city English hospitals. The study received ethical approval from the local NHS Research Ethics Committee.

Subjects

Interviews were conducted with respondents as couples, or individuals, depending on their stated preferences, recognising the benefits and constraints of individual versus dyadic interviews. Of the 18 participants, six couples agreed to a joint interview. Two couples provided individual accounts, and two men agreed to participate only without their partner.

Maximum variation sampling (18) encouraged a purposively heterogeneous demographic mix (*Table 1*), that included a diverse group of men with experience of a range of treatment options. Participants' ages ranged from 34 to 78 years and all were at least 2 years post surgery or radiation therapy, some were still on hormone therapy. The majority

of patients and partners were classified as White British, with the remaining 44% drawn from minority ethnic backgrounds including Greek, African Caribbean, Chinese, North African and Filipino. The sample was also purposively devised to ensure men had completed treatment at least two years before and were undergoing follow-up care at the time of the study. This allowed them, and their partners, to consider the enduring impact of cancer treatment.

Inclusion & exclusion criteria

Inclusion criteria were men (and their partners where possible) willing to provide written consent and living with a diagnosis of prostate cancer who have had experienced external beam radiotherapy, prostate surgery or androgen therapy at least 2 years previously. This was to ensure that short-term treatment effects were minimised and some adaptation to their situation had occurred. Exclusion criteria included men less than 2 years out of treatment, those unable/unwilling to provide written consent or those with co-morbidities that were likely to impact significantly on their experiences. Advice was sought from medical staff about the study and its aims, assistance with access to men was provided by Nurse Specialist or Consultants.

Procedure & interview focus

Interviews were carried out in participants' homes. They were intentionally exploratory in nature and all participants were invited to reflect on the cancer experience and its impact on their sex lives. They were advised at the time of recruitment that we wanted them to be as honest as possible and to use language that felt comfortable.

An experienced researcher with a background in mental health and communication skills education undertook all interviews (SML). The interviews followed a conversational style using series of prompts such as 'Can you tell me about the impact of prostate cancer on your relationship?'; 'How has this been experienced in terms of your sexual relationship?'; 'How has your partner been affected by this experience'; 'Can you tell us how this was dealt with by the health care professionals you have met?'.

Interviews were then transcribed in full and checked for accuracy by two members of the team (DK & SML).

Coding

Analysis proceeded with a full reading and discussion between two of the researchers to support the organisation of the interview transcripts. Codes were developed in the process of defining categories and identifying recurring and less usual themes. Thus, categories were formulated and overarching themes were identified (for example gendered aspects of the disease, the shared nature of the event), to describe and explain the couples' and individuals' experiences of prostate cancer outcomes. Data were coded and comparisons made across the transcripts about couples' experiences since treatment, expectations and views of current service provision regarding recovery following prostate cancer treatment. This approach allowed the interviewer to remain focussed on what might help people in their situation, as well as illuminate the experience they have been through as a couple (19).

Results

Four categories were identified within the transcripts that represented core components of intimacy and sexual recovery following prostate cancer treatment. These related to social influences and language used to describe the loss or recovery of sexual activities; discussing sexual activity with clinicians; the clash of individual versus couple paradigms of prostate cancer recovery and the re-integration of sexual activities into the relationship.

Social influences and language used in relation to sexual expression

Social influences and language emerged as recurring features in these interviews. This included, for instance, the use of metaphors, the vocabulary chosen to describe sexual dysfunction or activity, and inherent culturally-derived expectations. There was evidence of frequent use of metaphors by both patients and partners. One partner, who found it difficult to express her frustration at her husband's ED, and his complacency towards it, related her dissatisfaction in terms of "*I'm dying… of thirst… of food*" and "*A little cuddle?, No! I'm starving*" (Female partner, couple 9, post surgery).

The expression "*Rome wasn't built in a day*" (Female partner, couple 8, post radiotherapy) was also used to demonstrate a partner's understanding of the gradual process involved in resuming sexual expression after prostate cancer treatment. Much of the language used by participants referred to ED and the failure of achieving an erection, for example: "*it was floppy*", "*we had a small, fairly flaccid penis*"—it was noteworthy that women would often

speak of the problem in joint terms rather than just the man's. More technical terms such as "*masturbation*" and "*ejaculation*" were used during the interviews rather than colloquial or slang terms. In social terms, this might be a sign of not seeming to appear crude or disrespectful in the interview context but, does raises an important point about the type of language that may be considered acceptable in clinical situations.

Underpinning the use of these terms were cultural expectations in relation to sexual dysfunction experienced predominantly from the patients' perspective. The focus was usually on problematic erectile function. One of the men had asked his male friends about failing sexual ability. Although he had very strong sexual desire, his "*orgasm, erection does not last for a long time, one to one and a half minutes… not very strong*" (Patient, couple 3, post surgery). He was prescribed a PDE5 inhibitor and claimed that he didn't want pleasure for just himself but for his wife as well, and felt that he was the one who should "*fix this problem*". The expectations of all patients (except two) was to resume full sexual activity as soon as practicable and this appeared to be a higher expectation than that held by partners who expressed the belief in taking things at a more steady pace.

One female partner explained this eagerness to please and to meet perceived expectations within the sexual relationship:

"*He was too anxious to try to please me and nothing would happen at all and I would say, 'Don't be silly, you wait and see another couple of months will be fine and give it time and don't jump the gun too quick. You know, you're not ready yet'. After a while it was fine. I think it was a natural reaction.*" (Female partner, couple 1, post surgery).

At times, the choice of language of sex and intimacy could become a source of discomfort and tension within the medical consultation. The following quotation illustrates how one patient was asked to modify his language:

"*Do you remember he said 'Have I had sex?' and I said 'Oh I'd had a shag twice' and he said 'Don't talk like that' or something…so I said 'Oh well better call it sexual intercourse.'*" (Male patient, couple 4, post surgery).

This articulation of clinician discomfort is framed as related to the language that the patient chose to use, rather than avoidance of talking about sexual topics more generally. This does, however, signpost potential tensions in talking openly about sex and intimacy in a language which the patient, partner and clinician are comfortable with.

There were instances of remarkable frankness during data collection—one Arab couple spoke of their intimate relationship very openly with young children climbing over them, another couple was interviewed as they lay on their bed together and the interviewer sat beside them. There was no sense of awkwardness about these situations; perhaps because the interviewer was comfortable with the discussion herself and so the participants felt able to vent their concerns and experiences. The social context within which disclosure takes place, therefore, may need to be considered. The home setting for this study seems to have encouraged openness—more so than had been achieved in clinical settings.

Discussions with clinicians about sexual activity

Talking with clinicians about intimacy and sex—either in a positive or a negative sense—was a common theme within these interview data. This included reflections on treatment decision-making, the range of topics that were discussed, and the ways in which clinicians communicated whether sex was an acceptable and appropriate topic for discussion within oncology outpatient clinics. Information about sexual functioning and the likely changes post-treatment were an expressed interest of both patients and partners.

Health care professionals in outpatient clinics had communicated their understanding of the expected pattern of sexual recovery of patients and their partners:

"*We found … (the nurse practitioner) very good and explained about sex life. Her information helped a lot.*" (Female partner, couple 1, post surgery).

Participants reflected on who had initially raised the topic of sex and concerns about ED:

Female partner: *(My husband's) erection was getting worse and worse… Now this isn't something he brought up with (consultant) really.*

Patient: *(Consultant) asked me about it I think, as opposed to the other way around.*

Partner: *He (my husband) didn't really want to bring up that topic, did you?* (Couple 2, post surgery).

Couples felt that talking about sex and intimacy was an important topic; requiring a senior clinician to give the topic the gravitas it deserved:

"*I think you have to have a doctor or a nurse or somebody who's in a senior position who is quite sympathetic and knows how to be able to sit down and talk to a couple, because I think that's very important really, you know it's no good like the couple going in and seeing the consultant or the doctor or the nurse and they're just flippant with them, they don't explain things.*" (Partner, couple 1, post surgery).

The majority of this sample did not feel that clinicians

had discussed sexual functioning well, and reported that such concerns were not always appropriate to share with friends/family:

"*Unconsciously, I was really scared. I wish I'd had somebody like you (the researcher), what I would call a professional that's, because it's not something that you can talk about to close friends because, it's too intimate.*" (Partner, couple 6, androgen therapy).

Limited opportunities were offered to individuals or couples to talk about their psychosexual needs; this was felt to be a considerable constraint of current service delivery:

Interviewer: *Do you think that your psychosexual needs had been met in terms of the care that you received after the treatment?*

Patient: *Well once I went off the hormonal drug, there was no, shall we say, counselling or anything.*

Interviewer: *Did anyone talk to you about it or mention it or was it...?*

Patient: *No, no, my oncologist in [another country] did say to me that once you're off the drug things will get back to normal but what he didn't tell me was how long, it was only after I asked a question when I got the result that it will take a year to eighteen months for your body to eliminate all the effects of the [treatment], that was all I was given.* (Patient, couple 6, androgen therapy).

Thus, while some information was provided, all of the participants felt that this stopped short of what they required to enter psychological recovery feeling adequately prepared for the timeframes over which physical recovery might take place.

At times, discussions about sex included directives about what sexual activities were permissible at specific time points during recovery. A minority of participants had offered leaflets about sexual functioning, and aids to EF recovery such as vacuum constriction devices, while others had not been given advice but felt that this would have been extremely helpful. Rarer in the data was an expression that discussions of ED and incontinence had guided treatment decision making. The following participant describes photo dynamic therapy (PDT) and high-intensity focused ultrasound (HIFU):

"*He said 'What are you going to do?' so I said 'I got two choices I need to do the PDT again... or I can do the HIFU and I don't want to do the conformal, I don't want to do the surgery or any others' I said because 'I don't want to be impotent or incontinent and everything else'. And what I said was 'Is it better to have ten years of fun or fifteen years of hell?'*" (Patient 5, post radiotherapy).

Meanwhile, other patients were given so little information, that they were uncertain when they could resume regular sexual activity, without putting their health in danger:

"*The thing that stopped me [having sex] was, first of all I was, I couldn't pee at all so I had to put the catheter in every few hours, and, we saw there was lots of blood coming out and this sort of thing...so I thought maybe I'd do myself some damage and that's why... So it was a 'damage I might do to myself' scenario, and nobody said to me in the hospital, 'Oh you can have sex whenever you feel like' they just said 'You've got to wait a week or a month.'*" (Patient, couple 4, post surgery).

In other instances pre-treatment discussions of sexual functioning continued post-treatment, with some physicians asking about erectile functioning at follow-up appointments. The following participant reflects on how he was asked to describe his erections, following treatment:

"*And I said 'Oh it's', I said 'the first time was a bit soggy... And the second one was okay' I said 'but only half an hour.'*" (Patient, couple 4, post surgery).

The importance of transparent discussions with clinicians is underlined by the paucity of opportunities some participants seem to have had to talk about their sexual functioning elsewhere.

At an individual couple level, there were also examples of where the private impact had probably not been appreciated by health professionals. One of the members of the two gay couples who took part spoke of how his partner had become 'very fat after the hormone therapy' and said:

'*This was very hard to come to terms with, a fattish old man.*'

His partner does not now ejaculate but:

'*Joint ejaculation had been very important to us, now he says I'm coming, I'm coming!, but there's no visible signs.*' (Couple 6, androgen therapy).

The man also spoke of feeling guilty at finding his partner unattractive since hormone therapy but had felt unable to discuss this—certainly not with a health professional. He also had not been aware that it was possible for a man to have a powerful but dry orgasm without a full erection; this learning had been experiential rather than anything given to the couple as an information package associated with the treatment choices.

Another man spoke of having had erections and sexual dreams leading to orgasms in the post-surgical period in hospital. This had not been disclosed to staff but his attitude to this had been rather accepting and he described professionals doing such work in a matter of fact way:

"*If you go to the butcher he know how to cut meat, if you go to the mechanic he fixes your car, so it's your job…*'. (Patient 6, post surgery).

Regarding his erections and his sexual recovery, however, he spoke of masturbating into a cloth after surgery due to it being:

'*Bloody and so as not spread germ.*'

An individualistic model of care

Partners often reported feeling excluded from the events relating to the cancer and its impact. For instance, shortly after diagnosis one of the partners recalled:

"*I would have liked, just once in a while, for the consultant to say, "How is your wife getting on? I really feel out of it and the fact was of course, I was affected.*" (Partner, couple 2, post surgery).

Partners were rarely asked during clinics about their own needs in relation to the effects of the diagnosis and treatment outcomes. Thus, despite the sexual consequences of treatment having had a clear impact on the partner, they were rarely made to feel they had a genuine contribution to make to the medical consultation. The following excerpt illustrates this position of feeling under-supported, particularly in the context of being in a same-sex relationship:

Interviewer: *Did they ask you, questions in terms of you know, were you okay?*

Partner: *No they never did, they were very nice but I think the… medical establishment in general is still rather formal.*

Interviewer: *There's no guidance, for same sex couples?*

Partner: *No, that's right, and that really would have been very helpful.* (Partner, couple 6, androgen therapy).

Some couples told the interviewer that they had not talked with each other about sexual concerns either before or after treatment:

Interviewer: *Now did you talk about this before the operation?*

Partner: *No we didn't actually. (…) I was very patient, because I think you have to be like that, you can't just like click your fingers and everything's going to come back to normal because it's a big operation and he was very ill and it's just basically if you love somebody enough it's just, you just must sort of have patience and wait and then everything is fine.* (Female partner, couple 1, post surgery).

The above extract indicates that although the clinician had talked with him and his partner about sexual concerns, this did not mean that the couple would necessarily talk about it further together.

Re-integrating sex into the relationship

Couples found their own harmony in living with enduring changes such as moderate or severe ED and reflected on this with the interviewer:

"*The only way it's affected my sex life is not getting an erection, to begin, though now that's not a problem now.*" (Patient, couple 1, post surgery).

A further interviewee reflected on changes in erectile functioning as he recovered from treatment:

'*Well it wasn't really there because, there is no erection you know, as strong as I want it to be.* (Patient, couple 3, post surgery).

Participants indicated the importance of regaining sexual functioning, with one man stating that he yearned for sex before his physical capability returned:

"*I was dying for some sex*" (Male, couple 4, post radiotherapy).

Couples discovered that although penetrative sex may not be possible, other forms of intimacy and sexual activity were still available, and again the issue of orgasm without full EF was mentioned:

"*One thing that I picked up on, which I thought was quite extraordinary was that you can have an orgasm with a flaccid penis.*" (Partner, couple 2, post surgery).

For one man, although he regained erections very quickly after surgery, however this was not something his wife was aware of. Indeed, several years after treatment he was still sexually active, but not with his wife. The following quotation was also used above to illustrate pre-cancer relationships, but speaks equally powerfully to this sense of (re)integrating sex into current lifestyle choices:

"*Well she (wife) will believe, and she does still believe it, that, I am er...you know, I cannot make love. She accepted it...that the penis it never gets hard.*" (Patient 5, post radiotherapy).

Discussion

The final quotation above may reveal one of the reasons why some professionals, who may be under time pressures in clinical situations, find the discussion of sexual dysfunction a challenging topic. Such conversations bring them closer to the social and psychological complexities of people's lives in ways that go beyond the traditional focus of the biomedical model. By adopting a qualitative approach to this study we were exposed to aspects of the couple's sexual lives which would have not been revealed by questionnaire methods. However, both approaches have a role to play to reveal the needs of larger samples of couples across different disease trajectories.

Although only one person in a partnership experiences cancer, these data clearly indicated that the disease and its treatments also impacts on the partner. There is evidence that adjustment to ED takes time, but is a highly significant event in couples' lives following prostate cancer. Its importance should not be under-estimated (20). Consequently, there is not only a need for patient-centred care, but also relational models of care, whereby side-effects are recognised to impact on both members of a partnership (for example ED, or lack of ejaculation). Supportive care is

therefore important for both patient and partner but must be tailored using language that is appropriate to the context of their lifestyles and expectations (3).

A relational approach to couple-focused support will take into account an understanding of illness impact that extends beyond the individual (patient-centred) bio-medical model. Instead it views cancer as a life-changing event that affects not just individuals, but everyone in one's intimate, and wider, sphere of relationships. To truly understand and address the wider impact of prostate cancer, it is vital that it is accepted as a condition affecting both the patient and those with whom he relates. Importantly these relationships may be spousal, casual or committed (or multiple combinations of these possibilities). The danger for the professional is to make assumptions.

These data suggest that health professionals should be supported to develop a more nuanced understanding of how best to work with couples in the post-cancer situation. There are clear practice implications for professionals in being able to provide care for patients and partners in a morally neutral way, while maintaining confidentiality within and between the two (or more) parties in sexual relationships. Consequently, HCP education should explore culturally held assumptions related to aspects of social difference such as age, gender and sexual orientation. Beyond this ideological level, healthcare professionals should also receive practical support to raise sexual functioning and intimacy within clinical conversations, so that these topics are given adequate gravitas. In addition to the bio-medical model of resuming sex after-treatment using medication, assistive technologies and sex toys (20), there should also be an emphasis on the development of couple-working skills and disclosure of worrying symptoms; such as the impact of ED. Clinical trials are exploring ways to support men and partners after therapy (21,22).

Finding a vocabulary for these clinical conversations is critical, with the patient, partner and health professional finding a mutually acceptable lexicon for discussing sex and intimacy. This may include shifting between medicalised language and lay terms to accommodate varying levels of (dis)comfort at talking about sexual/intimate issues (19).

We must also acknowledge the limitations of this small, heterogenous sample. In qualitative studies the aim is to obtain depth of insight from smaller numbers. However, we also wanted to include as mixed a group as possible. Whilst we can offer insights here we recommend larger scale studies are needed, using mixed research methods.

To return to the original impetus for this study about the impact of prostate cancer on couples, we are reminded of the fact that sexual expression is one of the most fundamental of human pleasures. It allows people to engage intimately, to derive pleasure from each other's body and reinforces the bonds that exist—and that become so important—when a life threatening illness is diagnosed. When we set out to cure or control cancer but, in the process, leave individuals unable to experience the pleasure of sexual intimacy then we must ask if we have really promoted their wellbeing. To some individuals this outcome may be very acceptable, whereas to others they may be left with a sense of regret.

Qualitative research methods can allow us insights into these issues and can reveal nuanced examples of the impact of cancer treatments. The primary message of this paper is that information and communication can help clarify what expectations might be acceptable, and achievable, and which are less likely. Previous research revealed that prostate cancer consultations, although focussing on the area of the body most associated with sexual function, did not always encourage the topic to be addressed. Instead the topic was most often raised by the patient, if it was at all, and some men (such as those with co-morbidities) were least likely to be asked about sexual concerns (19).

By not including this issue into routine practice we risk diminishing the importance of sexual function in the mind of patients and partners, and in doing so their anxiety may be magnified. Mitchell (23) captures this final sentiment well:

'Sexual pleasure, is also relative, and is often as much a product of expectation, of the symbolic meaning of the act, and of the emotional and relational context as it is of the physical experience' (p.60).

This is a useful reminder as we engage in curative work with increasing numbers of men, and their partners, following the diagnosis of prostate cancer (23,24).

Acknowledgements

Footnote

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Couples-based interventions following prostate cancer treatment: a narrative review

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Background/Objective: Sexual dysfunction following prostate cancer (PC) treatment often results in sexual avoidance and a loss of sexual intimacy, which can lead to relationship distress. This review aims to evaluate six studies intended to address relational and sexual intimacy following PC treatment and discuss methodological concerns which may help produce more effective interventions.

Methods: Electronic databases used to conduct literature searches included Medline, PsychINFO, and Web of Science. Studies were included if they were: randomized controlled trials (RCTs) using samples of men diagnosed with PC of any stage, had a psychosocial intervention, and addressed at least one sexual and relational outcome.

Results: As a whole, the literature has produced mixed results. While significant findings were reported, many of the primary hypotheses were not achieved. The six studies show that men with PC may benefit from education and support related to treatment options for erectile dysfunction (ED), whereas their partners may benefit more from interventions focused on relational issues. Important methodological limitations included: selection of general outcome measures as opposed to measures specific to sexuality or intimacy outcomes, lack of assessing distress or bother of the patient/couples as study entry criteria, heterogeneity of study populations, and lack of innovative intervention content as the current studies tested standard educational interventions, sex therapies techniques, and couples therapy strategies with only marginal success.

Conclusions: Interventions based on innovative theoretical approaches as well as study designs that address the outlined methodological limitations are needed in this area.

Keywords: Prostate cancer (PC); erectile dysfunction (ED); sexual function; erectile rehabilitation

Introduction

In the U.S., over 200,000 new cases of prostate cancer (PC) will be diagnosed in 2015 (1,2), making it the most commonly diagnosed cancer in men. Of these cases, 90% will be diagnosed in the early stage due to effective screening and early detection (3). With early detection, survival rates continue to increase and close to 100% of cases diagnosed in the U.S. will survive five years post-diagnosis (4).

The combination of the large number of men diagnosed with PC, early detection, and effective treatment, has led to an increased focus on survivorship-related concerns following treatment for PC, of particular importance, erectile dysfunction (ED) (5). Data suggest that only 16% of men will return to their baseline erectile function following PC surgery (6) . Importantly, ED can have a significant negative psychological effect; men with ED report frustration and shame, an increase in depressive symptoms, and lower general life happiness (6). The impact of ED can also extend to the couple. The psychological burden related to difficulties with erections often results in a loss of sexual and non-sexual intimacy, which, in turn, can lead to relationship distress (7). Additionally, while men with PC may experience psychological distress, psychosocial research has emerged

suggesting that female partners may experience equal, if not more distress than their male partners with PC (8,9).

These findings suggest that high levels of distress may be present in both men with PC and their partners, and that this distress can have a negative impact on their relationship. Thus, there is a significant need for interventions that help the PC patient and his partner to manage and cope with the impact cancer treatment can have on their intimacy and relationship. The purpose of this paper is to review and to critically evaluate important intervention studies that intended to address relational and sexual intimacy following PC treatment. Possible methodological concerns are discussed in order to determine what is needed to produce more effective interventions in this area.

Methods

Identification of relevant studies occurred by a two stage process: (I) database search: electronic databases used to conduct literature searches included Medline, PsychINFO, and Web of Science (January 1, 2005~January 1, 2015). Key words used to search titles and abstracts included *prostate, AND randomized-controlled trial, AND psychosocial intervention OR psychological intervention* OR psychosocial support* OR psychological support* OR psychosexual* OR psychosexual support* OR intimacy enhancing intervention *OR education OR counseling**; (II) inclusion screening: abstracts were screened for relevance according to the inclusion criteria. Retrieved studies were included if they were randomized-controlled trials (RCTs) using samples of men diagnosed with PC of any stage. Studies were required to have a psychosocial intervention in at least one arm of the study design, which had to address at least one sexual and one relational outcome. Following this search, and through group consensus with the authors, six RCTs intended to increase intimacy and sexual functioning in couples following PC treatment were identified. We review these studies below.

Randomized clinical trial of a family intervention for prostate cancer (PC) patients and their spouses

Methods

The objective of the Northouse *et al.* [2007] study was to test if a family-based intervention could improve coping resources, appraisal variables, quality of life (QOL), and symptom distress in patients with PC and their spouses. Three groups of PC patients were recruited: those newly

diagnosed with PC after completion of their primary treatment, those in biochemical recurrence who had two consecutive rises in their PSA score, and those with advanced stage PC after the diagnosis of metastatic disease (10). Two hundred and thirty-five dyads (PC patients and their spouses or live-in partners) in total participated in either the control (n=123 for 4-month assessment, n=114 for 12-month assessment) or experimental conditions (n=112 for 4-month assessment, n=104 for 12-month assessment). The control condition was standard clinic care, whereas the experimental condition was standard care plus the FOCUS program, an intervention adapted from the stress-coping framework of Lazarus and Folkman. The participants all received assessments at baseline, four months, eight months, and 12 months. Northouse *et al.* hypothesized that couples who received the FOCUS program would report fewer negative appraisal variables, more positive outcomes on coping resources, and higher QOL than couples in the control group (10).

Intervention

The FOCUS Program, based off of Lazarus and Folkman's cognitive appraisal framework, consisted of three 90-minute home visits and two 30-minute telephone sessions spaced out between the baseline assessment and the four month assessment. FOCUS stands for the sessions of family involvement, wherein couples are encouraged to work as a team, communicate openly about the illness and be supportive of one another; optimistic attitude, in which couples are told to maintain hope and focus on short-term, attainable goals; coping effectiveness, wherein couples are taught techniques for stress reduction as well as active coping strategies and healthy lifestyle choices; uncertainty reduction, the focus of which is on how to obtain information, and how to live with uncertainty; and symptom management, which teaches couples how to cope with symptoms. The trained nurses who delivered this intervention also tailored the intervention to the individual couple's needs.

Results

While the patients received only minimal benefit from the FOCUS program, the partners in the intervention group demonstrated moderate advantages. The most robust result for the partners was the reporting of better communication with the patients across all three assessment points compared to control partners. The partners in the

intervention condition also demonstrated less negative appraisal of caregiving, including less uncertainty on the Mishel Uncertainty Illness Scale (11) and reduced hopelessness on the Beck Hopelessness Scale (12) than the control partners at 4 months. However, only the result for uncertainty remained significant at a later time point. Additionally, the partners in the intervention condition demonstrated benefit in general well-being or QOL when compared to controls. At four months, the partners in the intervention group reported significantly better scores on the Medical Outcomes Study 12-item short form (MOS SF-12) mental health QOL subscale (13) and better overall Functional Assessment of Cancer Treatment (FACT-G) QOL (14) scores compared to controls. For later time points, the intervention partners reported better physical QOL on the MOS SF-12 (13) at eight and 12 months compared to control partners. On the measures of coping resources, the partners in the intervention had higher self-efficacy to manage the illness at four and 12 months on the Lewis Cancer Self-Efficacy Scale (15) and more active coping at 12 months than those in the control condition on the Lewis Mutuality and Interpersonal Sensitivity Scale (15). Additionally, partners who had undergone the FOCUS program had significantly less general symptom distress than control spouses on the Symptom Scale of the Omega Clinical Screening Questionnaire (OSQ) (16) and fewer problems related to husband's urinary incontinence at four months and eight months.

The patient results stand in contrast to these partner results. The intervention patients only significantly differed from control patients on the measures of communication and uncertainty about their illness at four months. The patients in the intervention did not differ from those in the control condition on any QOL variables and the patients saw no significant differences in general symptom distress or PC specific symptoms, including patients' urinary, bowel, sexual, and hormone symptoms, as measured by the 50-item Expanded Prostate Cancer Index Composite (EPIC) (17). Therefore, the spousal benefit from the family-based intervention, FOCUS, proved to be far better than the benefit for the patients.

Pilot intervention to enhance sexual rehabilitation for couples after treatment for localized prostate carcinoma

Methods

A study by Canada *et al*. [2005] focused specifically on the

sexual rehabilitation aspect for couples where the man had either undergone surgery or radiation therapy (RT) for PC. Canada and colleagues developed an intervention that was either given to the patient alone or to the patient and his female partner (18). Eligible patients included those who had been treated for localized PC within three months to five years of starting the intervention, were unable to achieve and maintain an erection for sexual intercourse during ≥50% of attempts within the past three months, and had not been successful in using medical treatment for ED. A total of 26 men received the intervention without their partners and 25 men received the intervention with their partners. The purpose of this intervention was to enhance levels of sexual satisfaction and help men achieve successful utilization of medical treatments for ED. Assessments were given at baseline, after the last session of the intervention (after one month), and at three-month and six-month follow-ups.

Intervention

A trained interventionist administered four counseling sessions to the patient alone or to him and his partner. The sessions included education surrounding the sexual impact of surgery or RT for PC, medical and surgical treatments for ED, coping strategies to use during sexual activity for patients experiencing urinary incontinence or partners with postmenopausal vaginal atrophy. Additionally, couples were given skill training to enhance general communication of feelings, open expression of affection, and sexual communication. Cognitive behavioral techniques were also used to decrease negative beliefs about cancer and sexuality. As homework assignments, the patients and partners were asked to do a variety of behavioral exercises, and to make action plans for their use of medical treatments for ED.

Results

There were no significant differences between the two treatment groups (the participant attending the sessions alone compared to the couple attending together). Therefore, the data from both groups were combined and repeated measures analyses were conducted using the subjects as their own controls. There was no intervention impact on marital adjustment, as measured by the dyadic adjustment scale (A-DAS) (19), perhaps due to the fact that many couples already had high marital adjustment scores at baseline. The patients' scores on emotional distress did

significantly improve from baseline on the Brief Symptom Inventory (20) as did male sexual functioning/satisfaction in general as measured by the International Index of Erectile Functioning (IIEF) (21). The subscales of the IIEF of erectile function (mean score at baseline 7.6±8.7, mean score at 3-mos 15.3±11.2), orgasmic function, intercourse satisfaction, and overall satisfaction all were significantly improved at three months, however only overall sexual satisfaction remained significant at six months. The partners' scores on sexual functioning/satisfaction as measured by the Female Sexual Function Index (FSFI) (22) significantly improved on the global score as well as on all of the FSFI subscales for the post treatment time point. As with men, only overall sexual satisfaction remained significant at six months. Importantly, the use of medical treatments for ED improved from the 31% of men using them at baseline, to 52% at post treatment, and to 55% at three-month follow-up. At six months, the significant improvement in the use of ED treatment remained with 49% continuing to use the treatment.

A randomized trial of internet-based versus traditional sexual counseling for couples after localized prostate cancer (PC) treatment

Methods

A study by Schover *et al*. [2011] built upon Canada *et al*.'s [2005] study. Schover and colleagues compare a face-to-face format to an internet-based format of a revised version of the Canada *et al*. intervention entitled Counseling About Regaining Erections and Sexual Satisfaction (CAREss) (23). The study also included a three month wait list control condition. A second internet-based group was added to examine the relationship between website use and outcomes. The internet-based intervention was created with the hopes to be more convenient, to minimize the drop-out rate, and to play to the fact that many men already seek sexual content on the internet. Males who were married or living with a partner for over a year with localized PC who had either definitive surgery or RT three months to seven years previously were included in this study. The men had to be unable to achieve and maintain an erection for sexual intercourse for ≥50% of attempts within the past three months. The 112 couples (waitlist n=43 (waitlist randomized after three-month period to FF n=20 and WEB1 n=22), FF n=40, WEB1 n=41, WEB2 n=43) were given assessments at baseline, posttreatment (after 12 weeks), and at three-months,

six-months, and 12-months follow-up.

Intervention

The content of these interventions were based on the Canada *et al*. [2005] intervention described above. The face-to-face and internet-based formats of CAREss had the same content and homework and both were three sessions in length. For participants in the WEB condition, their therapists were available through email and to give feedback on homework. For participants in the FF condition, therapists discussed the homework in the next session. The exercises were designed to boost expression of affection and comfort in initiating sexual activity, enhance sexual communication, and aid in resuming sex without performance anxiety. The education provided gave suggestions regarding coping with postmenopausal vaginal atrophy and coping with male urinary incontinence. Participants learned cognitive reframing techniques to identify negative beliefs about sexuality, and received a decision aid for choosing ED treatment together.

Results

There were no differences for any of the variables compared to the wait list controls. Additionally, there were no differences between the face-to-face CAREss group and the internet-based CAREss group. Therefore, these two groups were combined for repeated measures analyses. Men who received the CAREss intervention had significant gains on the subscale of erectile functioning (EF) on the IIEF between baseline and six-month follow-up as well as between baseline and one-year follow-up, with 16% having near-normal function (a score of ≥22 on the EF subscale of the IIEF) at baseline increasing to 39% at six months, and slightly declining again to 35% at one year follow-up. Men in the intervention conditions also improved significantly on the subscales of orgasmic function, intercourse satisfaction, and overall sexual satisfaction from baseline to one year. The rates of ED treatment use did not change significantly within any group. However, men who intensified their ED treatment [the use was defined as (I) none; (II) using oral medication only; and (III) using invasive ED treatment] had large, significant increases in IIEF scores across time. There were no significant differences in marital happiness, as measured by the A-DAS, or overall distress, as measured by the Brief Symptom Inventory (BSI-18) (24), for men in either of the intervention conditions. However, the sample

of men was not particularly distressed at baseline, which could have been the reason for the lack of change. Women as a whole in the intervention conditions did not improve significantly on sexual functioning/satisfaction, but when divided into the categories of those who had abnormal versus normal scores at baseline, the women who had abnormal scores at baseline in the intervention conditions did have significant improvement over time. Interestingly, normal FSFI scoring women in the intervention conditions at baseline actually declined and then recovered to baseline by one year. The baseline sexual functioning of women predicted the efficacy of CAREss in improving men's IIEF scores.

Intimacy-enhancing psychological intervention for men diagnosed with prostate cancer (PC) and their partners: a pilot study

Methods

Manne *et al*. [2011] conducted a pilot evaluation of an intimacy enhancing therapy for men diagnosed with PC and their partners. The aim of this study was twofold; to determine whether IET proved efficacious in a small sample and to identify couples for whom IET would be most beneficial (25). To achieve both aims, the impact of IET versus usual care (UC) on survivor and partner psychological outcomes was evaluated, as was the impact of IET on dyadic communication. The participants were men diagnosed with localized PC within the past year, who were married or living with a significant other of either gender. Seventy-one couples were randomized to receive either five sessions of IET (n=37) or UC (n=34), which consisted of standard psychosocial care, such as social work consultations. Couples were assessed at two time points; baseline, and at eight weeks following baseline assessments (IET patients n=31, partners n=30; UC patients n=29, partners n=26).

Intervention

Utilizing the Relationship Intimacy Model of Cancer adaption (26), a 90 minute, five session intervention coined IET was developed to improve communication amongst PC survivors and their partners. The ultimate goal of IET is to address the effects of cancer and it's treatments on relational intimacy. Each session of IET focuses on didactic content, and includes in-session skill practice as well as homework practice assignments. IET aims to enhance couples' emotional intimacy by promoting the use of techniques that focus on the maintenance of mutual understanding and support, as well as reciprocal disclosure. By providing techniques that facilitate constructive discussions regarding patients' and partners' concerns of the experience and impact of cancer, IET sessions aim to provide greater overall relationship satisfaction. To achieve improved communication skills and enhance emotional intimacy, couples utilize a variety of techniques derived from cognitive-behavioral and behavioral marital therapy. Rudimentary communication skills techniques were adapted to the context of PC from the Prevention and Relationship Enhancement Program, and from Gottman and colleagues' communication skills intervention (27).

Results

The significant results for this study were found following moderator analyses. When comparing the groups without moderation analyses, no significant effects were found for couples general distress on the Psychological Distress scale of Mental Health Inventory (28), cancer-specific distress on the Impact of Events Scale (29), cancer concerns, relationship satisfaction on the Dyadic Adjustment Scale (30), and relationship intimacy on the Personal Assessment of Intimacy in Relationships scale (30). Similarly, no treatment differences were observed for patients or partners on relationship communication outcomes. For patients, there were marginally significant treatment effects following IET on psychological well-being on the Psychological Well-Being Scale of Mental Health Inventory (28), while no significant treatment differences for partners were observed.

Moderator analyses of baseline variables revealed that patients with greater cancer concerns and poorer communication showed an increase in self-disclosure, perceived partner disclosure and perceived responsiveness following IET compared to UC, using scales adapted from Laurenceau and colleagues (31). While no significant effects following IET were found for mutual constructive communication on The Mutual Constructive Communication subscale of the communication Pattern Questionnaire (32) and demand-withdraw communication on The Demand-Withdraw subscale of the CPQ (32) for patients, significant effects were found for partners. Interestingly, patients who reported high levels of self-disclosure at baseline showed a reduction in self-disclosure at the eight-week follow-up after IET. Partners who reported greater cancer-specific distress, higher relationship

satisfaction and intimacy, and poorer communication benefited more from IET than UC, specifically with cancer-specific distress, relationship satisfaction, and relationship intimacy. Partners who reported low levels of pre-intervention cancer-specific distress and high levels of relationship satisfaction and intimacy at baseline reported an increase in cancer-specific distress, and lower levels of relationship satisfaction and intimacy following IET.

A randomized controlled trial (RCT) of a couples-based sexuality intervention for men with localized prostate cancer (PC) and their female partners

Methods

While support for patients with PC and their partners include nurses, social workers, psychologists and sex counselors, research emerging from the PC community has highlighted the benefits of peer support (33). To date, no research has been done to examine whether peer support is equally, if not more beneficial, than current professional care. With this in mind, Chambers and colleagues (2014) conducted a study that compared the efficacy of a couples-based, peer-delivered telephone support (n=63) versus couples-based, nurse delivered telephone counseling (n=62) versus UC (n=64) in improving patients' and their partners' psychosexual adjustment after the diagnosis and treatment of PC (33). In total, 189 couples were randomized to one of the three arms. The couples who received UC received standard medical management and a set of published educational materials. The participants were men who were scheduled for, or who had undergone surgery for PC within the last 12 months and their female partners. Assessments were conducted at four time points: baseline and at 3 (peer-delivered n=53, nurse delivered n=54, UC n=54), 6 (peer-delivered n=53, nurse delivered n=54, UC n=52), and 12 months follow-up (peer-delivered n=52, nurse delivered n=53, UC n=54).

Intervention

The couples-based, peer-delivered telephone intervention was oriented to empathic mutual support and education, which is consistent with a peer support framework in which couples bolster support based on shared personal experiences. Content included psycho-education about PC diagnosis, treatment and recovery, ED management, and maintaining intimacy and constructive communication

between couples. Managing and reviewing goals was also specifically focused on, and in doing so, couples were able to move beyond any setbacks experienced during the intervention. The couples-based, nurse-delivered telephone counseling followed theoretical principles and techniques of cognitive-behavioral sex and couples therapy, in which couples self-selected goals. Intervention content included education about PC, menopause, and sexuality. Behavioral homework consisted of aiming to increase the expression of affection and non-demanding sexual touch, challenging negative beliefs, and helping the couple collectively choose a medical treatment for ED that each would feel comfortable incorporating into their intimate relationship. It is important to note that for both intervention arms, couples recruited post-surgery received six sessions, while couples recruited pre-surgery received eight.

Results

No significant treatment effects were found for patients or partners for either intervention arm on sexual function on the IIEF (21) and the FSFI (22), sexuality needs on the sexuality needs subscale of the Supportive Care Needs Survey (34), sexual self-confidence on The Psychological Impact of Erectile Dysfunction-Sexual Experience scale (35), masculine self-esteem on The Masculine Self-Esteem Scale (36), marital satisfaction or intimacy on The Revised Dyadic Adjustment Scale (37). To examine whether beginning the intervention pre-or post-operatively had a significant effect, longitudinal analyses were run for all continuous variables. While no significant effects were found for partners, significant effects were found for patients for sexual function and sexual self-confidence. At 12-month follow-up, there were significant differences among intervention arms for overall use of medical treatments for ED. Patients in the peer intervention were 3.14 times more likely to use medical treatments for ED than those in UC, and patients in the nurse-delivered intervention were 3.67 times more likely to use medical treatments for ED than those in UC.

Androgen deprivation therapy (ADT) and maintenance of intimacy: a randomized controlled pilot study of an educational intervention for patients and their partners

Overview

Walker *et al.* [2013] conducted this pilot study to evaluate an

educational intervention designed to help couples anticipate and manage ADT generated changes, and to investigate whether such intervention impacts couple's relationships favorably. The participants were Caucasian men with PC who had either just started, or were scheduled to begin ADT, and showed no evidence of metastatic disease (18). Partners of any age were permitted to participate as long as they had English fluency and were either married or common-law female partners to the patient. Following consent, couples were given their own baseline questionnaire packets and instructed to complete each questionnaire and seal them in individual envelopes. Upon completion, 20 couples were randomized as a unit to either a treatment arm or UC (number of participants in experimental and control arms were not listed). Couples were assessed at two time points: baseline and at six months following baseline assessments.

Intervention

The educational intervention involved reading a 70-page booklet entitled "Androgen Deprivation Therapy: a Guide for Prostate Cancer Patients and their Partners," which discussed different ways to manage the side effects of ADT that directly affect patients (e.g., hot flashes and fatigue), as well as those that impact the couple (e.g., reduced libido and emotional liability). Each couple had two weeks to read the booklet, and subsequently received a one-hour private educational review session. The educational review session was headed by a male and female team to ensure that couples' individual needs were adequately met. The educational review session served as an opportunity for couples to address any remaining concerns that may have come up while reading the booklet, and to address any issues that may have not been brought up in the booklet. By providing a combination of an educational booklet as well as a review session, Walker and colleagues [2013] intended to help couples maintain a co-supportive bond that includes emotional and sexual intimacy.

Results

With the small sample size in each group, the authors focused on reporting effect sizes as opposed to statistical significance. A medium effect size (d=0.58) for patients' changes in the Personal Assessment of Intimacy in Relationships (PAIRS) (30) was observed favoring the treatment group, while partners in the treatment group scored lower (d=0.04) on PAIRS than the controls at

follow-up. Thus, patients in the educational intervention demonstrated gains in intimacy, while partners in the intervention evidenced no important change. For patients, a large effect size (d=1.02) was seen in DAS (38) scores at the six month follow-up, indicating that patients in the intervention arm had better dyadic adjustment following the educational intervention. A medium effect size (d=0.50) was observed for partners' scores on DAS at six months follow-up, indicating that partners in the intervention group also had more improvements on dyadic adjustment. While both patients and partners experienced improvements in dyadic adjustment, partner's scores following the intervention eventually attenuated. Secondary analyses of sexual activity revealed that controls had a 42% decline in sexual activity from baseline, while couples in the intervention group reported only a 32% decrease in sexual activity at six months follow-up. Taken collectively, couples who did not receive the educational intervention experienced greater losses in intimacy, dyadic adjustment and sexual activity following ADT.

Discussion

Taken as a group, these studies have produced mixed results. While there are clearly significant findings reported, many of the primary hypotheses were not achieved, and at times mediator or moderator analyses were needed to demonstrate effectiveness. Additionally, only two of the six studies (Northouse and Chambers) were large randomized controlled studies (10,33). To organize the summary of results, the manuscripts can be grouped loosely into two types of studies. First, the Canada, Schover, Chambers and Walker studies all focused on sexuality and ED treatments (18,23,33,39). These studies addressed: (I) educating participants about ED treatments; (II) educating participants about how to initiate sexual activity; or (III) managing side effects of PC treatment, with a focus on engaging in sexual relations (18,23,33,39). Although the results from these studies indicated an increase in the utilization of ED treatments, the primary aim of improved EF was generally not sustained. When significant results were reported, the effect of the intervention was not encouraging as the mean Erectile Function Domain of the IIEF improved but stayed within the "moderate" ED range. Additionally, these studies generally did not find significant outcomes for the partners. The second group of studies utilized couple's interventions that primarily addressed relationship aspects. The Manne and Northouse studies addressed a variety of concerns

regarding relationship variables such as communication and intimacy (10,25). The results from these studies were mixed but suggest better relationship outcomes and reduced distress for the partners. There were not many significant outcomes for the patients, suggesting that partners benefit more from relational aspects of interventions (10,25).

When this literature is considered as a whole, it is clear that future studies are needed. Since no one study stood out, using the lessons learned from these studies, and assessing their strengths and limitations, can provide valuable guidance for the next generation of interventions in this area. We outline what we believe to be important methodological and intervention considerations that when addressed, may help to produce more effective interventions for these men and their partners.

First, innovative theoretical approaches are needed to continue to push this literature forward. While the above literature has provided a sound foundation of intervention content and techniques, the studies have tested standard educational interventions, sex therapies techniques, and couples therapy strategies with only marginal success. According to the Complex Intervention Framework outlined by the Medical Research Council, in order to produce an effective intervention, the intervention must be grounded by a strong theoretical base (40). Therefore, changes that are expected, or changes that are likely to be achieved will have been tailored by the specific needs of the population. For example, in a recent qualitative study, Nelson *et al.* (in press) develop a theoretical argument that avoidance of sexual situations is an important construct to address with new interventions (6). The authors outline a theoretical justification to using Acceptance and Commitment Therapy techniques as the main intervention component to help men utilize ED treatments. A similar approach related to preventing avoidance of sexual situations is also being tested by Wooten and colleagues (41). Developing more specific interventions, based on sound theoretical foundations, would also have the benefit of helping us understand which components of the interventions are most effective for both the patients and the partners. Conducting qualitative research prior to intervention development is one way to understand which theoretical framework may be most useful. The studies reviewed above relied on previous research to guide their interventions; however, they did not conduct their own qualitative research before running their RCTs. Interviewing men with PC and their partners would have given the authors an opportunity to explore theoretical frameworks, develop a better understanding of the needs

of men and their partners, and address any potential study barriers (40).

A second consideration is the selection of outcome measures. The assessment of sexual function is well defined in the field. The IIEF for men and the FSFI for women are gold standard measures. However, assessing secondary distress variables can be a challenge. Many of these studies used relatively general assessments of "distress", depression, or relationship functioning, and found no change on these variables. More focused assessments targeting specific constructs related to sexuality may be needed to see beneficial effects. Examples of more specific outcomes are constructs such as sexual bother, sexual self-esteem, or sexual relationships.

These studies also prove that greater attention needs to be paid to assessing the level of distress of the patients/couples prior to entry into the study. Canada *et al.* found no changes in marital adjustment on the A-DAS most likely because the couples were not distressed at baseline (18). Similarly, Schover *et al.* found no change in marital happiness or overall distress because there was high marital happiness and there were low-distress levels at entry into the study (23). Even more discouraging was the outcome that intervening on these low-distress couples can actually have unintended negative effects. Manne *et al.* found couples with low distress levels at baseline, after the intervention to have an increase in distress, lower intimacy levels, and poorer communication (25). The intervention may have been making couples more aware of problems, thus heightening their distress. Additionally, future studies should take into account the individual couples' needs in order to focus on important issues for that couple. A study protocol by Robertson *et al.* addresses this issue by including a qualitative interview to get an in-depth understanding of the specific challenges of each couple and what they would hope to gain from the intervention (42).

Other patient selection criteria, beyond levels of distress, are also important. It is essential to distinguish eligibility criteria related to such variables, such as: type of treatment for PC, the amount of time following treatment, and stage of disease. The distinction between men who were treated with surgery compared to men treated with RT can be very important for research in this area. These men differ on the trajectory of EF following treatment, types of ED treatments that will be effective at different time points following treatment, and important patient characteristics such as age and co-morbidities. Many of these studies discussed above grouped men who had surgery and men

who had RT together, without addressing the distinct needs between these two groups. This limits the effectiveness of interventions and may dissipate their treatment results. Second, the length of time following treatment should be addressed as patient and partner concerns may differ based on this time frame. In the Canada study, participants were eligible if they had received treatment between three months and five years prior to entry into the study (18). This gap in time is especially important when addressing the individual needs of each participant, as sexual side effects of PC treatment may vary largely depending on the length of time post-treatment. The distress level within a couple may also be related to time following treatment. Clinical observation suggests that couple distress may be lower following the completion of early stage treatment when support related to the diagnosis/treatment is high and the couple is relieved with the completion of treatment, yet there is no current data available tracking the level of the couples' distress following treatment. It may not be until several months following PC treatment that the impact of ED and frustration of loss of intimacy is felt by the couple.

The largest complication of these interventions appears to be that men and women may need different types of interventions to see benefits. The six studies illuminate the fact that men who have undergone treatment for PC may benefit from education about treatment options for ED and avoidance of sexual situations, whereas their partners may gain more from interventions focused on relationship issues. In the interventions where sexual functioning was the main concern—Canada *et al*., Schover *et al*., Chambers *et al*., and Walker *et al*.—patients were more likely to report benefit and sustained increases in ED treatment use (18,23,34,40). However, the partners in these studies did not see many benefits and neither patient nor partner saw gains on measures of marital satisfaction. Conversely, in the interventions focusing on intimacy support for couples after PC treatment—Northouse *et al*., and Manne *et al*.—the patients reported far fewer benefits, if any, as compared to their partners, while the partners reported gains (10,25). Taken altogether, this suggests that interventions in the future should be developed to target the patient and partner separately, as well as together, so that the couple receives the intervention necessary to improve its sexual functioning and intimacy. Addressing the needs of the partner and the patient as individuals, as well as together, will be vital in successfully giving support to patients and their partners after treatment for PC.

While the six RCTs intended to address relational and sexual intimacy following PC treatment, the methodological limitations of these studies reduce the effectiveness of these interventions. If the aforementioned areas of concern are considered and individual needs of participants are taken into account, interventions in the future have the potential to be more effective.

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Footnote

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The argument for palliative care in prostate cancer

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Palliative care uses a team approach to improve the quality of life of patients and families faced with long-term and progressive illnesses through prevention and relief of suffering with symptom management. Palliative care focuses on three main realms to achieve these goals: morbidities associated with the disease, morbidities associated with the treatment, and quality of life of the patient. Oncology guidelines suggest initiating palliative care "early in the course of illness in conjunction with other therapies that are intended to prolong life, including chemotherapy and radiation" (1). Possibly due to the association with end of life care, palliative care is underutilized for prostate cancer despite evidence showing benefit in several patient populations.

Prostate cancer is particularly amenable to palliative symptom management because of its long disease course and, often, non-lethal progressive nature. It is the most common cancer diagnosis in American males and there are expected to be almost 240,000 American men diagnosed with prostate cancer in 2013 (2). Of those staged at diagnosis, 80% will have localized disease, 12% will have regional disease, and 4% will have metastatic disease (3). Disease specific morbidity worsens as prostate cancer progresses and can include bony metastases, spinal cord compression, lymphedema, urinary obstruction, fatigue, anemia, and significant psychological effects including depression, anxiety, poor coping ability, altered view of self and future, lack of empowerment, and disrupted partner intimacy (4). The treatments for prostate cancer vary by stage but all may be associated with morbidity. Localized treatment with surgery and/or radiation is associated with side effects including pain, erectile dysfunction, incontinence, bowel dysfunction, fatigue, dysuria, gross hematuria, and urethral stricture development (5). Men who

develop biochemical recurrence after localized therapy and men who are diagnosed with regional or metastatic disease may be treated with androgen deprivation which can cause side effects including nausea, vomiting, diarrhea, hot flashes, loss of libido, gynecomastia, insomnia, gastric ulceration, immune suppression, psychiatric effects, myalgias, weight gain, osteoporosis, and lower urinary tract symptoms (6). A number of novel therapies including chemotherapies, targeted-hormonal therapies, and immunotherapies have been developed for castrate-resistant prostate cancer in recent years that can prolong survival on average two to four months, however they may be associated with a number of severe side-effects (*Table 1*). In light of the significant disease and treatment morbidities associated with prostate cancer it is not surprising that Torvinen *et al.* found markedly worsened quality of life across the three realms of palliative care as prostate cancer progressed from localized to metastatic disease (7).

The literature overwhelmingly supports the utilization of palliative care in both long-term illness and various forms of cancer. Multiple randomized trials have demonstrated significantly improved quality of life, decreased symptom intensity, improved patient satisfaction, longer hospice stays, lower health care costs, and less aggressive end-of-life care (8,9). One of the most widely discussed and intriguing trials by Temel *et al.* showed significantly longer median survival (11.6 *vs.* 8.9 months, $P<0.02$) after implementation of palliative care for patients with non-small-cell lung cancer (10). The limited data examining palliative care specifically in prostate cancer also supports its use. A retrospective review at MD Anderson found that the most common symptoms reported by men with advanced prostate cancer included fatigue, drowsiness and pain.

Table 1 Castrate resistant prostate cancer treatment side effects

Drug	Median OS (months)	S/E
Mitoxantrone	No improvement, but improved symptom palliation	Dyspnea, mucositis, cytopenias, cardiac dysfunction
Docetaxel	2.9 (mitoxantrone/prednisone)	Neutropenia, neuropathy, fatigue, N/V, alopecia, diarrhea, dyspnea, tearing, peripheral edema, nose bleeds
Sipuleucel-T	4.1 (placebo/prednisone)	Chills, HA, fever
Abiraterone	4.6 (post-docetaxel, placebo/prednisone)	Fatigue, arthralgia, peripheral edema, anemia, back pain, bone pain, hypokalemia
Cabazitaxel	2.4 (post-docetaxel, mitoxantrone/prednisone)	Neutropenia, diarrhea, N/V
Enzalutamide	4.8 (post-docetaxel, placebo/prednisone)	Fatigue, diarrhea, hot flashes, seizures
Radium-223 dichloride	2.8 (placebo)	Nausea, vomiting, peripheral edema

OS, overall survival; S/E, side effects; N/V, nausea, vomiting; HA, headache.

After palliative care intervention patients had statistically significant improvement in those symptoms as well as sleep, well-being, anxiety and depression (11). Rabow *et al.* found that men with prostate cancer undergoing palliative care in addition to their oncologic or surgical management had significant improvements in fatigue (P=0.02), anxiety (P<0.01), depression (P<0.01), quality of life (P<0.01) and spiritual well-being (P<0.01) (12).

However, referrals to palliative care typically occur late in the disease process because of its inappropriate association with end-of-life care and failure to recognize that symptom management can be utilized throughout the course of a disease. Dalal *et al.* found that one barrier to care was the name "palliative care" itself. After changing their group's name from "palliative care" to "supportive care" they received an increase in consultations and shorter duration from the time of diagnosis to consultation (13). Similar to the change in terminology from "watchful waiting" to "active surveillance" indicating use in different patient populations and treatment interventions, perhaps palliative care would benefit from a name change to broaden its recognition and allow patients with non-life-threatening disease to benefit from its incorporation into routine care.

Palliative care used in conjunction with prostate cancer treatment can significantly improve patient quality of life however it is rarely implemented early in the disease process. With nearly 38,000 men expected to be diagnosed with regional or metastatic prostate cancer this year and an expected 30,000 deaths from prostate cancer in 2013 it is imperative that we begin to follow the guidelines and initiate symptom-modifying palliative care along with disease-modifying therapies.

Footnote

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Prevention of bone metatasis in prostate cancer by denosumab: Unneeded endpoint or unmet need?

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Targeting bone metastases in prostate cancer (PCa) is a major goal since bone metastases are present in >90% of advanced PCa patients causing significant morbidity and mortality (1). Treatment strategies used for "bone targeted" therapies including bisphosphonates and radionuclides mainly focused on the treatment of existing bone metastases and were not deemed to delay the development and formation of new bone metastases. Preclinical evidence suggests that the RANK-Ligand plays an important role for the development of bone metastasis by influencing cell migration and the tissue-specific metastatic behavior of cancer cells. Targeting the RANK-Ligand may therefore be effective in preventing the development of new bone metastases in prostate cancer patients (2). Denosumab is a monoclonal antibody that binds the RANK-Ligand thereby inhibiting interaction with its receptor on the cell surface of osteoclasts and prostate cancer cells. After demonstrating efficacy in the prevention of treatment induced bone loss and prevention of skeletal related events, denosumab has already been licensed for the treatment of prostate cancer patients (3,4). Most recently, the results of a phase-III clinical trial investigating the effects of denosumab on the development of bone metastases have been published (5). The trial recruited 1,432 patients to randomly receive either denosumab (120 mg s.c. 4-weekly) or placebo. Patients with castration-resistant prostate cancer and a high risk of developing bone metastases (i.e. PSA >8 ng/mL and/or PSA doubling time <10 months) were included into the trial. Treatment was continued until occurrence of bone metastases as evidenced by bone scan that was confirmed by a second imaging modality (CT, MRI or plain radiography). Patients were then taken off study and treated per investigator discretion to receive standard treatment for bone metastasis.

Primary endpoint of the trial was bone-metastasis-free survival, as determined by time to first occurrence of bone metastasis (symptomatic or asymptomatic) or death from any cause. Secondary endpoints included time to first bone metastasis and overall survival. The results showed that denosumab significantly improved bone-metastasis-free survival by 4.2 months compared to placebo [HR 0.85 (95% CI: 0.73-0.98), P=0.028]. Median time to first bone metastasis was 29.5 (95% CI: 25.4-33.3) and 25.2 (95% CI: 22.2-29.5) months with denosumab and placebo, resulting in a risk reduction of 15% [HR 0.85, (95% CI: 0.73-0.98), P=0.028] for the development of bone metastasis (*Figure 1*). Furthermore time to first bone metastasis improved significantly (33.2 *vs.* 29.5 months, HR 0.84 with P=0.032) and denosumab led to a 33% reduction in the risk to develop symptomatic bone metastasis (HR 0.67, P=0.01). There was no difference in the time to overall prostate cancer progression (22.4 *vs.* 21.9 months, P=0.13) and median overall survival (43.9 *vs.* 44.8 months, P=0.91) between treatment groups. Overall toxicity and the rate of serious adverse events did not differ significantly, although patients receiving denosumab showed a higher incidence for osteonecrosis of the jaw (5%, any grade) and hypocalcemia (2%, any grade).

By meeting its primary endpoint, denosumab can be regarded as the first "bone targeted" agent that prevents the development of bone metastasis in patients with PCa. This clearly demonstrates the role of RANK and its ligand for the process of bone metastasis formation and leads the way for new treatment strategies in PCa. Despite the positive results of the trial the FDA (food and drug administration)
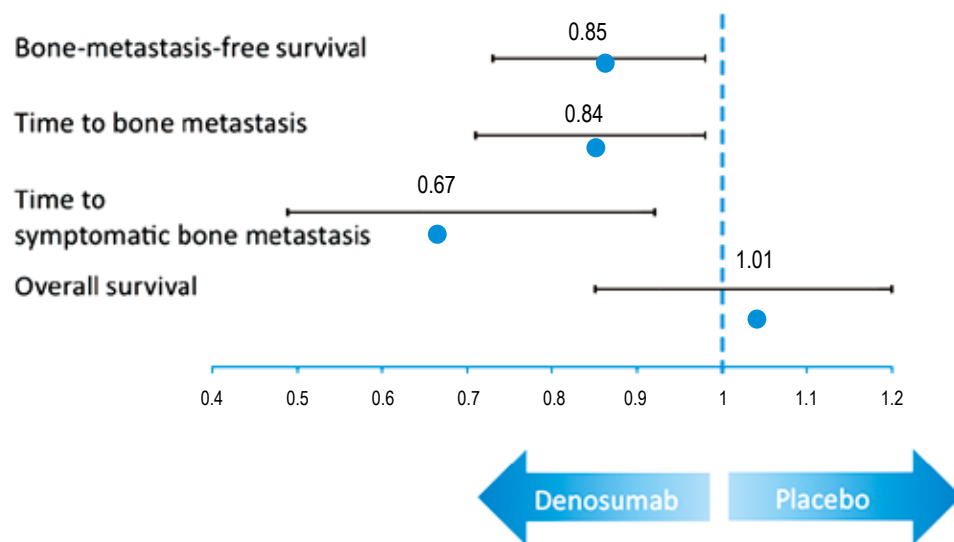
Figure 1 Hazard Ratios (HR, CI 95%) of the primary (bone-metastasis-free survival), secondary (time to bone metastasis, overall survival) and exploratory endpoints (time to symptomatic bone metastasis)

did not agree to expand the indication of XGEVA for the prevention of bone metastasis. The FDA assessed overall survival, patterns of metastases, and the development of symptomatic metastases as important review issues prior to the initiation of the trial. Time to symptomatic bone metastasis was evaluated in the trial by a *post-hoc* analysis and the FDA therefore considered this endpoint of little value. Furthermore overall survival did not show a difference between groups. Given the fact that denosumab had to be stopped at the time of first bone metastasis and the various subsequent treatments it seems not surprising that an overall survival benefit was not shown for denosumab. The FDA further questions whether time to first bone metastasis is a clinically relevant endpoint given the fact that denosumab showed efficacy in prevention skeletal related events in the metastatic setting with a similar delay. These seemingly limitations of the trial and its results lead to an underestimation of the clinical benefit of denosumab rather than provoking a too optimistic interpretation. It would be not surprising if a delay in the development of bone metastasis as the leading cause of morbidity and death from prostate cancer has an impact on the clinical course and survival of the patients. Even if not proven by the results of this trial it will hopefully prompt further investigations of therapies directed against the development and formation of bone metastasis. Unfortunately the trial of denosumab *vs.* zoledronic acid in metastatic PCa patients did not report on

the prevention of subsequent bone metastasis since is seems unlikely that the development of the first bone metastasis abrogates the preventative effect of denosumab. Apart from the new insights in androgen signaling and the integration of the new anti-hormonal into modern therapeutic strategies, treatments targeting bone metastases will clearly have the capability of improving the prognosis of patients with prostate cancer.

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Contemporary and future insights into fertility preservation in male cancer patients

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Abstract: In recent years, survival rates of cancer patients have increased, resulting in a shift of focus from quantity to quality of life. A key aspect of quality of life is fertility potential; patients suffering from iatrogenic infertility often become depressed. Since many cancer therapies—chemotherapy, radiotherapy and/or surgery—and even cancer itself have detrimental effects on the male reproductive system, it is important to preserve fertility before any treatment commences. Currently, the only reliable method of male fertility preservation is sperm banking. For patients who are unable to provide semen samples by the conventional method of masturbation, there are other techniques such as electroejaculation, microsurgical epididymal sperm aspiration and testicular sperm extraction that can be employed. Unfortunately, it is presently impossible to preserve the fertility potential of pre-pubertal patients. Due to the increasing numbers of adolescent cancer patients surviving treatment, extensive research is being conducted into several possible methods such as testicular tissue cryopreservation, xenografting, *in vitro* gamete maturation and even the creation of artificial gametes. However, in spite of its ease, safety, convenience and many accompanying benefits, sperm banking remains underutilized in cancer patients. There are several barriers involved such as the lack of information and the urgency to begin treatment, but various measures can be put in place to overcome these barriers so that sperm banking can be more widely utilized.

Keywords: Cancer; male infertility; sperm banking; fertility preservation

Introduction

Advances in anti-cancer therapies and supportive care have led to increased survival rates in cancer patients (1,2), with 5-year survival rates surpassing 70% in children and adolescents (3). This has resulted in a shift of focus from merely lengthening the patient's lifespan to improving his quality of life (4,5). Fertility preservation is deemed an important aspect of post-treatment quality of life (6,7), especially since anti-cancer therapies are known to have gonadotoxic side effects (1,3). Approximately 15% to 30% of male cancer survivors lose their reproductive potential after treatment (2,6), causing much distress and unhappiness (8-10). Moreover, among those who recover, sperm parameters are likely to be reduced, thus having a negative impact on future fertility (3,11).

The World Health Organization (WHO) defines infertility as "the inability of a sexually active couple (at least three times per month), not using contraception, to achieve pregnancy within one year" (12). Since there is hitherto no cure for infertility, the only way to preserve male fertility is by sperm banking before treatment (4,5). Sperm banking involves sperm retrieval, usually by masturbation, and cryopreservation of the semen sample. Subsequently, the sample can be thawed and used in various assisted reproductive technologies (ART) to achieve pregnancy. Not only is sperm banking non-invasive and safe, but it has also been reported to be very effective (13). This option should therefore be offered to all patients before treatment commences because it is very difficult, if not impossible, to predict who will be rendered permanently sterile post-
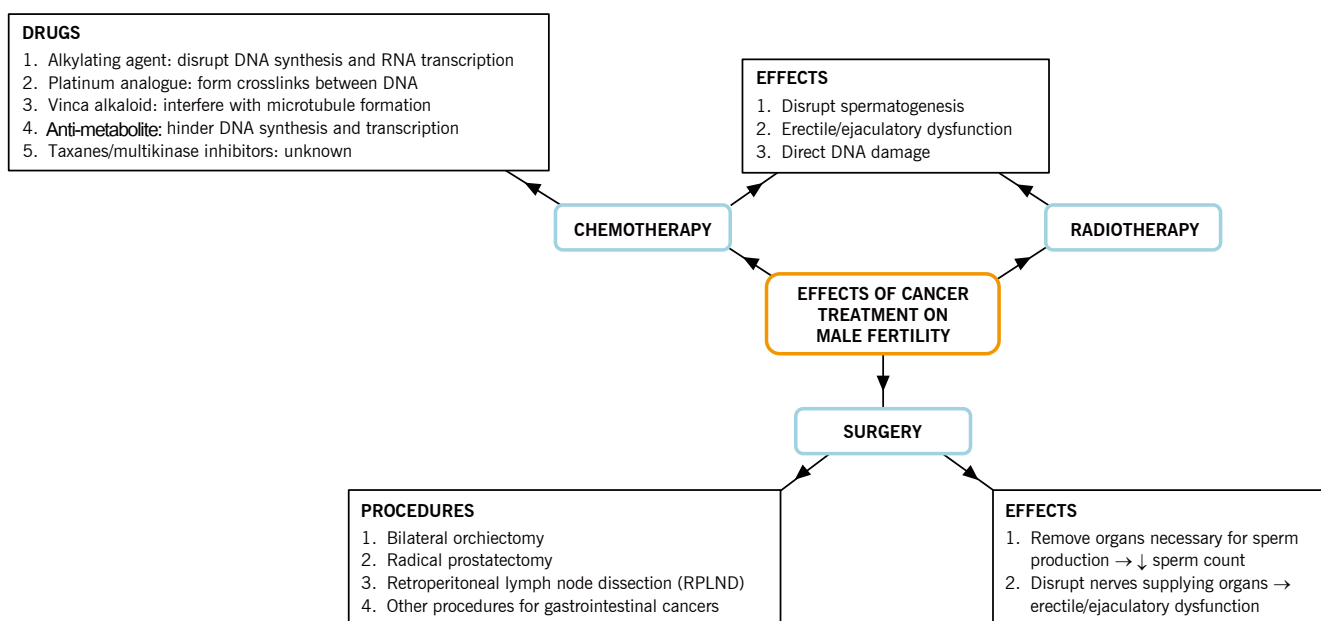
Figure 1 Effects of the three main modalities of cancer treatment on the male reproductive potential.

treatment (14,15). In fact, only 20% to 50% of patients regain their fertility within three years after treatment (16). At present, indications for sperm banking include, but are not limited to, couples who are physically separated (10,17), men with high-risk occupations (18,19), men about to undergo vasectomy (18,20) or potentially gonadotoxic therapies (17,20), sperm donation (21,22), and men with reproductive problems such as anejaculation, severe oligozoospermia and obstructive azoospermia (17,18).

In this review, we will discuss the effect of cancer and its treatment on male fertility, explain how and when sperm banking should take place, and explore current and future alternative strategies that can be employed should sperm be unobtainable due to the inability to masturbate or in cases of azoospermic and pre-pubertal patients. In addition, we will also elaborate on the benefits of sperm banking and possible barriers that may exist, resulting in the low utilization of sperm banking despite its effectiveness (23,24).

Effect of cancer and its treatment on male fertility

Cancer treatment involves cytotoxic chemotherapy, radiotherapy or radical surgical procedures (19,25), and these have the potential to affect one's reproductive capacity by impairing spermatogenesis, damaging sperm DNA, and/or causing erectile or ejaculatory dysfunction (3,26). An outline of these effects can be seen in *Figure 1*. The

presence of cancer itself can also impair fertility, and this will be elaborated upon in the following section. Iatrogenic infertility caused by anti-cancer treatment can be temporary or permanent and differs in severity between patients (4). A myriad of factors—pre-existing defects, endocrine disturbances, type of cancer, and dosage and duration of treatment—contribute to the patient's likelihood of regaining fertility (27,28), making it practically impossible to predict who will be severely affected (11,29). Some patients may regain fertility in a few months' time while others may take several years, but usually with suboptimal sperm quality (16,30). To date, the most gonadotoxic regimen is the combination of intensive chemotherapy and total body irradiation in bone marrow transplantation procedures (25).

Cancer

The presence of cancer may affect a patient's fertility potential via different possible mechanisms even before any gonadotoxic treatment is given, and this is summarized in *Table 1* (16,36). Men with testicular cancer and Hodgkin's lymphoma are known to have impaired spermatogenesis and are likely to be oligozoospermic or azoospermic at the time of cancer diagnosis (29). It is also interesting to note that testicular cancer seems to affect the quantity, rather than the quality, of sperm produced (4). A study conducted

Table 1 Possible mechanisms by which common cancers in the male could impair fertility

Type of cancer	Effect on male fertility	Possible mechanism
Testicular cancer	↓ Sperm quantity > quality (4) ↓ Sperm DNA integrity and compaction (31)	Pre-existing defect due to flawed development of testes (32,33)
Hodgkin's lymphoma	↓ Sperm quantity and quality (29) ↓ Sperm DNA integrity and compaction (31)	Secrete β-human chorionic gonadotrophin (34)
Testicular germ cell tumours (TGCTs)	↓ Spermatogenesis (35)	Secrete β-human chorionic gonadotrophin (35)
Other tumours	Prevent proper sperm function (35)	Production of antisperm antibodies that bind to sperm (35)

by O'Flaherty *et al.* showed that sperm DNA integrity and compaction were compromised in patients with testicular cancer and Hodgkin's lymphoma before chemotherapy (31). Although the exact mechanism by which cancer affects semen quality is not known (19), it is likely that pre-existing defects due to flawed development of the testes could contribute to testicular cancer (32,33), while abnormal cytokine secretion in the presence of cancer could result in Hodgkin's lymphoma (34).

Cancer can also affect spermatogenesis via autoimmune, endocrine or systemic effects (5,17). For instance, testicular germ cell tumours (TGCTs) secrete β-human chorionic gonadotrophins, which depress spermatogenesis, while other tumours spur the production of antisperm antibodies, which could bind to sperm and prevent proper sperm function (35). Moreover, it has been established that the emotional stress experienced by patients who receive a diagnosis of cancer impairs spermatogenesis (5,30). It is therefore evident that cancer itself, prior to any treatment, can affect male fertility.

Chemotherapy

Chemotherapy regimens target proliferating cancer cells and thus, exert their effects on rapidly dividing spermatogonia as well (10,37). These drugs penetrate the blood-testes barrier and interrupt spermatogonial differentiation, hence hindering spermatogenesis (5) and causing oligozoospermia or azoospermia (38,39). Spermatogonial stem cells (SSCs) in the germinal epithelium, though comparatively less active, are also susceptible to permanent damage at higher doses (35,40). More mature germ cells such as spermatocytes and spermatids are less sensitive to chemotherapy because they have stopped dividing, and hence, the effects are only temporary. This may be the reason why some sperm can be found immediately after chemotherapy but gradually decrease in numbers over time (4). Due to their

low proliferation rates, Leydig cells are relatively resistant to chemotherapy (35,36). However, there has been some evidence of damage to Leydig cells—increased luteinizing hormone (LH) levels with normal to low testosterone levels (41). In addition to disrupting spermatogenesis, cytotoxic chemotherapy may also contribute to erectile or ejaculatory dysfunction (42) or directly damage sperm DNA (10), resulting in the transmission of defective DNA and abnormal chromosomes to offspring (43).

The severity of damage depends most importantly on the type and total dosage of drug used, as well as the patient's age (5,42,44). As expected, a higher cumulative dose of drugs given over a longer time period will result in more extensive damage (8). Alkylating agents, such as cyclophosphamide, procarbazine and chlorambucil (45), are the most gonadotoxic drugs because they interfere with DNA synthesis and RNA transcription, thus causing new mutations that may lead to apoptosis (46). Cisplatin, a platinum analogue, is also equally harmful as it causes crosslinks to form between DNA (23,46). Whereas vinca alkaloids interfere with microtubule formation thereby preventing mitosis from occurring, anti-metabolites hinder DNA synthesis and transcription (46). Furthermore, different combinations of drugs are usually given simultaneously in chemotherapeutic regimens, thus making it more challenging to predict their additive effects on reproductive function (27,47). Unfortunately, the effect of newer drugs like the taxanes and multikinase inhibitors are still unknown (19,46), although there have been indications that when used as an adjuvant, taxanes may enable cyclophosphamide to become more toxic (25).

To combat this problem, less gonadotoxic alternatives or lower doses of drugs are used whenever possible (5,48). Also, since chemotherapy targets rapidly proliferating cells, it has been proposed that hormonal manipulation such that the hypothalamus-pituitary-gonadal (HPG) axis is suppressed may cause spermatogenesis to slow down or even

stop, hence protecting spermatogonia from the cytotoxic effects of chemotherapeutic drugs (16,49). In studies conducted on rats by Cespedes *et al.* and Kangasniemi *et al.*, the administration of flutamide and a luteinizing hormone releasing hormone (LHRH) agonist successfully prevented chemotherapy from damaging the germinal epithelium (50,51). However, both Johnson *et al.* and Fosså *et al.* had earlier found that the results could not be produced in humans (49,52). As such, hormonal manipulation is not clinically recommended for patients (53).

Radiotherapy

As in chemotherapy, the rapidly dividing cells in the germinal epithelium of the testes are most susceptible to damage and can be permanently destroyed by irradiation (17,48). Radiation doses as low as 0.1-1.2 Gray (Gy) can damage spermatogonia morphologically, hence preventing spermatogenesis from occurring (19,25,37). This can be caused by direct DNA damage or by disturbing the HPG axis (19,35). Exposure to 2 to 3 Gy permanently damages spermatocytes (46), giving rise to azoospermia (19), while doses exceeding 4 Gy generally affect the spermatids and cause an even longer period of azoospermia (5,46). Again, the Leydig cells are more resistant to radiotherapy (48,54) and are only affected by doses above 15 Gy (19,25). In addition, radiation may also play a role in causing erectile dysfunction (39).

The extent of damage depends on various factors such as the total dose, radiation source, field of treatment, and whether it is fractionated (37,54). A higher dose of radiation not only causes more damage, but also increases the time needed for recovery, if at all (5). Radiation damage occurs when radiotherapy is used directly on the testes in testicular cancer (35,36) but is more commonly caused by scatter radiation from radiotherapy directed at the lower abdominal and pelvic regions (38,55). Although lead shields are always used to protect the testes, some scatter radiation is inevitable and can often be extremely gonadotoxic (16,37). Hormonal manipulation via administration of gonadotrophin releasing hormone (GnRH) agonists was used to decrease the rate of spermatogenesis and to reduce the gonadotoxic effects of radiotherapy without success (56).

Surgery

Cancer surgery may decrease the patient's fertility potential if the organs necessary for reproduction need to be removed or the nerves supplying these organs are disrupted (42). In both cases, sperm counts decrease and erectile or ejaculatory dysfunction occurs (10,29). Bilateral orchiectomy in patients with testicular cancer will result in permanent azoospermia (38,55), whereas radical prostatectomy in patients with prostate cancer can lead to erectile dysfunction (38,39). Retroperitoneal lymph node dissection (RPLND) in testicular cancer patients may damage the autonomic pelvic plexus (4,46), causing retrograde ejaculation or anejaculation (55,57). However, nerve-sparing RPLND can be successfully carried out with the maintenance of normal ejaculatory function post-surgery (23). Other surgical procedures for gastrointestinal cancers in the lower abdomen and perineal regions may also damage nerves and affect ejaculation, resulting in infertility (4).

Process of sperm banking

The entire process of sperm banking is complex and involves many steps from the initial cancer diagnosis to semen collection, sperm cryopreservation and eventually, the use of ART to hopefully result in a pregnancy. This is illustrated in *Figure 2* and will be further elaborated on in the following sections.

Sperm retrieval

The first step of sperm banking involves collecting semen samples from patients by self-stimulation and masturbation (35,54). Not only is ejaculated sperm of the best quality, but masturbation is also inexpensive and safe (46). However, men must understand that masturbation cannot be carried out with lubrication, and that the entire ejaculate has to be collected in the sterile specimen cups provided (19). This is because the first part of the ejaculate usually contains the most sperm (30,35). Should the patient wish to masturbate in the privacy of his home, he must be instructed to keep the specimen at body temperature and bring it to the laboratory within the next hour (35). After collection, the samples will be left to liquefy at room temperature (22,30,58).

Men are usually encouraged to bank three samples with at least 48 hours of sexual abstinence between samples for maximal concentration of healthy sperm (14,19). However, patients with low sperm concentrations or poorer sperm parameters may be asked to provide more samples in order to pool a sufficient number of sperm for cryopreservation (5,35). In some cases, men who are unable to produce more than one sample due to urgency of treatment or health
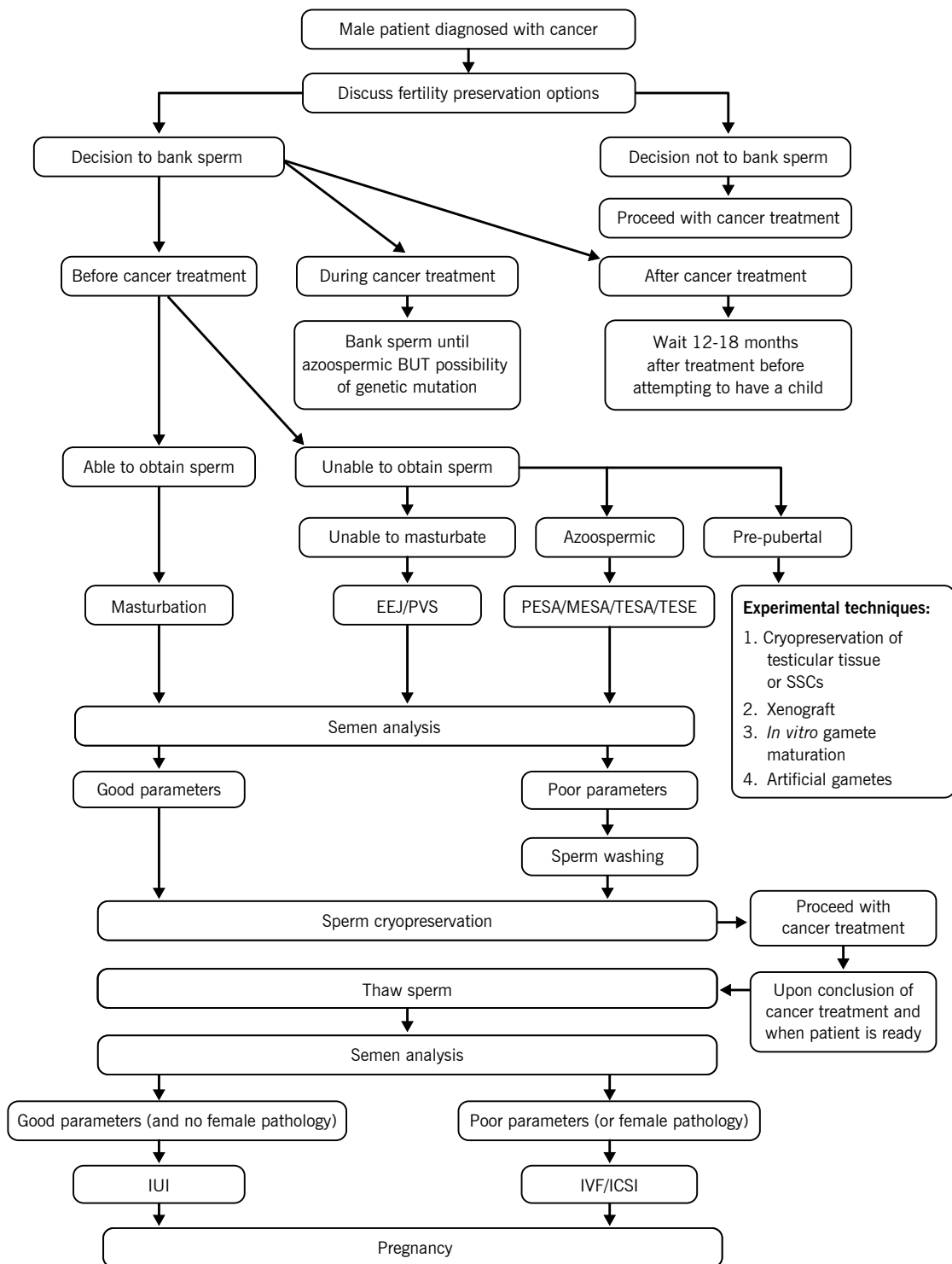
Figure 2 Algorithm showing the process of sperm banking from initial diagnosis of cancer to possible methods of sperm collection, followed by sperm cryopreservation and thawing, and depending on the sperm parameters obtained, its use in suitable assisted reproduction techniques. EEJ, electro-ejaculation; PVS, penile vibrostimulation; PESA, percutaneous epididymal sperm aspiration; MESA, microsurgical epididymal sperm aspiration; TESA, testicular sperm aspiration; TESE, testicular sperm extraction; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; IUI, intrauterine insemination.

reasons should still bank their sperm as more advanced techniques are now available that enable a single motile sperm to fertilize an egg (14,55). Finally, for patients who are unable to masturbate or produce viable sperm, other alternative options of sperm retrieval are available, and this will be expanded upon later.

Cryopreservation

After liquefaction and before cryopreservation, the semen samples are analysed and the colour, viscosity, and semen parameters such as sperm count, motility and morphology are recorded (4,22,55). In the event that a sample has poor sperm characteristics, the sample can be enhanced via sperm washing procedures like swim-up or density gradient centrifugation (35,46). Swim-up involves centrifuging the sample and adding culture medium on the top—only motile sperm will be able to swim up into the media. On the other hand, density gradient centrifugation involves centrifuging the semen sample on top of a density gradient, allowing only the motile sperm to move in the direction of the sedimentation gradient and thus forming a pellet at the bottom (10). Both these techniques will allow only healthy, motile sperm to be selected from the seminal plasma and other cellular debris, hence improving the sample's quality and concentration (10,35,46).

Cryoprotectant is then added to the sample to prevent the formation of ice crystals—inside or outside the cell—during cryopreservation (46). This is because cryoprotectants contain glycerol (and egg yolk), which helps reduce salt levels, decreases osmotic stress, and ultimately maintains the integrity of the sperm cell membrane (22). After equilibration, small aliquots of the mixture are frozen in separate vials for ease of thawing (16,25). Usually, an aliquot is frozen separately, then thawed and analysed again the following day. This 'test-thaw' will give a good indication of the quality of that particular semen sample after cryopreservation (4,22,35).

There are two methods for conducting cryopreservation—slow or controlled freezing and vitrification. With slow freezing, the most conventional and commonly used method, freezing medium is slowly added to the sample, which allows dehydration to occur during cooling (10). The vials are immersed in –20 ℃ for 15 to 30 minutes, then in –79 ℃ for another 15 to 30 minutes, and finally dipped into liquid nitrogen and stored at –196 ℃ until they are needed (10,30). These steps can be done manually or in a programmable freezer (10,35). Despite the effectiveness of this method,

slow freezing takes up to 1.5 hours and exact protocols differ between labs (22,35).

In contrast, vials are quickly plunged into liquid nitrogen with vitrification (21), and this decreases the protocol time to five minutes (22). Vitrification completely avoids freezing, and consequently the formation of ice crystals, by causing the sample to form an amorphous solid state. However, vitrification is still a novel procedure and is not part of standard clinical practice (10,35).

In the process of cryopreservation, it is inevitable that sperm parameters will be drastically affected, especially that of motility (21,22). It is not uncommon to see a decrease in motility of 25% to 75% after thawing, and the acrosome structure and sperm nuclei may also be damaged (40,44). Furthermore, sperm concentration will be reduced due to dilution with the cryoprotectant (20). As such, in order to attempt a pregnancy, the vials may have to be pooled together to obtain enough viable sperm (5). Nevertheless, semen samples can be stored for up to 50 years in liquid nitrogen with no further damage incurred (59).

Use of sperm in assisted reproductive technologies (ART)

There are three main techniques used to achieve a pregnancy with thawed sperm—intrauterine insemination (IUI), in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). The decision to use a particular technique depends on the number and quality of thawed sperm available, female factors and individual preferences (19,35,40).

IUI is only used when the number of viable sperm post-thaw exceeds five million and the woman has at least one normal fallopian tube (35,60). In this procedure, a thin catheter is used to introduce the semen sample into the woman's uterus (55). Two inseminations—one given two days prior to ovulation and the other on the day of ovulation itself—are necessary to increase chances of fertilization because sperm can only survive for 48 hours in the female (30). In some cases where ovarian stimulation is also employed, a few IUI cycles are sufficient to achieve pregnancy in 15% to 30% of women (60).

On the other hand, IVF and ICSI are more rigorous techniques that are used when sperm count and/or quality is too low, both of which are commonly seen in cancer patients (3,25), or when the woman has some abnormality in her reproductive tract (38). In both IVF and ICSI, oocytes are removed from the woman and fertilized *ex vivo* in the laboratory (47). The fertilized egg is allowed to develop

486

Agarwal et al. Fertility preservation in male cancer patients

into an embryo, which is then returned to the uterus to be implanted (55). In IVF, all motile sperm in the sample are added to the same petri dish as the oocyte in the hopes that fertilization will occur, but ICSI is more complicated (55). ICSI only requires a single viable sperm which will be directly injected into the oocyte (35,60). This circumvents the need for sperm to be of good quality and hence, patients are still encouraged to bank sperm even if there is a very small number of good quality sperm in the sample (9,20,26). In fact, this novel technique can also be employed to enable less mature sperm retrieved from the epididymis or testes to be of reproductive use (60). A study conducted by Chung *et al.* revealed that 75% of patients, including one with only a few motile spermatozoa, who attempted to father a child post-treatment were successful, thus lending credence to the feasibility of ART with cryopreserved sperm (61).

When sperm banking should occur

According to the American Society of Clinical Oncology (ASCO) [2006], "fertility preservation should be considered as early as possible during treatment planning" (6,62). Ideally, sperm banking should be completed before any potentially gonadotoxic treatment—chemotherapy, radiotherapy or surgery—commences (14,30,63). Patients who start on low-dose treatments should also be advised to bank sperm in case stronger treatment is indicated before their testes are able to fully recover from the initial milder gonadotoxic therapy (7,8). A study conducted by Ginsberg *et al.* reported that 60% of patients who banked sperm after treatment began were azoospermic (64). This highlights how susceptible the testes are to gonadotoxic treatments—even a single low dose of therapy can severely affect spermatogenesis (19,42). Moreover, providing semen samples before treatment also ensures that sperm DNA already affected by the cancer is not further damaged by therapy (5,20).

If treatment has already begun, patients can still bank their sperm until they become azoospermic (4,14). Although chemotherapy is capable of causing gene mutations, it is not known whether gonadotoxic treatments have any detrimental effect on existing sperm (11). However, animal studies have shown that young produced when the male is undergoing gonadotoxic therapy tend to have many genetic mutations (14). As a precautionary measure, preimplantation genetic diagnosis (PGD) is recommended when reproduction is attempted with sperm obtained during treatment (11). Therefore, it is safer to bank sperm

before initiation of gonadotoxic therapy.

In cases where treatment has ended, patients are advised to wait 12 to 18 months before banking sperm or attempting to father a child (19,35,65). This is due to the fact that increased genetic and chromosomal abnormalities have been reported to last up to 18 months post-treatment (14,19,35).

What to do when sperm cannot be obtained

Unable to masturbate

Patients may find it difficult to masturbate due to physical, psychological, cultural or religious reasons (5). Some may be on medications or may feel too ill, stressed or anxious to perform the act (30,42,46) while others may have been brought up in a conservative environment in which masturbation is frowned upon by their culture and/or religion (19,55). Men with ejaculatory dysfunction due to spinal cord injuries, anejaculation or retrograde ejaculation are also unable to produce a semen sample (10,46).

In cases where oral sympathomimetics fail to result in ejaculation (14,19), electro-ejaculation (EEJ), penile vibrostimulation (PVS) or retrieval of sperm from post-coital urine can be carried out to obtain sperm for cryopreservation (27). EEJ is a painful procedure which is performed under general anaesthesia (1,35). A probe is inserted via the anus and placed against the anterior rectal wall. The application of electricity stimulates the prostate gland and seminal vesicles, causing ejaculation (35,42,55). However, EEJ should not be performed when patients are thrombocytopenic or leukopenic as the procedure may give rise to excessive bleeding or infection (4,55). Samples obtained by EEJ usually have a normal concentration but individual sperm are likely to have poor motility, morphology and viability (16,22). These samples are therefore more effectively used in IVF or ICSI rather than IUI (19,35). PVS is simpler and does not require anaesthesia (35). A vibrator is placed against the frenulum of the penis to stimulate the dorsal penile and pudendal nerves and cause ejaculation (35,46). However, this should not be used on boys who have not masturbated previously as it might have psychological side effects. As for patients suffering from retrograde ejaculation, sperm can be obtained from the urine after orgasm (5).

Azoospermia

As mentioned earlier, some patients are azoospermic even

before therapy commences because of the effects of cancer (42). Hence, novel techniques have been developed to extract sperm directly from the testes or the epididymis (5,25). For obstructive azoospermia, percutaneous or microsurgical epididymal sperm aspiration (PESA/MESA) can be carried out to obtain sperm from the epididymis. In cases of non-obstructive azoospermia, testicular sperm aspiration or extraction (TESA/TESE) must be carried out under anaesthesia to extract sperm from the testes (3,5,10).

PESA is the easiest technique because no microsurgical equipment or skill is needed. Under local anaesthesia, a 21-gauge butterfly needle is inserted into the caput epididymis and fluid is drawn up into the attached tube. This procedure is repeated until sufficient fluid is collected. However, due to the lack of visual guidance with a microscope, it is easy to inadvertently puncture blood vessels and cause bleeding (10,46). Alternatively, sperm can be extracted from the epididymis via MESA, and this is the preferred technique for patients with obstructive azoospermia (42,46). In MESA, patients are anaesthetized and the procedure is performed with the aid of an operating microscope. This allows for easy identification and directed insertion of the needle into individual epididymis tubules for aspiration of the fluid into a syringe. Again, this is repeated until sufficient fluid is collected (10,46). The fluid collected via PESA or MESA is then analysed and processed in the lab (10). Sufficiently motile and viable sperm can usually be obtained for ART via MESA (14,46).

In TESA, a needle is inserted into three different locations of the testes (upper, centre and lower segments) and the samples are extracted via negative pressure. The extracted fluid is then analysed for sperm in the lab (10). TESE is the more commonly used option for patients with non-obstructive azoospermia (46). After being cut transversely at its centre and at its upper and lower poles, each testis is then lightly squeezed so that some of the tissue bulges outward. The protruding tissue is excised, transferred into culture media, and sent to the lab to extract sperm cells (10,46). This technique can also be used in cases of testicular cancer where the testes have been removed from the body by orchiectomy (5,66). Sperm obtained from TESE can only be used for ART as only certain sections of the testes will contain sufficiently mature sperm (4,10).

A more recent improvement to TESE is microdissection TESE (mTESE). This technique uses microsurgical equipment to identify larger seminiferous tubules that are more likely to be active in spermatogenesis (10,46). Not only does mTESE minimize the loss of testicular tissue

(especially in patients with atrophied testes), but it also prevents the accidental puncture of neighbouring blood vessels (10). Moreover, mTESE has been shown to be more effective than regular TESE, obtaining approximately 18% more healthy, viable sperm from the testes (5).

Pre-pubertal

Another subset of patients from whom sperm cannot be extracted from is pre-pubertal boys, whose reproductive systems have not begun spermatogenesis. There is currently no known method of preserving fertility in such patients, but research into various techniques is being carried out (25,54). These include the cryopreservation of testicular tissue or SSCs, xenografting gonadal tissues, *in vitro* gamete maturation, and the use of artificial gametes (20,25,47). Although results have generally been encouraging, there are still safety, ethical and legal issues that must be addressed before they can be implemented clinically (54).

Cryopreservation of testicular tissue or spermatogonial stem cells (SSCs)

Cryopreservation of testicular tissue or SSCs is the most promising method (5,67). This involves the extraction of testicular tissue, prior to gonadotoxic therapy, to be cryopreserved. When the patient desires to have children, the tissue can be thawed and re-implanted into the patient. Theoretically, SSCs will be recognized by the Sertoli cells and due to their innate ability to self-renew and differentiate, spermatogenesis will resume, restoring gonadal function to the patient (11,37,63). Otherwise, it is expected that by then, advances in technology will find a way to stimulate spermatogenesis from cryopreserved tissue or SSCs (5,35,54).

Furthermore, it was found that cryopreservation of testicular tissue instead of SSCs alone is more likely to preserve the natural function of SSCs. This is because freezing the tissue allows for the SSCs' surroundings to be preserved as well, hence maintaining the support system they need for proper functioning (5,46). At present, this method has only been successfully carried out in rodents and its use in humans is still experimental (11,19). In these animal models, spermatogenesis was successfully re-initiated when the SSCs were returned to the animal post-treatment (16).

Despite the advantages of this method, there are certain safety and ethical issues that need further consideration. Firstly, there is a possibility that in the process of returning thawed testicular tissue to the cured patient, malignant cells may be transplanted as well (14,47). To circumvent

488

Agarwal et al. Fertility preservation in male cancer patients

this problem, it may be safer to isolate SSCs from the testicular tissue and only transplant those back into the patient (37,42,68). However, as explained above, this will compromise the ability of the SSCs to produce sperm. Alternatively, the SSCs can be allowed to mature *in vitro* and only the mature sperm will be used in ART (14,68). Another ethical issue is that the procedure may be too invasive for young patients who may not be of age to give consent for themselves (5,37).

Xenograft

Testicular tissue extracted from a patient may also be transplanted into a host animal to provide a suitable environment for sperm maturation, after which sperm can be extracted for use in IVF or ICSI (35,47). Nagano *et al.* showed that human SSCs were able to persist and proliferate in mouse testes (69), thus lending support for this method. However, in the use of sperm derived from xenotransplanted SSCs, there is a risk of interspecies transmission of animal DNA, viruses or infections to humans (11,47,54). Therefore, more measures have to be implemented to address these issues before xenotransplantation of gonadal tissue can be considered for clinical use.

In vitro gamete maturation

In vitro maturation (IVM) of SSCs is yet another method that may solve the problem of infertility in pre-pubertal cancer patients. As briefly mentioned above, SSCs extracted from the patient before treatment can be developed into mature sperm cells for IVF or ICSI (46,63). Although this removes the possibility of returning cancer cells to the cured patient, the full intricacies of the support network and environment of the cell culture required for proper maturation are hitherto not understood (37,47). As such, there are concerns about the possibility of improper sperm maturation and subsequent birth defects (5,37).

Artificial gametes

Finally, a newer technique that has been proposed is the creation of artificial gametes (47,70). Geijsen *et al.* showed that mouse embryonic stem cells can be manipulated in the laboratory to produce sperm cells (71). Nayernia *et al.* further demonstrated that these sperm cells could be used to produce live offspring. Unfortunately, the offspring in that study were unhealthy and died young (72). In addition to these safety issues, there are ethical concerns regarding the creation of artificial gametes that result in live births (47).

Benefits of sperm banking

Aside from the obvious benefit that sperm banking will preserve the reproductive potential of the patient after cancer treatment and enable him to have biological children (46,73), there are also many positive psychological and emotional effects that will aid the patient in coping with his cancer diagnosis (13,28).

Firstly, it is known that the loss of fertility is a significant cause of anxiety and distress in many patients, especially for those who have yet to complete their family (5,74). Knowing that they have cryopreserved sperm in case they are rendered infertile by treatment will assuage their worries and reduce their fears of being childless (16,20,38). Not only will this help them to cope better, but they will also have a better quality of life after treatment (15,75,76). Additionally, when a physician discusses sperm banking with a patient, this reinforces the belief in long-term survival and reassures the patient that his diagnosis is not fatal (54,73). With the mindset that they will eventually be cured of cancer, patients and their families will be more optimistic and cooperative in the treatment plan too (25). Moreover, in the midst of all the uncertainties and feelings of helplessness, sperm banking gives patients a sense of achievement and control over their lives (77). Therefore, it can be seen that sperm banking has many psychological and emotional benefits, and this is further supported by the fact that 80% of cancer patients who banked their sperm were happy with their decision (4).

Barriers that prevent sperm banking

Despite the relative ease and reliability of sperm banking as a method of preserving fertility potential and its accompanying benefits, it continues to be underutilized among cancer patients. For example, Babb *et al.* found that only 42 out of 79 patients' banked sperm, and only half of those who banked sperm proceeded to use their samples in ART (78). Furthermore, in a separate study where questionnaires were given to patients undergoing radical prostatectomies, only 20% of them wanted to bank their sperm although 84% of them felt there was a need for sperm cryopreservation to be offered (24). As such, this section will discuss the barriers that exist from the physician's and patient's perspectives as well as more general barriers such as legal issues and the fate of unused sperm.

Physician

One of the reasons that physicians fail to offer the option of sperm banking to patients is lack of time—both during consultation and before treatment begins. During consultation, the oncologist must not only break the news of the cancer diagnosis to the patient, but he must also explain the effects of cancer and the treatment required. With the tight schedule of a busy clinic, physicians have insufficient time to explain and discuss the issue of sperm banking with their patients (79-81). Additionally, there is often a need to start life-saving treatment as soon as possible and hence, physicians are reluctant to advise sperm banking, which will postpone treatment (48,57).

Many physicians also lack knowledge regarding sperm banking and its benefits as well as the facilities that are available for patients. Oncologists may not be aware of the latest developments in fertility techniques and do not have relevant education materials for the patient (9,18). For example, not knowing that only a single motile sperm is required in ICSI may cause physicians to prematurely dismiss a patient's suitability for sperm banking (38,62,82). Physicians also tend to underestimate the importance of fertility and subsequently leave it out of routine discussions with their patients (13,63). Moreover, oncologists are unaware of the nearest and most convenient sperm banking facilities that they can refer their patients to (57,59,83).

Another barrier faced by physicians is the sensitivity of the issue. Physicians may feel uncomfortable discussing fertility with their patients, especially with adolescents, and therefore choose to completely avoid the topic (13,45,82). Finally, the last barrier elucidated from interviews and surveys is the perceived high cost of sperm banking. Oncologists tend to overestimate the costs of sperm banking and therefore, knowing a patient's financial situation, may refrain from suggesting the option at all (59,81,83).

Patient

Even if sperm banking is offered, patients may not choose to take the option. The main reason cited is the lack of information (25% of interviewees) that hindered patients from making an informed decision (57,58,79). Even if patients proactively searched the Internet for information, Merrick et al. found that resources had incomplete information and were not reader-friendly in terms of design and language (84). As such, patients had insufficient information regarding the effects of cancer on fertility (18)

and were equally uninformed about the procedure (45,83).

The next most common reason is patient uncertainty over the desire for biological children (especially for adolescents) (8), or the need for additional children, especially if they have already completed their families (78,79). Moreover, patients may be anxious that offspring produced from cryopreserved sperm will be abnormal, unhealthy, have birth defects, or have a higher risk of cancer (31,47,85).

Additionally, some patients fear that sperm banking will postpone life-saving cancer treatment (8,84,86), while others may feel too ill to provide a sample (6,8) or too stressed to make such a decision (30,38,77). Sperm banking is also often considered too sensitive for discussion, especially with adolescents (8,27,87), and is deemed to be immoral in certain cultures and religions such as the Evangelicals (8,27,75,88). Finally, some patients are unable to afford the cost of sperm banking (18,30,55), which includes freezing, storage, as well as the type of ART and the number of cycles required to achieve pregnancy (66).

General

It has also been found that very few patients return after gonadotoxic treatment to use their cryopreserved sperm in ART procedures. In a study conducted by Girasole et al., only 3 of the 31 patients had used or were intending to use the sperm (23), while in another study by Menon et al., a mere 2.2% of patients used their sperm (81). Tournaye et al. established the possible reasons for low utilization—recovery of normal reproductive health (41%), death of patient (37%) and no desire for biological children (7%) (11). Other suggested reasons include the fear that offspring will inherit the disease, uncertainty of their prognoses and the cost of ART (16,25,40). Moreover, some patients refuse to dispose of their sperm even when fertility was regained because they wanted it as backup should there be a relapse (73). With such low utilization rates, sperm banking appears to be a waste of resources, hence physicians and patients may feel it is unnecessary (85).

In cases of patient death, it is also difficult to determine if it is legal and/or ethical for surviving relatives to use sperm posthumously to produce a child (69). For now, this is only allowed if unambiguous consent to do so was given by the patient when he was alive (16,27,55). Moreover, laws regarding sperm cryopreservation differ across countries. For example, the United Kingdom and Canada allow donation and cryopreservation of gametes and embryos for young cancer patients, but more conservative countries like

Switzerland and Italy have outlawed procedures like gamete donation and embryo freezing (47). As such, complex legislations may hinder the process of sperm banking too.

How to overcome these barriers

Not all barriers are insurmountable. Other members of the oncology team, such as nurses can be trained to discuss fertility options with the patients and counsel and support them where needed (59,73). Additionally, appropriate and useful education materials using various platforms such as pamphlets, videos or interactive media can be designed to help patients make decisions about sperm banking (3,74,83). As previously highlighted, the introduction of ICSI eliminates the need for multiple samples of good quality to be collected (11). Hence, the collection of a single semen sample should not delay treatment significantly (57).

Physicians' lack of knowledge regarding fertility issues can be improved by education and training (6,55,82). A simple Internet search will identify the locations of nearby sperm banking facilities (6,59). Alternatively, some sperm banks provide cryopreservation kits that can be returned via post after the semen sample is collected at home. This makes the entire process very convenient and comfortable for the patient (6,19). Furthermore, in order to standardize the level of care provided by all physicians, protocols can be implemented for the discussion of sperm banking with patients (2,82). In fact, ASCO's recently updated guidelines state that all health care providers should be willing to discuss fertility preservation options and "present sperm cryopreservation as the only established fertility preservation method" as other methods are still experimental (53).

Although costs differ among banks, it is projected that the approximate annual cost of storing three ejaculate samples is between $300-$500 (6). A part of it may be covered by insurance, especially if the patient has cancer, and some banks also offer payment plans (6,59). To avoid awkward situations where adolescents are too embarrassed to talk about fertility in front of their parents, separate discussions should be conducted (59,74). Parents should also be advised on how to approach the topic with their children in an appropriate manner (1).

Conclusions and future directions

In conclusion, cancer and its treatment (chemotherapy, radiotherapy and/or surgery) can potentially impair fertility and therefore, it is important to cryopreserve sperm samples before any form of gonadotoxic treatment commences. In cases where sperm cannot be retrieved by the conventional method of masturbation, there are alternative techniques that can be employed such as EEJ, MESA and TESE. With the numerous benefits of sperm banking and its relative ease and convenience, more effort should be put into overcoming the barriers that prevent its utilization so that post-treatment cancer patients can enjoy a better quality of life. Most importantly, there is a need to increase awareness and knowledge of sperm banking among healthcare providers (physicians, nurses and counsellors alike) and the general public as the whole process requires extensive coordination between all parties (89). Also, more research is needed to develop techniques of preserving fertility in adolescent pre-pubertal patients.

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Footnote

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492

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A second opportunity to come for anti-angiogenics in prostate cancer?

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Abstract: Similar to the previous experience combining the anti-angiogenic agent bevacizumab with docetaxel, the use of sunitinib and prednisone (as compared to placebo and prednisone) did not result in prolongation of overall survival in patients with metastatic castration resistant prostate cancer (mCRPC) who had failed one previous docetaxel-based regimen. However, both progression-free survival (PFS) and response rate were significantly improved. Possible explanations to these findings and strategies to optimize the clinical application of anti-angiogenics in prostate cancer are discussed.

Keywords: Angiogenesis inhibitors; prostate cancer; sunitinib

Better understanding of the biology of advanced prostate cancer has led to unprecedented progress in its therapy over the past few years. The androgen biosynthesis inhibitor abiraterone acetate, the androgen receptor (AR) antagonist enzalutamide, the cytotoxic chemotherapeutics docetaxel and cabazitaxel, the immunotherapeutic sipuleucel-T and the alpha particle-emitting radiopharmaceutical radium-223 have all been shown to extend survival (OS), and in some cases provide symptomatic improvement in phase III clinical trials in patients with metastatic castration resistant prostate cancer (mCRPC) (1-7). While androgen signaling inhibitors and chemotherapy primarily target tumor cells, the effects of radium-223 and particularly sipuleucel-T are likely mediated in part by modulation of the tumor microenvironment, including immune and other stromal cell constituents of the primary tumor and metastatic sites. Thus, targeting components of the microenvironment in prostate cancer can meaningfully affect the rate of cancer progression and survival outcomes.

Tumor growth is often critically dependent on its ability to sustain an adequate blood supply, which, to a different degree depending on cancer type and state, is facilitated by newly developed blood vessels through the process known as tumor angiogenesis. Anti-angiogenic drugs were developed to "starve" tumors by primarily affecting tumor-associated blood vessels. These agents have been mostly designed to inhibit vascular endothelial growth factor (VEGF) signaling, a key mediator of tumor angiogenesis. Several VEGF-targeted drugs (the anti-VEGF monoclonal antibody bevacizumab, the synthetic VEGF trap aflibercept, and the multi-tyrosine kinase inhibitors (VEGFR TKI) sorafenib, sunitinib, pazopanib, axitinib, vandetanib, cabozantinib and regorafenib) have been approved as single agents for solid tumors such as some that respond poorly to conventional chemotherapy (e.g., advanced renal cell, pancreatic neuroendocrine, medullary thyroid and hepatocellular carcinomas), and also in combination with chemotherapy (8). However, the results of controlled clinical trials using anti-VEGF therapies in prostate cancer have so far been disappointing.

A few VEGF inhibitors have been tested in combination with standard first-line docetaxel-based chemotherapy in mCRPC. In phase II clinical trials, bevacizumab and sunitinib showed seemingly modest additional activity when combined with docetaxel (9,10). Yet neither bevacizumab nor aflibercept in combination with docetaxel and prednisone led to improvement in OS as compared with docetaxel and prednisone alone in respectively the

CALGB 90401 and VENICE phase III clinical trials, even though bevacizumab resulted in extension of progression-free survival (PFS) and a higher rate of objective responses (ORR) (11,12).

As single agents, sorafenib, sunitinib, and cediranib (a separate VEGFR TKI) showed noticeable but limited activity in phase II studies, both in chemotherapy-naïve patients and after progression to docetaxel (13-16). Interestingly, responses (in pain and scans) were frequently discordant with changes in PSA, which tended to increase during treatment (4 weeks on, 2 weeks off schedule) and drop off it (17). Some of those early trials were designed using PSA response as the primary endpoint, leading to early study closure. Bevacizumab monotherapy, however, did not show clinical activity in mCRPC (18).

Marc Dror Michaelson *et al*. recently published the results of the international phase III trial of sunitinib plus prednisone versus placebo plus prednisone in patients with progressive mCRPC (SUN 1120) (19). Different to the CALGB and VENICE studies above, sunitinib was used as mainstay treatment without chemotherapy, and following failure of one previous docetaxel-based regimen. Patients (n=873) were randomly assigned 2:1 to receive the study drug or placebo continuously in combination with oral prednisone. OS, the primary endpoint, did not differ significantly between treatment arms (13.1 *vs*. 11.8 months respectively for sunitinib and placebo, P=0.168), leading to early termination of the trial after a second interim analysis on the basis of futility. Sunitinib was however comparatively better in secondary endpoints, such as PFS (5.6 *vs*. 4.1 months, P≤0.001) and ORR in patients with measurable disease (6% *vs*. 2%, P=0.04). Also to note, the rate of discontinuation of sunitinib before objective disease progression was an important 37%, mostly due to toxicity but also to a high censoring rate from patient termination before disease progression in relation to the early study closure. How the interpretation of PSA raises by the treating oncologists may have influenced their evaluation of response and thus the study results was not formally evaluated.

Together, the data from the sunitinib and the docetaxel and bevacizumab/aflibercept combination phase III trials strongly suggest that there is no general role for limited anti-VEGF therapies in patients with mCRPC, either alone or in combination with docetaxel. The clinical experience suggests that the multi-targeted TKI, particularly sunitinib, are more active in mCRPC than the VEGF ligand blocking drugs as single agent, and that only subsets of individuals seem to obtain benefit. Important challenges therefore remain. What characterizes the disease of the responsive patients? And at what point in the natural history of the disease are anti-angiogenics most beneficial? In the current state of knowledge, the answer to neither question is obvious. In spite of the established relevance of angiogenesis in tumorigenesis, prostate cancer is characterized by a dominance of androgen signaling-related evolutionary and adaptive changes in its castration resistant progression. How each of those changes alters the balance of pro- and anti-angiogenic drivers in the microenvironment and the host, and in the end the relative contribution of tumor angiogenesis through prostate cancer progression remain poorly depicted in patients. Moreover, the consistent tropism of prostate cancer for bone and the inherent difficulty in reliably evaluating disease and treatment-related changes in the osseous environment make this characterization particularly challenging.

Still, it is speculative but probable that a subset of mCRPC patients exist in whom angiogenic mechanisms of progression are important, thus potentially rendering them more responsive to angiogenesis inhibition. For instance, an estimated 10-20% patients treated with sunitinib demonstrate sometimes dramatic bone scan responses, although the translation of these findings into survival outcomes is not available (20). Moreover, the biology of the disease in specific metastatic sites may rely heavily on angiogenesis. This could be the case of lymph nodes, which are the most frequent site of measurable metastasis in mCRPC patients. Both the CALGB 90401 and SUN 1120 studies demonstrated significant improvements in ORR compared to control (11,19).

Regarding timing for application, the limited existing data for bevacizumab and sunitinib in castration sensitive patients (mostly with high-risk prostate cancer) do not suggest that early introduction of VEGF-targeted therapy results in meaningfully better outcomes than in the castration resistant phase, at least in what concerns the primary disease site. However, even complete pathologic responses occur in rare cases (21), suggesting that angiogenesis inhibition can be useful in treating prostate cancer patients.

So what are the possible venues to improve the efficacy of these agents? An obvious one is the definition of predictive molecular and/or genetic markers that enrich for microenvironmental dependence on angiogenesis. Years of research in the field of biomarkers have not yet resulted in the identification of any prospectively validated

molecular or cellular surrogate of anti-angiogenic treatment benefit in prostate or any other cancer type. Because of the limited relevance of animal models in prostate cancer and the disease's inherent heterogeneity, information should originate from characterization of angiogenesis mediators in clinical specimens serially obtained from individual patients. Another comes from the development and application of multi-targeted drugs or combinations that block pathways complementary to VEGF in driving angiogenesis and metastatic progression, or mechanisms of adaptive resistance to angiogenesis inhibition. A very relevant even if *a priori* unexpected example of this comes from cabozantinib, which inhibits VEGFR and the hepatocyte growth factor (HGF) receptor potently and can result in striking radiologic and pain-relieving responses in mCRPC (22). HGF receptor has been shown to participate in escape from VEGFR inhibition (23) and mediate cross-talk signaling between prostate cancer and host cells in bone metastasis (24). Not surprisingly, cabozantinib is being evaluated as single agent in phase III clinical trials in mCRPC. Combinations of anti-angiogenics with immune and bone metastasis modulatory drugs may also prove useful.

Last is the issue of toxicity, which is quite relevant because mCRPC patients are generally older and more comorbid than those with other tumor types. In CALGB 90401, the number of treatment-related deaths (4% *vs.* 1.2%) was greater in the experimental bevacizumab arm (11). In SUN 1120, 27% patients abandoned sunitinib therapy because of toxicity before progression, probably affecting the OS results (19). Studies have shown that sudden discontinuation of anti-angiogenic treatment may result in "rebound" production of potentially tumor supportive pro-angiogenic factors. Therefore, it may be useful to sustain or even expand angiogenesis inhibition to other relevant targets beyond disease progression (8). Eight percent of the patients in SUN 1120 had their sunitinib dose escalated, resulting in no apparent effect on clinical outcome. Whether more conservative and thus less toxic doses and schedules than those used in cancers more dependent on angiogenesis would be sufficient to achieve anti-tumor effect in prostate cancer has not been formally tested and warrants consideration.

In spite of so far limited effectiveness and significant toxicity and cost, the available data suggests that angiogenesis inhibition should still be considered a potentially useful strategy for the treatment of prostate cancer. Upcoming clinical trials should be based on rational combinations that include potent but narrow in spectrum (and thus likely less toxic) angiogenesis inhibitors, targeting only specific prostate cancer patient subsets and clinical states. The hope is that next generation profiling technologies soon result in better understanding of the driver genetic and molecular networks in prostate cancer, allowing for optimization of the use not only of angiogenesis inhibitors but of all other treatment options for the welfare of the patients.

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Footnote

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Treatment of localized prostate cancer in elderly patients

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Abstract: Prostate cancer is a disease of the elderly. According to National Cancer Institute, more than 56.7% of incident cases are diagnosed and more than 90% of cancer specific deaths occur in men greater than 65 years of age. Despite equivalent oncologic outcomes with treatment, primary local therapy is often deferred in elderly men with high-risk prostate cancer, in part due to concerns that post surgery quality of life (QOL) functional outcomes compare poorly to younger men. Our aim in this editorial is to discuss the functional and oncological outcomes in management of elderly with localized prostate cancer.

Keywords: Prostate cancer; local therapy; elderly patients

Introduction

Treatment decisions for men newly diagnosed with localized prostate cancer are complex, and require careful consideration of the malignant potential of the primary tumor, patient life expectancy (LE), baseline quality of life (QOL), and expected change in QOL following definitive therapy. The purpose of the study by Hampson *et al.* (1) was to examine differences in QOL outcomes by age following treatment for localized prostate cancer.

Expert summary

Hypothesizing that declines in QOL after treatment would be less meaningful to older compared to younger men, Hampson *et al.* investigated changes in QOL outcomes over time by age using the CaPSURE database. CaPSURE is a longitudinal, observational cohort of approximately 15,000 men with all stages of biopsy proven prostate cancer enrolled at 43 community urology practices, academic medical centers, and VA Hospitals since 1995, and is unique in the fact that it predominantly represents outcomes for patients treated in community practice (2).

The analytic cohort included patients newly diagnosed with clinically localized (≤ cT3aN0M0) prostate cancer during 1999-2013 undergoing local treatment [radical prostatectomy (RP), brachytherapy, EBRT] versus no local therapy [ADT, active surveillance/watchful waiting (WW)]. To meet inclusion criteria, all patients completed QOL questionnaires (RAND-36 short-form health survey, UCLA-Prostate Cancer Index) at the time of diagnosis and/or within 2 years after treatment. Following adjustment, QOL changes over time between age groups were compared using repeated-measures mixed models, utilizing an interaction term (age*time) to assess if the trajectory of QOL over time differed by age category. Secondary analyses adjusting for the same covariates were used to assess three-way interactions between age, time, and primary treatment.

Among 9,945 patients identified, 6,522 patients reported QOL data within 2 years meeting study criteria. Stratified by age (<60, 60-70, >70 years), older men had higher PSA at diagnosis, increased number of co-morbidities, higher clinical T-stage, higher biopsy Gleason Grade, and higher CAPRA clinical risk strata (all P values <0.01). A total of 44% of patients in the >70 years group underwent no local therapy compared to <5% in men <60 years of age and 11% in men 60-70 years of age (P<0.01).

Compared to younger men, men >70 years of age had lower baseline un-adjusted QOL scores in all domains (urinary function, urinary bother, sexual function, sexual bother, bowel function, bowel bother, physical function) except mental health. Over time, following adjustment for

clinical characteristics and treatment type, QOL differed by age group for all domains. For sexual and urinary domains, younger men had higher baseline scores, which declined at one year and then improved (but not to baseline values). Bowel function and bother domains were stable across age groups, except in men >70 years of age who reported less improvement in bother. At 2 years, declines in QOL were evident for sexual function, sexual bother, and urinary function regardless of age group, but the differences in QOL change were greatest in men <60 years of age.

Secondary analyses evaluating the impact of treatment type (local versus non-local) on change in QOL demonstrated that the largest differences in were noted in sexual function, sexual bother, and urinary function, most notably in those undergoing local treatment. At 2 years, more men <60 years experienced a decline in sexual function following local treatment (42% *vs.* 34%), whereas rates of decline in sexual function for men >70 years of age were similar between those undergoing local therapy and those who did not (43% *vs.* 45%). Adjusted scores for sexual bother and urinary function worsened after local versus no local therapy across age categories.

Summarizing these findings, the authors noted that older patients had lower unadjusted QOL scores both before and after treatment for all domains except mental health. However, in general, older and younger men experienced QOL declines in different ways. Men undergoing local therapy had lower post treatment urinary function scores compared to the no local therapy group regardless of age category. With respect to sexual outcomes, younger men had greater declines and better recovery in function, but experienced more bother over time when compared to older men. The authors concluded that age has a variable effect on QOL after treatment for localized prostate cancer, which has important implications for patient centered discussions regarding treatment options and patient's preferences regarding impact on QOL.

Expert comments

With the growing recognition that over diagnosis has resulted in the over treatment of early stage, screen detected prostate cancers, the dilemma of how best to treat an older patient with clinically significant prostate cancer has become over shadowed. However, as a gradual increase in the proportion of men presenting with locally advanced cancers is an anticipated consequence of the Unites States Preventive Services Task Force decision to issue a Grade D recommendation against PSA screening in asymptomatic men (3), most experts agree that more focused recommendations for treatment of older men with high risk localized disease are needed.

It is an undisputable fact that elderly men with low risk disease and a limited LE are over treated with either RP or radiotherapy, and the most appropriate management strategy may be active surveillance (4). However, elderly patients show less effect from lead-time bias as they are screened and diagnosed at a later age and often present with more advanced disease compared to younger patients (5). High risk, clinically localized prostate cancers are not indolent and can have a significant deleterious effect on cancer specific survival in the absence of definitive local therapy. However, while RP and XRT are commonly employed for older patients with low and intermediate risk disease, older men with higher risk disease are less likely to be offered curative treatment (6), despite strong evidence to suggest a survival benefit with active treatment compared to conservative therapy or androgen deprivation alone (7-12). Although treatment decisions in elderly men are complex, reluctance to employ curative treatment in more elderly patients may be due to underestimation of LE, lack of definitive evidence demonstrating a survival benefit, and concerns regarding negative impact on QOL.

Estimating LE

Current guidelines are unclear when to offer primary treatment to elderly patients and likely impacts utilization of definitive therapy. The American Urological Association recommends RP or radiation therapy (RT) when the patient would have a reasonably long LE (13). "Reasonable" is left up to the discretion of the clinician. In comparison, the National Comprehensive Cancer Network guidelines recommend RP as a treatment alternative in men who have a LE of 10 years or more. RT is recommended as an acceptable strategy in patients with LE more than 20 years in low-risk, and an option in intermediate to high-risk regardless of LE (14). Although NCCN guidelines are more clearly defined, accurate LE calculations are still very difficult. Clinicians tend to grossly under-estimate LE and accuracy of clinician-predicted survival is limited (15-17). There is no definitive methodology of calculating accurate LE, which is based on both age and comorbidities. Life-tables themselves have a limited ability to predict LE in screened patients with prostate cancer, as healthier men than the general populations are usually screened (15).

Risk stratification and oncologic outcomes

Rather than relying on LE alone, prostate cancer risk stratification is paramount prior to offering treatment, as age may have less of an impact than tumor characteristics on mortality outcomes (18). Two randomized studies have showed a survival benefit from radiotherapy in combination with androgen deprivation therapy for men with high-risk prostate cancer, with a similar effect for men younger and older than 67 years of age (7,8). However, the comparative effective evidence base for RP in men with high-risk disease is lacking regardless of age.

   The Scandinavian Prostate Cancer Group Study Number 4 (SPCG-4), a randomized clinical trial of RP and WW in men with localized prostate cancer diagnosed during the pre-PSA era, revealed a mortality benefit favoring surgery in men <65 years old and no benefit in men >65 years of age (19). However, application of the age cut-offs from SPCG-4 trial are challenging in the PSA era as these patients were not screened and therefore had a lower lead-time bias. While this trial demonstrated a survival benefit with treatment in patients with intermediate-risk prostate cancer, men with poorly differentiated prostate cancer on histology were excluded in the SPCG-4 study. As a result, it is very difficult to extrapolate these results to guide decisions in elderly men diagnosed in the contemporary PSA era with intermediate and high-risk disease. In comparison, the Prostate Cancer Intervention Versus Observation Trial (PIVOT), a trial randomizing men to RP or WW performed in the United States during PSA era, revealed no mortality benefit with RP at any age (20). In comparison to the SPCG-4 study, the PIVOT study included more men with cT1c disease with a PSA <10, and in post hoc analyses they observed that reductions in prostate-cancer mortality in the radical-prostatectomy group were more demonstrable in men with a PSA value that was greater >10 and in those with high-risk disease. In part due to misinterpretation of the existing evidence base, a recent study from Prostate Cancer Data Base Sweden (PCBaSe) illustrated that only 10% of men with high-risk prostate cancer aged 75-80 with Charlson Comorbidity Index (CCI) 0 received RT or RP despite 52% probability of 10-year LE, compared with approximately 52% of the men younger than 65 years with CCI 3 with similar 10-year LE (21).

Functional outcomes and QOL

Oncologic outcomes aside, localized treatment for prostate cancer can have effects on urinary, sexual, and bowel function even up to 15 years after RP or RT (22) and these effects can vary by age. Historically, it has been assumed that younger men had a quicker and more durable return to function following RP. Retrospective review of a large single surgeon series reported improved long-term continence and sexual function outcomes in men less than 60 years of age (23,24). A large study from Germany evaluating 8,295 patients with normal continence and International Index of Erectile Function (IIEF) >18 who underwent RP between January 2009 and July 2013 showed similar trends among the elderly. One-year continence rates were 93.2% in men <65 years of age compared to 86.5% in men >75 years of age. Additionally, 1 year potency rates were 59.3% in mean <65 years of age versus 31.3% in men >75. In multivariate analysis, older age showed a significant negative effect in both functional outcomes (25). Other large series have similarly showed the negative effect of age on sexual and urinary function (26,27).

   In comparison, a number of studies have demonstrated that functional decline following RP may not be age dependent. Namiki *et al.* evaluated QOL outcomes in 143 men >70 years of age undergoing RP, and demonstrated improved emotional, mental health, and social functioning post-surgery compared to pre-surgery (28). While only 25% of patients returned to baseline sexual function level, 83% had reached baseline sexual bother. Herkommer *et al.* conducted a prospective single-center study to evaluate QoL using EORTC QLQ-C30 questionnaire preoperatively and every 3 months postoperatively in 374 patients with localized prostate cancer undergoing RP (29). Sexual and urinary functions were not assessed but the group assessed global health, cognitive function, social function, emotional function, physical function and role functioning. Comparing patients <60 and >70 years of age, no differences were demonstrated post operatively with respect to global health and cognitive functioning. Physical function remained stable postoperatively in men >70 years while it declined at 3 months and then returned to baseline in men <60 years. Social functioning and emotional functioning scores were higher in patients >70 years of age both preoperatively and postoperatively.

   The findings reported by Hampson *et al.* nicely illustrate that changes in functional status following prostate cancer treatment are strongly influenced by pre-treatment QOL, and that the absolute differences when comparing pre and post treatment may not be as large as previously assumed. It is clear that use of absolute or unadjusted post treatment

outcomes will favor younger patients with improved pre treatment functional status, but when rigorously measured over time and adjusted appropriately, age alone does not predict decline in QOL in most cases.

While the effect of localized treatment on functional outcomes can be quantified, it is more difficult to assess the natural progression of functional outcomes after WW or non-localized treatment. Furthermore, secondary procedures such as channel TURP, ureteral stents, and nephrostomy tubes are commonly performed to relieve obstruction from advanced prostate cancer, and the total burden of these events is poorly described in the literature. In addition, an analysis of patients in SPCG-4 (both RP and WW arms) age-matched against a non-cancer control group revealed the prevalence of erectile dysfunction to be 84% in RP and 80% in men treated with WW compared to 46% in the control arm. Additionally, prevalence of urinary leakage was documented in 41%, 11% and 3% of patients treated in the RP, WW, and control group respectively (30). These results indicate that functional outcomes can also be negatively affected by progression of untreated local disease and it is very likely that these outcomes are underestimated.

Conclusions

To summarize, age should not be the primary motivator in driving the decision to undergo primary therapy in patients with localized prostate cancer. Treatment decisions for localized prostate cancer are complex, particularly in men with high-risk disease who are at significant risk for development of local symptoms and metastases. Discussions should be patient centered and focus on individualized assessment of malignant potential, baseline functional status, and estimation of LE. Careful elucidation of each and every patient's QOL priorities as well as understanding of expected changes to QOL should be an integral part of these discussions regardless of age.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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The primary health care physician and the cancer patient: tips and strategies for managing sexual health

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Abstract: There is a large and growing population of long-term cancer survivors. Primary care physicians (PCPs) are playing an increasingly greater role in the care of these patients across the continuum of cancer survivorship. In this role, PCPs are faced with the responsibility of managing a range of medical and psychosocial late effects of cancer treatment. In particular, the sexual side effects of treatment which are common and have significant impact on quality of life for the cancer survivor, often go unaddressed. This is an area of clinical care and research that has received increasing attention, highlighted by the presentation of this special issue on *Cancer and Sexual Health*. The aims of this review are 3-fold. First, we seek to overview common presentations of sexual dysfunction related to major cancer diagnoses in order to give the PCP a sense of the medical issues that the survivor may present with. Barriers to communication about sexual health issues between patient/PCPs in order are also described in order to emphasize the importance of PCPs initiating this important conversation. Next, we provide strategies and resources to help guide the PCP in the management of sexual dysfunction in cancer survivors. Finally, we discuss case examples of survivorship sexual health issues and highlight the role that a PCP can play in each of these case examples.

Keywords: Primary care; behavioral health; cancer survivors; sexuality; quality of life

Cancer related sexual dysfunction

Worldwide, an estimated 14.1 million patients are diagnosed with cancer annually (1) and a majority of these individuals will become long-term cancer survivors (2). As greater numbers of survivors are living long after diagnosis and treatment, there is growing recognition that primary care physicians (PCPs) need to play an increasingly important role in addressing the numerous treatment-related side effects that impact quality of life for millions of cancer survivors (3). As PCPs are often in position to provide the majority of post-treatment medical care when survivors transition out of the oncology setting (4), they are, in a sense, on the "front lines" when it comes to managing long-term side effects for many survivors (5).

Sexual health is one of the most fundamental and long-lasting aspects of function that can be negatively affected by cancer treatment (6). Estimates of the prevalence of sexual dysfunction after cancer range from 40-100% (7-9), and affect both sexes. For both men and women, common problems includes disorders of sexual response (e.g., arousal, erectile dysfunction, ejaculatory dysfunction, reduced lubrication in females, chronic dyspareunia, orgasmic dysfunction etc.), and disorders of sexual desire and motivation (e.g., hypoactive sexual desire, reduced sexual motivation, body image disturbances, loss of sexual self-esteem etc.) (10). Without intervention, the detrimental impact of sexual dysfunction on cancer patients is significant and evidence suggests that these problems often get worse over time (6,11).

Because the sexual side effects of treatment are both profound and enduring, PCPs have a particularly important role to play in helping cancer survivors address and manage

504

Zhou et al. The PCP and the cancer patient

these problems. However, it has been shown that PCPs often do not feel comfortable managing sexual side effects (12) and that a lack of discussion with patients about sexual dysfunction has been associated with PCPs' self-report of not receiving adequate preparation and/or formal training around survivorship care (13). Communication about sexual health is also hampered by a concrete lack of material resources such as clinical checklists, and educational materials (14,15). Thus it is imperative for PCPs to have a range of efficient strategies including language for communication, simple checklists for clinical inquiry and access to useful resources in order to facilitate communication with patients about sexual problems after cancer.

For the purpose of providing an overview of commonly reported cancer-related sexual health issues that PCPs can expect to encounter, we will begin by briefly describing frequently reported sexual symptoms and side effects related to common cancers. In other sections covered within this special journal issue, there is more in depth coverage of the specific sexual dysfunction that providers can expect to encounter with regard to particular diagnoses and treatments. Subsequently, we will address strategies for enhancing communication about sexual health between PCPs and survivors, offer tips for use of a model for clinical inquiry and clinical checklists, and make recommendations for resources to offer patients who are struggling with sexual problems after cancer. Finally, we will highlight clinical case examples that PCPs may encounter in their practice, and overview clinical next steps that a PCP might consider for the cases described.

Overview of common cancers and treatment-related sexual problems

Breast cancer

Treatment for breast cancer can involve surgery, chemotherapy, radiation, and/or hormone therapy. The experience of any one of these treatments is likely to impact the survivor's sexual health (16). Therefore, research indicates that breast cancer survivors are significantly more likely to suffer from sexual problems when compared to the general population (17). Breast cancer survivors are likely to report libido changes, vaginal dysfunction (dryness, stenosis), different orgasm experiences, changes to body image, loss of intimacy, and a different relationship with their partner (16,18-20).

Gynecologic cancer

The treatment of gynecologic cancers is very likely to result in some form of sexual dysfunction (21). Survivors of gynecologic cancers experience worse sexual problems than women in the general population (22), regardless of specific site of their gynecologic cancer (e.g., endometrial, vulvar, cervical), treatment type, time from diagnosis and age (23,24). Their sexual dysfunction tends not to improve over time, and can intensify in nature (25). Surgery is a common component of treatment for gynecologic cancers and can result in long-term sexual health issues of pain, loss of sensation, changes in body image, vaginal dryness, difficulty reaching orgasm (26), and can trigger premature menopause symptoms including dyspareunia, and low libido (27). In addition, these women report that their treatments can alter their feelings of femininity, mood, self-esteem, and the way they relate to and discuss sexual issues with their partners (28-33). The addition of radiation and/or chemotherapy to the treatment regimen puts the patient at an increased risk of developing more severe sexual problems (34).

Prostate cancer

Because of the direct effect of treatment on sexual organs, men surviving prostate cancer commonly experience a significant impact on their sexual function as a result of treatment (9,35,36). Depending on a number of disease specific characteristics, prostate cancer patients may undergo a range of treatments including surgery, radiation, and hormonal therapy. Post-treatment, prostate cancer survivors commonly report symptoms of sexual dysfunction including erectile dysfunction, dry orgasms, urinary incontinence during orgasm, decreased satisfaction with orgasm, decreased penile length, body feminization and avoidance of sexual activities (37-54). As all active treatment options for prostate cancer are associated with compromised sexual functioning (55), the implications that these issues have on quality of life for prostate cancer survivors is critical for a provider to consider (36,55-59). In addition, prostate cancer survivors commonly endorse sexual bother even when function recovers, indicating that significant struggles remain in their efforts to cope with their functional decrements (60,61).

Testicular cancer

Sexual problems commonly reported by testicular cancer survivors include difficulties with sexual desire, ejaculatory difficulties and erectile dysfunction (62-65). These may be related to structural and emotional (body image) issues related orchiectomy, as well as retrograde ejaculation due to pelvic lymph node dissection. Following treatment these

men often report reductions to sexual activity levels, and general sexual dissatisfaction (66,67).

Bladder cancer

Treatment for bladder cancer includes surgery, radiation, immunotherapy, and chemotherapy, with surgery being the most common course of action. For men, standard radical cystectomy is often associated with the loss of sexual function, most notably erectile dysfunction (68). For women, radical cystectomy is likely to create prominent sexual dysfunction including reduced libido, dyspareunia, decreased lubrication, and diminished ability or inability to achieve orgasm (69,70). In addition, men and women also suffer from the psychological impact of treatment in addition to their physical challenges. They often report body image concerns after urinary diversion following radical cystectomy (71-74), which is associated with significant loss of sexual function and satisfaction up to five years later (75).

Colorectal cancer

Surgery for colorectal cancer often causes nerve damage, and can cause erectile and ejaculatory dysfunction in men and desire, pain, and orgasm difficulties in women (76-82). Colorectal cancer survivors can report that their sexual function is affected by emotional reactions and adjustment to colostomy and their stoma, with notable concerns related to odor, flatulence, and diarrhea.

Head and neck cancer

Treatment for head and neck cancers can cause facial alterations/disfigurement, as well as persistent changes to saliva quality and/or quantity, breathing and speech (83). As the function and appearance of the head and neck region plays such a critical role in our social interactions (84), treatment can have implications for relationship and sexual function in survivors (85,86). Consequently, head and neck cancer survivors report feeling less attractive, reduced libido, and decreased satisfaction with their sexual relationships (87-91).

Hematologic malignancies

For those diagnosed with a hematologic malignancy, chemotherapy, total body irradiation, stem cell transplantation, and even the placement of a central venous catheter can significantly impact the patients' body image, intimacy, and sexuality (92-95). Survivors can report erectile dysfunction in men, vaginal dryness in women, and pain and

difficulty with orgasm for both men and women (94,96-98).

Childhood cancer

Those diagnosed with cancer at a young age are exposed to treatments that can impact their sexual health during critical developmental periods. Physically, treatment can impair their hormonal, vascular, genitourinary and neurological function, placing these survivors at risk for both sexual dysfunction (99-104), and infertility (105,106). Further, evidence suggests that even when young adult survivors of pediatric cancer report generally good health, they still have increased prevalence of sexual dysfunction (104). Psychosocially, they tend to be less sociable and more isolated, are less likely to marry, show greater restriction in their sexual behavior (e.g., masturbation, talking to friends about sex), delays in reaching sexual milestones (e.g., dating, intercourse), and decreased sexual interest and satisfaction with sex (107-113).

Communication about sexual dysfunction

A consistent theme across the literature has been that cancer patients and survivors rarely discuss issues of sexual function with their medical providers (114). As the patient begins their cancer treatment, the main focus for both the patient and oncology provider is on ensuring their survival. Therefore, it is not surprising that conversations with oncology providers about the short and long-term sexual health consequences of treatment either do not take place or are not well remembered (115,116). An estimated 0-37% of cancer survivors report that they had a discussion about sexual health with any member of their medical team (117-119). Similarly, oncology professionals report that they do not often discuss issues related to sexual health with their patients. In a survey of gynecologic oncologists, less than half reported that they took a patient sexual history at least 50% of the time (120). Similarly, providers treating women with ovarian cancer indicated that the overwhelming majority did not discuss sexual issues, despite acknowledging that these patients were likely to experience some form of sexual dysfunction following treatment (121). Like their colleagues in oncology, PCPs are also unlikely to discuss sexual health issues with cancer survivors who have completed active treatment. In a survey of primary health care physicians, over 60% of providers reported that they "never" or "rarely" addressed sexual dysfunction issues, and more than half were unlikely to initiate a conversation about sexual dysfunction with cancer survivors (122). Even when

506

Zhou et al. The PCP and the cancer patient

discussions occur with cancer survivors, they are often limited to the discussion of functional status, and rarely do issues related to the impact of sexual dysfunction on mood, quality of life, relationship functioning etc. get discussed (123,124).

This lack of discussion about sexual health stands in direct contrast to what medical professionals report regarding the importance they place on such issues, and their capability of delivering this care. For instance, nearly all gynecologic oncologists surveyed in one study reported that they were comfortable with taking the sexual history of their patients (120), and that issues related to sexual health consequences of cancer treatment should be discussed with patients (125).

Despite the lack of communication about sexual health after cancer, cancer patients/survivors report a consistent desire to have open dialogue with their medical providers about sexual issues (117,126). They are interested in conversations ranging from physiological changes that result from treatment, to the safety of sexual activity to psychological issues such as reassurances that their sexual issues are commonplace. Perhaps more importantly, cancer patients/survivors indicate that they are amenable to discussing how to resolve the impact of the changes to their sexual function and intimate relationships following cancer (116,117,123).

Challenges in sexual health communication

Despite the medical provider's awareness of the importance of discussing sexual function, and the patient's interest in receiving further information about sexual health issues, assessment and counseling about sex is not commonly a part of routine medical care across the world (114,127). There are numerous barriers to this important conversation on both sides of the examination table, and often neither the patient nor the PCP feels comfortable initiating conversations about sexual health.

From a patient's perspective, he or she may experience challenges due to the patient/provider relationship, and a lack of accurate knowledge about sexual function and cancer. Moreover, patients report that if his or her provider does not bring up a medical issue, then it must not be of significant concern. Therefore patients are cautious about bringing up sexual dysfunction concerns because they are uncertain about its validity if their provider does not initiate (123,124,128) and they may be worried about feeling disrespected in such an interaction (128,129). During the particularly stressful period of time soon after a cancer diagnosis, patients are often overwhelmed with information and treatment planning and simply do not have the capacity to consider the sexual health implications of their cancer treatment (128,130). In addition, cancer patients may possess inaccurate beliefs about sexuality that reduce the likelihood that they will raise such issues with medical providers. For example, they may worry that cancer is contagious and can be spread through sexual acts, that sexual activity may impact their cancer recovery, or that side effects from cancer treatment make sexual activity impossible (131,132).

For the PCP, the literature points to types of barriers that bar effective communication about sexual health: patient characteristics, provider characteristics, and systems-based challenges. First, there are a number of patient characteristics that can discourage a medical provider from discussing sexual health; for example, age, gender, race/ethnicity, sexual orientation and partner status can all impact the provider's initiative (121-123,133). In addition, the patient's health prognosis, particularly in a palliative care setting, is likely to play a role in impacting whether the medical professional believes that the patient is interested in having a conversation about sexual health (123,124). Second, provider characteristics including their training background, knowledge about sexual health issues, and attitudes towards sex can negatively impact the likelihood of a conversation about the topic. Some medical providers recognize that they lack the experience and/ or knowledge about sexual health issues that would allow them to feel confident with discussing it with their patients (121,122,133). Furthermore, medical providers report that it is frequently unclear as to which member of the multi-disciplinary medical team is responsible for initiating the conversation (121,123). Given that sex is often considered a taboo subject in many cultures not to be discussed openly, medical providers admit that they are sometimes embarrassed to openly speak about sexual issues, and consequently, avoid such intimate conversations (121,123). Finally, the medical system itself can make conversations about sexual health issues challenging. Physicians often have overloaded patient schedules and do not have sufficient time to thoroughly explore sexual functioning with each and every patient (116,120). Even when patients and providers discuss sexual dysfunction, there may be systemic difficulties surrounding the lack of resources available for the patient, and whether their health insurance would provide coverage for these issues (116,132). Given that cancer survivors may have complicated medical histories and a variety of other late effects of treatment, barriers such as time constraints

and lack of experience and/or knowledge may be even more problematic for the PCP.

Strategies to address sexual health in a primary care setting

We suggest that the Five A's Framework, a counseling model built on five basic components (*ask*, *advise*, *assess*, *assist*, and *arrange*), can provide an efficient and flexible structure for helping PCPs address sexual function with their patients (13,134). The first A (*ask*) underscores the PCP's primary role in ensuring that patients know that sexual dysfunction is a medical issue that is commonly experienced by cancer survivors, and that this is a topic area which will be discussed during the course of their medical visit. We recommend that conversations about sexual dysfunction should be considered part of the routine review of systems. PCPs should aim to inquire about sexual function in an open-ended fashion. Common non-judgmental questions to begin initial inquiry and offer validation of the problem may include:

- "Many patients that I see express concerns about how their (treatment, disease) has affected sexual function. How has this been for you?"
- "Do you have concerns or worries about how your intimate relationship has been affected by your cancer treatment? Is this something you would like to talk about?"
- "In my experience, many people find that the kind of treatment you received can affect sexuality or intimacy. Do you have any questions for me about your experience?"

Closely related to providing validation is the PCP's responsibility to let the patient know that he or she is willing to *advise* the patient as needed. That is, this initial communication also conveys an important implicit message that treatment for sexual problems after cancer is available. The next step involves the need to adequately *assess* the problem in a manner which is efficient and also allows the PCP to identify next steps for intervention. Initially, PCPs can consider the use of paper and pencil screening tools as part of their regular intake paperwork. This serves the purpose of briefly assessing sexual function in cancer survivors evaluating which patients may require further evaluation. The use of such a screening measure can help to address barriers such as providers who have limited time, feel uncomfortable screening certain patients (e.g., an older patient who is recently widowed) or feel embarrassed

by bringing up sexuality directly with patients. Providers can swiftly review patient's responses and utilize endorsed items as a starting point for a more thorough conversation about sexual dysfunction. Even for providers who currently feel comfortable having a discussion with patients about sexuality, screening tools may serve as a guide to review symptoms that might be present post-cancer treatment.

PCPs can consider several easily accessible and widely utilized instruments for the evaluation of sexual dysfunction. For female patients, the Female Sexual Function Index (FSFI) (135) is a commonly used 19-item self-report measure originally developed to assess female sexual function in women of any age, including pre- and post-menopause, in the general population and takes approximately 15 minutes to complete. The scale assesses function over the past month in several domains: desire, arousal, lubrication, orgasm, satisfaction, and pain (136) and has been utilized and validated in cancer patients and survivors (137,138). For providers who are particularly conscious of patient burden, they can consider the abbreviated 6-item version of the FSFI, though this is not recommended as it does not provide as much clinical information as the full scale (139). Those seeking further information about this scale can find additional resources at www.FSFIquestionnaire. com (135). In male patients, providers can consider the International Index of Erectile Function (IIEF) (140). The scale is a 15-item self-report measure developed to assess erectile function in men in the general population and has been utilized in studies with cancer patients and survivors, particularly with prostate cancer populations (137). The IIEF measures function over the past month in the following domains: erectile function, orgasm, desire, intercourse satisfaction, and overall satisfaction. As with the FSFI, there are also briefer versions of the IIEF which may be considered for use as a screening tool (137,141). Though potentially useful for the PCP, the IIEF is limited as it primarily evaluates for erectile dysfunction, and other sexual dysfunctions in men could be overlooked. Providers should be aware that there are a large number of other sexual function measures available. Other screening checklists of sexual dysfunction in men have been developed, and are of value for the PCP. However, providers should exercise clinical judgment as these measures may not have been empirically validated in cancer patients. For example, a brief general screening tool (versions for both men and women) has recently been provided by Hatzichristou *et al.* (142) and can serve as a guide for PCPs looking to incorporate such a checklist with their patients. When making a decision as to which measure to

508

Zhou et al. The PCP and the cancer patient

utilize, the validity and reliability of the measure, as well as the time it takes to complete and the breadth of the assessment are crucial factors for the PCP to consider.

After the PCP has clarified the problem, patients then need to be *assisted* by receiving necessary resources such as information sheets, and access to educational books and websites or potentially PCPs may need to *arrange* further intervention other providers. It is possible that patients may need to see a specialist such as mental health professional, a sexual health counselor, and an urologist specializing in sexual medicine or a menopause specialist. It is also our belief that the final step of making arrangements for additional evaluation with specialists also includes arranging to follow-up with patients at subsequent visits. Such follow-up communicates the message that the PCP takes these issues seriously, is willing to communicate about sexual health, and reassures the patient that remaining challenges can be addressed. The following section contains specific recommendations and tips that PCPs can use in the context of assisting and arranging care for patients.

It is important to consider that the optimal delivery of survivorship care, including attention to sexual health, may require more time than the PCP has with his or her patient. Other comparable models for intervention, such as the ALLOW algorithm (143) (ask, legitimize, limitations, open up for further discussion/evaluation, work together to develop a treatment plan) encourages the PCP to discuss sexual health issues, and help the patient find adequate resolution for their identified dysfunction. Similar to the five A's Framework, this model also acknowledges that whatever the concrete constraints of clinical practice, the key elements for interaction revolve around inquiry, validation and provision of resources as needed.

Just as it is important for PCPs to have access to basic information about managing common sexual side effects, it is equally important that they have access to patient education and self-help resources which can be made available to both male and female patients. First, one should identify local professionals capable of providing more specialized treatment for sexual health issues. These professionals could include members of the following disciplines:

- Urologist/sexual medicine specialist/uro-gynecologist;
- Gynecologist/menopause specialist;
- Endocrinologist;
- Clinical psychologist/sexual health counselor;
- Pelvic floor physical therapist.

Often it is helpful to build a resource list of local specialists which may be cultivated through a number of professional societies that specialize in sexual medicine/ sexual therapy. Many of these societies not only have listings to find providers but also have excellent educational material related to sexuality after cancer. Examples of such societies that a PCP can consider include:

- International Society for Sexual Medicine (http://www.issm.info);
- North American Menopause Society (http://www.menopause.org);
- International Society for Sexuality and Cancer (http://www.issc.nu);
- Women's Health and Research Institute of Australia (http://www.whria.com.au);
- American Association of Sexuality Educators, Counselors and Therapists (http://www.aasect.org).

There are also a wide range of websites that are aimed at patient education and yield a significant amount of high quality information. Examples of patient websites include:

- American Cancer Society: Sexuality for the Woman with Cancer: http://www.cancer.org/treatment/treatmentsandsideeffects/physicalsideeffects/sexualsideeffectsinwomen/sexualityforthewoman/sexuality-for-the-woman-with-cancer-toc;
- American Cancer Society: Sexuality for the Man with Cancer: http://www.cancer.org/treatment/treatmentsandsideeffects/physicalsideeffects/sexualsideeffectsinmen/sexualityfortheman/sexuality-for-the-man-with-cancer-toc;
- Macmillan Cancer Support: Effects of Cancer on Sexuality: http://www.macmillan.org.uk/Cancerinformation/Livingwithandaftercancer/Relationshipscommunication/Sexuality/Effectofcanceronsexuality.aspx.

Acknowledging that PCPs often work under multiple constraints, including the need to address a very wide range of issues in a very compressed amount of time, it is ideal if primary care practices can have resource or "tip" sheets for cancer survivors that overview common problems such as vaginal dryness or lack of libido. Such resource sheets can save time and be an enormous resource for patients. It is our recommendation to work with either a nurse in the primary care practice or a local partner (pelvic floor therapist, sex therapist) to create a resource sheet. An excellent example is the suggested patient handout created by Carter *et al*. [2011] (144) for women to promote vaginal health after cancer (*Table 1*).

Table 1 Vaginal health handout example [adapted from Carter *et al.* (144)]
Vaginal moisturizers
Available in gels, tablets, or liquid bead
Administered either in a tampon-shaped applicator or as a vaginal suppository
Used to hydrate the vaginal tissues and improve vaginal pH
Decreases vaginal dryness and increases vaginal comfort
Vaginal moisturizers are non-hormonal, over-the-counter products that need to be used several times a week regularly
Vaginal moisturizers last for up to 2 to 3 days; then they need to be reapplied
The best absorption occurs when used prior to bedtime
Types of moisturizers include polycarbophil-based gel (e.g., Replens), hyaluronic acid-based vaginal gel (e.g., Hyalo-Gyn) and/or vaginal Vitamin E (capsule needs to be punctured prior to insertion)
Vaginal lubricants
Available in liquid or gel form
Applied in the vagina and around the genitals prior to sexual activity. The lubricants may need to be reapplied during sexual activity. It is important to also apply to a partner's genital area, especially before penetration
Used to minimize dryness and pain during sexual activity and gynecologic exams
Water- and silicone-based lubricants recommended; water-based lubricants wash away more easily
Avoid petroleum-based lubricants; they do not wash away easily, do not allow skin to breathe, and can increase the risk of infection
Use caution with perfumed or flavored lubricants; they may irritate or be atrophic to delicate tissues
Common brand names or types of lubricants can be found in drugstore chains, but online web sites and sexual boutiques can offer greater variety
Saliva is a natural lubricant
When a woman has cancer treatment that results in premature menopause or increased menopausal symptoms, the vagina can become dry and lose its elasticity. Simple strategies can help to improve the moisturization in the vagina and its ability to stretch without discomfort.

Clinical case examples

Case example 1—Mariel, a 28-year-old non-Hodgkin lymphoma survivor

Medical history

Mariel was diagnosed with diffuse large cell non-Hodgkin lymphoma at the age of 14 after presenting with a neck mass. She experienced a long course of treatment which included focal radiation and multi-agent chemotherapy. Unfortunately, Mariel relapsed at age 16, and required a stem-cell transplant with additional chemotherapy and total body radiation. Thus, Mariel was in and out of cancer treatment for most of her high school years.

Sexual health challenges

While in college, Mariel began noticing that she was behind her peers in terms of her social development. Despite being an excellent student academically, Mariel started to become aware that her friends were choosing to spend more time with their boyfriends, rather than with her. She wants to start dating more regularly, but does not feel comfortable in this domain. She has sexual desire, but has felt awkward during previous sexual interactions. Mariel says: "*I was not really depressed or anything during my treatment, I was a pretty happy person no matter how bad things felt. When I got sick and left school everyone was starting to date, and when I got better and came back it was like everyone was just having sex—I couldn't figure out what I missed and didn't know what I was supposed to do about even talking to a guy without feeling uncomfortable. Socially it has been a total disaster. I went from being in a protective bubble to being dropped in a huge college campus that was like one big party. I feel so out of place sometimes.*"

Considerations for the primary health care physician

This patient faces issues that are common among childhood cancer survivors. While cancer treatment may have untoward effects on physical function that can lead to sexual problems, this case highlights the psychosocial late

510

Zhou et al. The PCP and the cancer patient

effects for this vulnerable population. Despite coping very well with the immediate stressors of her cancer treatment, Mariel's social and psychological development was dramatically interrupted during her adolescence. Losing out on opportunities to be a "normal" teenager can make sexual milestones (e.g., dating, physical intimacy etc.) seem more intimidating especially if the survivor is aware that he/she is "behind". Despite presentation of overall robust health, the PCP needs to be aware that childhood cancer treatment often results in psychosexual developmental delays. Especially because young adults may not be comfortable initiating this conversation, the PCP's inquiry signifies an important first step to helping young adult survivors begin to readjust their expectations and return to normative function. The PCP can play an important role in encouraging that the childhood cancer survivor seek active psychotherapy, or to consider connecting with other childhood cancer survivors both in-person and via the internet in order to normalize their experiences.

Case example 2—Carson, a 62-year-old prostate cancer survivor

Medical history
Carson is a 62-year-old semi-retired, married attorney who was diagnosed 18 months earlier with high-risk prostate cancer. He underwent nerve-sparing radical prostatectomy, external beam radiation and was placed on androgen-deprivation therapy for 18 months. Carson has been on this therapy for 6 months now.

Sexual health challenges
Carson initially presented to his PCP for consultation about difficulty with his sleep. Secondary to this, he casually reported to his PCP that he had been experiencing low mood, lack of energy and erectile dysfunction for several months now. Upon further inquiry, Carson recalled that although desire is diminished, it is not absent and that his lack of sexual function is primarily related to impaired erectile function. He is otherwise healthy and is not taking any medications. He reports to be happily married with a supportive wife.

Considerations for the primary health care physician
Often, patients will present to their PCP with health-related concerns that are not directly tied to their sexual health. As discussed previously, patients are often reticent to speak about their sexual dysfunction for a variety of reasons.

It will be important for a PCP to be mindful of potential opportunities for further evaluation related to sexual health concerns. In Carson's case, erectile dysfunction may be a side effect of his surgery and/or radiation therapy (due to local damage to the nerves and/or blood vessels). While this may be long-lasting for some patients, many can recover their erectile function to a certain extent. Androgen deprivation may further result in erectile dysfunction due to depletion of testosterone. In addition, anxiety and depression may also play a role. Carson's case presents an excellent opportunity for the PCP to collaborate with a multi-disciplinary team in order to help Carson effectively manage his dysfunction. In addition to communicating with his oncologist, it may be helpful to connect with a urologist who specializes in men's sexual health, and a clinical psychologist who can help him and his wife navigate his adjustment to post-cancer sexual functioning.

Case example 3—Mackenzie, a 47-year-old breast cancer survivor

Medical history
Mackenzie is a 47-year-old female breast cancer survivor who has come for her yearly physical. She was diagnosed with ER + PR + HER2 breast cancer at age 45 and was treated with unilateral mastectomy, radiation therapy and chemotherapy including doxorubicin, cyclophosphamide and trastuzumab. She is currently taking tamoxifen. Mackenzie stopped menstruating during her active therapy and has since not resumed her menses.

Sexual health challenges
Mackenzie has been married for 15 years, and has two school-age children with her husband. She works full-time as a pharmacist, and often comes home tired after a long day at work. She notes that sex is very painful despite copious use of lubricants. Because of this discomfort, she has stopped having regular sexual activity with her husband. She is concerned about the pain and remarks that since ending her treatment the situation with her sex life has not improved. As a result of the cessation of regular sexual activity, she has noticed a decline in the quality of her marital relationship.

Considerations for the primary health care physician
Sexual health concerns can often be the tip of the iceberg for a PCP, as sexual dysfunction can be associated with work-life distress, as well as marital dysfunction. Strictly from a sexual health perspective, Mackenzie is likely suffering from

pain during intercourse due to vaginal dryness and vaginal atrophy. This is likely related to cessation of menses/early menopause (typically with cyclophosphamide) leading to loss of lubrication and/or as a side effect of the ongoing treatment with tamoxifen. Consequently, addressing this issue through the use of non-hormonal moisturizers, local topical estrogen use, and/or vaginal dilators with the assistance of a gynecologist and/or pelvic floor physical therapists could be beneficial for the patient. Also important for Mackenzie are the relationship sequelae of this dysfunction. The PCP is in an ideal situation to recommend that Mackenzie explore marital therapy in order to address the discord that resulted from her sexual dysfunction, and to help her understand that relationship function and sexual satisfaction are highly correlated.

Conclusions

Parallel to rapid advances in care, the number of long-term cancer survivors worldwide continues to grow at a significant pace. Consequently, these survivors bring a range of long-term side effects to their PCPs that need to address. As data demonstrate, PCPs want to provide excellent survivorship care yet often feel unprepared to do so. It is imperative that PCPs received straightforward recommendations and strategies to help their patients. Fortunately, the issue of sexuality after cancer is garnering greater attention and there are now more resources available for both professionals and patients than ever before. It is our hope that when PCPs appreciate the multifactorial etiologies for sexual symptoms in cancer survivors, have straightforward language and confidence to inquire about such symptoms, and have the tools to address and manage them and/or have readily available resources to call upon for additional evaluation, that they will feel more encouraged and empowered to not only assess patient function as it relates to sexuality post-cancer, but to also directly address sexual problems with patients as they are presented.

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Footnote

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512

Zhou et al. The PCP and the cancer patient

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514

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Future perspectives of prostate cancer therapy

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Abstract: We summarize several recent laboratory advances to tackle the problem of tumor-stroma-immune cell microenvironment interaction with the hope of developing and advancing new concepts and therapeutic strategies for prostate cancer therapy by improving bone and soft tissue metastases in prostate cancer patients. Given the emerging enthusiasm for immunotherapy in prostate cancer due to (I) improved understanding of the role of immune cells in the tumor microenvironment, (II) approval by the FDA of an immunotherapeutic drug to treat prostate cancer, and (III) recognition of immunotherapy as a novel approach to treat solid tumors by the Nobel Prize Committee (for discovery of dendritic cells that are used in immunotherapy), the field of tumor immunology is poised for growth in the next decade with the hope of developing new immunomodulatory drugs which will compliment and perhaps eventually replace traditional chemotherapeutic drugs. In this article, we provide a timely review of recent advances in the field of immunotherapy for prostate cancer, lessons learned from successes and failures, the contributory factors in the tumor microenvironment that could be rendered hostile to cancer cells, an exciting area of future research.

Keywords: Prostate cancer; cancer cells; stromal fibroblasts; vascular endothelial cells; immune cells

Introduction

Prostate cancer is the second most common cause of cancer-related death in American men. Along with surgery or radiation therapy, hormonal therapy is a main mode of treatment. For men with metastatic disease, chemotherapy provides a significant survival advantage. Therefore, new treatment options are being actively pursued to extend the survival of metastatic cancer patients. In this review, we will focus on current advances in therapies that target cancer cells, outline recent advances in our understanding of the tumor microenvironment and its therapeutic implications for advanced metastatic prostate cancer patients and discuss the current therapeutic modalities, highlight new treatment options and offer future perspectives on prostate cancer therapy. We will discuss therapies that target: (I) cancer cells; (II) stromal fibroblasts; (III) vascular endothelial cells; (IV) immune cells and (V) less well-defined population of cells that contribute to the effectiveness of immunotherapy and cancer vaccines.

Targeting prostate cancer cells

Hormonal agents for prostate cancer therapy

Androgen and androgen receptor (AR) are required for normal prostate development and carcinogenesis. Castration-resistant prostate cancer tissues (CRPC) express AR and remain responsive to low levels of androgens. AR mutation, truncation and/or amplification may confer differential ligand and antagonist affinity and specificity. Thus, even low levels of testosterone could still activate the AR and confer the growth and survival advantages for prostate cancer cells. Several studies have demonstrated that low levels of testosterone are present in prostate cancer cells. Mohler *et al.* studied testosterone levels in clinical specimens collected from castrated patients who underwent prostatectomy and found that intratumoral testosterone levels were elevated despite an overall reduction in serum testosterone (1). Intracellular androgen in prostate cancer tissues has demonstrated clinical significance as

518

Gururajan et al. Future perspectives of prostate cancer therapy

treatment with agents that reduce their levels have impacted overall survival for men with castrate-resistant disease. Abiraterone (Zytiga, Janssen Biotech, Horsham, PA) is a selective 17,20 lyase inhibitor, which inhibits the conversion of 17-α-hydroxyprogesterone to androstenedione. This agent was brought to a randomized phase III clinical trial against placebo in men with castrate-resistant disease who had received prior docetaxel. In this study, treatment with abiraterone was associated with a 35% reduction in death from prostate cancer with an improvement in median survival from 10.9 to 14.8 months (2). The survival benefit was observed across all subgroups analyzed, including number of prior chemotherapeutic regimens (one or two), type of progression (PSA versus radiographic), and patients with visceral metastatic diseases. Orteronel (TAK-700, Millennium Pharmaceuticals, Cambridge, MA) is another compound targeting 17,20 lyase and endogenous testosterone biosynthesis. In a phase I/II study of orteronel, of 43 patients with RECIST-evaluable disease, 6 showed a partial response, 23 had stable disease, and 9 showed progression (3). Phase III clinical trials with Orteronel are currently in progress in chemotherapy naïve and post-docetaxel settings. They will evaluate tumor response rate and survival benefit attributed to Orteronel therapy. Unlike abiraterone, it may be possible to administer orteronel without the use of concominant prednisone given the higher specific inhibition of this agent against CYP17.

MDV3100 (Medivation, Inc., San Francisco, CA) is an orally bioavailable anti-androgen lacking the agonist properties of conventional non-steroidal antiandrogens such as bicalutamide (4). MDV3100 antagonizes AR action by preventing the translocation of the AR from cytoplasmic to nuclear compartment and by inhibiting DNA binding of AR and hence repressed the expression of androgen-regulated genes. In a phase I study of docetaxel-naïve and docetaxel-treated patients, 62% and 51% of patients, respectively, had at least a 50% PSA decline (5). Phase III randomized trials have been completed evaluating MDV3100 in both the pre- and post-docetaxel clinical spaces (1,3,6-10). The results from the completed post-chemotherapy studies (AFFIRM) will be presented in February 2012.

Novel cytotoxic chemotherapeutics to treat prostate cancer

Cabazitaxel (Jevtana, XRP6258, RPR 116258A; Sanofi-Aventis, Paris, France) is a semisynthetic taxane that has been shown to have activity against multidrug-resistant prostate cancer cell lines *in vitro* (11). This preclinical observation led to a randomized trial in patients with CRPC who failed

docetaxel-based chemotherapy. Patients were eligible for study if they had PSA progression, or with soft tissue and/or new lesions on bone scan. In this phase III trial, 720 patients were randomly assigned to receive either cabazitaxel, or mitoxantrone, every 3 weeks. The median survival for patients treated with cabazitaxel was 15.1 months, compared to 12.7 months in those patients treated with mitoxantrone with an overall 30 reduction in death from prostate cancer (12). In order to compare the efficacy of cabazitaxel/prednisone as first-line chemotherapy to the current therapeutic regimen, docetaxel/prednisone, an international randomized study is currently being designed at the mandate of the US Food and Drug Administration (12).

Antiapoptotic agents in prostate cancer

One unique feature of the androgen-independent prostate cancer cells is that the regression of prostate tumors still required an activation of apoptotic machinery. In many cases, AR blocking is capable of inducing apoptosis. Therefore, identifying a cure for prostate cancer requires identification and reversal of the apoptotic avoidance mechanisms, either AR-related or unrelated, responsible for drug resistance and/or newer therapies that bypass the apoptosis-resistance pathways. A number of antisense oligonucleotides targeting several anti-apoptotic genes, including BCL-2, BCL-XL, clusterin, the inhibitors of apoptosis (IAP) family, MDM2, protein kinase C-alpha, c-raf, insulin-like growth factor binding proteins and the AR, are being tested for potential clinical use in prostate cancer. Clusterin is a proapoptotic protein expressed in prosate, kidney, bladder, ovarian, lung, colorectal, and breast cancers. Clusterin expression increases with Gleason score, and is upregulated after androgen blockade (13,14). Clusterin modulates resistance to androgen blockade, radiation therapy, and chemotherapy. OGX-011 (Custirsen) is an investigational antisense compound that downregulates clusterin expression and enhances apoptotic death of prostate cancer cells (15). Increased apoptotic index of prostate cancer cells have been reported subsequent to clusterin inhibition. OGX-011/docetaxel/prednisone has been evaluated in combination with docetaxel/prednisone in men with CRPC (16). Although there was no difference was observed in time to disease progression (7.3 *vs.* 6.1 months), a superior survival was noted with OGX-011 (23.8 *vs.* 16.9 months) (7,17). In a randomized trial of Custirsen with docetaxel or mitoxantrone in patients who have progressed through docetaxel chemotherapy, the addtition of Custirsen was well

tolerated and appeared to improve pain response. In the population of men who had previous docetaxel, the addition of OGX-011 yielded 23% partial radiographic responses by RECIST criteria and PSA declines in excess of 30% in 55% of the treatment arm (17).

Targeting tumor stromal fibroblastic microenvironments in prostate cancer

Tumor-stroma interactions are crucial for normal prostate development and neoplastic prostate progression. It has been demonstrated that fibromuscular stroma and stromal fibroblasts play a regulatory role in prostate development and prostate carcinogenesis. In these studies, urogenital sinus mesenchyme (UGM) or embryonic/adult stromal fibroblasts were shown to drive the growth of UG epithelium (UGE) and prostate cancer (18-22). Using a tissue recombination technique, it has been demonstrated that while UGM derived from AR-negative testicular feminized mice failed to induce prostate morphogenesis and cytodifferentiation, UGM isolated from AR-positive wild-type mice is competent in conferring growth and differentiation signals to UGE tissues by responding to androgen-regulated growth (23-28) and expression of differentiation-related genes regardless of their AR status. Results of these studies in aggregate suggest that AR signaling from the stroma is critical for the development and differentiation of the normal prostate epithelium (19,21). The inductive role of adult prostate stromal fibroblasts, promoting prostate cancer progression, was first demonstrated by our laboratory using cell recombination models (19,20,23-28). Specifically, the progression of prostate cancer from androgen-dependent to androgen-independent state and the acquisition of bone and soft tissue metastatic phenotypes can be achieved through cellular interactions between prostate cancer cells and organ-specific stromal fibroblasts including prostate or bone stromal cells in mice *in vivo* or when co-cultured these interactive cells under three-dimensional (3D) conditions (29-34). These findings, taken together, emphasized the important role of the stromal and tumor microenvironment in prostate cancer progression and hence the rationales for co-targeting tumor and stroma (20,22,34,35).

Stromal cells surrounding the cancer cells, including stromal fibroblasts, endothelial cells, and inflammatory cells in the primary and bone cells at the metastatic sites have been shown to exert directive action on prostate cancer cells by modulating reciprocally cancer cell growth, migration, invasion and metastasis. Impairment of reciprocal stromal or bone cell function and their communication with cancer cells could significantly impact the growth and progression of prostate cancer within the tumor microenvironments. *Table 1* summarizes several co-targeting strategies of cancer-associated stroma, either in the primary tumor or in bone metastases that have been implemented in the clinic for improving the mortality and morbidity of prostate cancer patients. Future research on the specific mediators and cell signaling pathways regulating the reciprocal cellular communication between cancer cells and their immediate microenvironments and circulating factors in cancer and microenvironment cell milieu could further significantly improve our ability to target the progression of cancer and its lethal metastatic progression. For example, it has been established that immortalized stromal fibroblasts or cancer-associated fibroblasts (CAF) adjacent to tumors are morphologically and functionally distinct from normal stromal fibroblasts adjacent to normal epithelium (18,31). These cells exhibit marked differences in gene expression profiles and have been shown to predict the progression of prostate cancer (66). We demonstrated the reciprocal cellular interaction between prostate cancer and CAF or stromal fibroblasts from different zonal origin (31). Using marginally androgen responsive tumorigenic LNCaP human prostate cancer cells, we demonstrated that co-culture of the cancer cells with microcarrier beads previously seeded with prostate or bone stromal cells of the human prostate gland or human bone, under 3D culture system, led to permanent nonrandom genetic and phenotypic changes in both the cancer and the stroma. LNCaP cells derived from these growth conditions became androgen-independent and gained the ability to metastasize. Stromal fibroblasts that interact with cancer cells, also gained increased levels of brain derived neurotropic factor (BDNF), chemokines (e.g., CCL5 and CXCL5), versican, tenascin, stromal cell derived factor-1 (SDF-1/CXCL12), and transcription factors like HIF-1α. These were validated using clinical tissue or serum samples obtained from prostate cancer patients with bone metastases. Studies from our group and others have demonstrated the role of stromal soluble factors such as VEGF, bFGF, HGF/SF, TGF-β, IGF-1, IL-6 and KGF, interacting with receptors on prostate cancer cells (refs). These studies highlight the bidirectional interactions and co-evolution of tumor–stroma in cancer progression (67). Therapies that target many of the stromal factors have been tested in preclinical models and in clinical trials to eradicate or delay the lethal progression of prostate cancer and other solid tumors to the metastatic phenotype.

Table 1 Summary of pre-clinical and clinical studies in prostate cancer therapy

Agent	Mechanism of action	Clinical status	References
Abiraterone (anti-testosterone)	17,20 lyase inhibitor	Phase II studies completed	(1,2)
TAK-700 (anti-testosterone)	17,20 lyase inhibitor	Phase III trails ongoing	(3)
MDV3100 (anti-androgen)	Prevents androgen receptor translication	Phase II trails ongoing	(3,6-10)
Cabazitaxel	Cytotoxic anti-microtubule agent	EU approved for CPRC patients	(11,12)
Docetaxel	Cytotoxic anti-microtubule agent	FDA approved for CPRC patients	(12)
OGX-011	antisense compound against clusterin	Phase II clinical trails complete for CRPC patients	(7,13-17)
Bevacizumab	Angiogenesis inhibitor (anti-VEGF antibody)	Phase II clinical trails ongoing for CRPC patients	(36-39)
Aflibercept	Angiogenesis inhibitor	Phase II clinical trails ongoing for CRPC patients	(40)
Cabozantinib	c-Met and VEGFR2 inhibitor	Phase III trails ongoing in bone metastatic patients	(41-43)
Atrasentan	ET-1A inhibitor (Endothelin inhibitor)	Phase II trails ongoing for CRPC patients	(44)
Dasatinib	Src kinase inhibitor	Phase III trails ongoing for CRPC patients	(45-48)
Denosumab	anti-RANK antibody	FDA approved for bone metastatic	(49-52)
Radium-223	alpha-emitter radioisotope	Phase III trails ongoing in bone metastatic patients	(53,54)
Tenascin inhibitors	anti-stromal agent	Clinical trails planned	(55)
Anti-β2-microglobulin antibody	Blocks activity of β2-M growth factor	Preclinical trails completed	(56)
AMD3100, NOX-A12, or CCX2066	anti-CXCL12 agents (targeting the stroma)	Clinical trails planned	(57)
CNTO 888	CCL2 chemokine inhibitor	Phase I clinical trails ongoing	(58,59)
Provenge	Immunotherapy (GM-CSF and PAP loaded DCs)	Approved by FDA for CRPC patients	(60-62)
PROSTVAC-VF	Gene therapy to deliver Poxvirus based PSA expression	Phase III trails ongoing	(63,64)
Ipilimumab (anti-CTLA-4 antibody)	Immunotherapy (checkpoint inhibitor)	Phase I clinical trails completed	(65)

Targeting angiogenesis in prostate cancer

Angiogenesis is essential for the growth and dissemination of prostate cancer cells. The process of blood vessel formation is regulated by complex interactions of vascular growth factors, including VEGF, matrix metalloproteins, and integrins. Inhibition of these proteins that support angiogenesis can block tumor growth as well as inhibit metastasis. Several studies demonstrate that circulating levels of VEGF were increased in patients with CRPC and serve as prognostic markers for patient survival (68). Microvessel density has been found to be increased in patients who have metastatic disease in comparison to those who have clinically localized cancer (36,37). Thus, the tumor vasculature appears to be a rational therapeutic target for men with prostate cancer. Significant work has been undertaken evaluating putative antiangiogenic agents. Early work with thalidomide showed activity as a single agent (38). This work has developed into a series of clinical studies supported by the intramural program of the National Cancer Institute including recent work with a combination of docetaxel, bevacizumab, and thalidomide (39). Bevacizumab, an antibody which blocks the biding of VEGF-A to the VEGF-R, is approved for use in non-small-cell lung and colorectal cancer (69). Other potent anti-angiogenic agents such as sorafenib (Nexavar), sunitinib (Sutent) (70), and aflibercept (VEGF Trap) (40) have shown the potential for benefit in this disease that is still under evaluation.

Targeting tyrosine kinases in prostate cancer

The efficacy of receptor tyrosine kinase inhibitors such as sunitinib or sorafenib has been disappointing in clinical trials for prostate cancer. Unlike other therapies, these agents have been associated with prolonged progression-free survival but no potent anti-tumor effect. A receptor tyrosine kinase inhibitor has a unique clinical phenotype that may potentially translate to therapeutic benefit. Cabozantinib (XL184, Exelixis, San Francisco, CA), is an orally available, multiple tyrosine kinases inhibitor. It inhibits activation of the c-MET protooncogene, as well as VEGFR2. In preclinical animal and cell models, cabozantinib exhibited potent dose-dependent cancer growth inhibition and tumor regression against a variety of solid tumors (41,42). Studies with prostate cancer specimens derived from primary tumors as well as bone, lymph node, and soft tissue metastases reveal that 51% of primary prostate cancer tissues expressed c-MET. In particular, osseous metastates from prostate

cancers have been found to express significantly more c-MET than even soft tissue specimens (41,42). A 9-arm randomized discontinuation trial of cabozantinib which included patients with metastatic CRPC has been reported (43). In the CRPC arm, of the 168 patients enrolled, 100 were evaluable for response by RECIST (Response Evaluation Criteria in Solid Tumors). Fifty-five of the 65 (85%) patients with serial bone scans showed complete or partial resolution of lesion as early as 6 weeks after starting therapy. Cabozantinib continues to be evaluated in prostate cancer as Exelixis has planned two phase III studies with this agent in prostate cancer that should begin in 2012- one evaluating pain response, the other evaluating the survival benefit associated with this agent.

Bone-directed targeting for treating prostate cancer bone metastasis

The endothelins (ET-1, ET-2, and ET-3), consisting of 21 amino acids, are expressed by endothelial cells; kidney and intestine; and brain, respectively. This class of peptides is known to control vasoconstriction, mitogenesis, and bone matrix formation with their actions mediated by ET receptors, ET_A and ET_B. The endothelin receptors are expressed in a variety of human tumors, including prostate cancer and osteoblasts. Interaction of endothelins with their receptors results in enhanced cell proliferation, bone matrix synthesis and deposition, and resistance to apoptosis in prostate cancer (41,44). Atrasentan, a specific $ET-1_A$ inhibitor, exhibits anti-mitogenic activity, anti-osteoblastic activity, decreases rates of bone metastases, anti-angiogenesis activity, and blocks nociceptive effects.

Dasatinib, a tyrosine kinase inhibitor that inhibits the Src-family kinases (SFKs) has been studied in CRPC. SFKs are known to play an important role in bone resorption (71) and appear to be upregulated in advanced prostate cancer (45). Work by our group and others have pointed toward SFKs as regulators of metastatic behavior (46). A phase II study in metastatic CRPC demonstrated that 41% of patients have greater than 50% PSA decline with 35% reduction in bone turnover in 46% of patients (47). Bone alkaline phosphatase levels also were decreased in dasatinib-treated patients. Docetaxel was combined safely with conventional docetaxel therapy (48) showing again, potent effects on bone turnover. Based upon the preliminary data, a randomized phase III trial comparing docetaxel/prednisone in 1500 patients with CRPC, either with or without dasatinib was executed. Results from this study are pending and may be available in 2012.

The receptor activator of nuclear factor-κB (RANK)/

RANKL axis has been shown to play a critical role in maintenance of osteoclast and osteoblast function. Given the imbalance of activities between these cell populations in prostate cancer, RANKL has been considered an attractive target for therapy. This has been borne out in preclinical models of prostate cancer metastasis (49). Denosumab (Xgeva, Prolia; Amgen, Thousand Oaks, CA) is a fully humanized monoclonal antibody that targets RANKL. The FDA initially gave approval for this agent in 2010 for the treatment of osteoporosis related to menopause. Subsequently, denosumab received approval for the treatment of skeletal related events in prostate cancer (50) and other solid tumors (51) and osteoporosis due to hormonal anti-cancer therapies in breast (52) and prostate cancer. Denosumab, targets RANKL axis was shown to delay the onset of bone metastasis and skeletal related events including the relief in the bone pain in men with CRPC who develop bone metastasis. Given the putative impact of RANKL on progression to osseous metastasis, a phase III trial of denosumab was initiated to test the hypothesis that treatment with denosumab would delay the onset of bone metastases in patients who were currently metastasis free (72). This study focused on a population of men at high risk for osseous metastasis (CRPC with serum PSA >8.0 ng/mL and/or PSA doubling time of <10 months). Treatment with denosumab was associated with a 15% reduction in the risk of bone metastasis with a median time to metastasis of 29.5 *vs*. 33.2 months in favor of denosumab.

Radioisotopes, such as strontium-89 (Metastron) and samarium-153-EDTMP (Quadramet), are approved for the palliation of bone pain in men with CRPC (73,74). Radium-223 chloride (Alpharadin; Algeta) is a selective α-emitter that has been evaluated in patients with CRPC. In contrast to the approved isotopes mentioned, improved survival was noted in patients treated with radium-223 when compared to placebo (65.3 *vs*. 46.4 weeks) in a phase II trial (53). As such a formal phase III study (ALSYMPCA) was initiated comparing radium-223 to placebo in men with bone metastases who had previously received docetaxel or were ineligible for docetaxel therapy (54). This trial was closed early by the independent data monitoring committee as criteria for a significant treatment benefit were reached. Treatment with radium-223 was associated with a 30% decrease in prostate cancer related death compared to placebo with median survivals of 14.0 *vs*. 11.2 months in favor of radium-223. Algeta has moved forward with their new drug application with hopes of approval in 2012.

522

Gururajan et al. Future perspectives of prostate cancer therapy

Molecular therapeutics to co-target prostate cancer and cancer-associated bone cells

Cancer-host interactions play a fundamental role in directing cancer plasticity, progression, responsiveness, and resistance to treatments such as hormone therapy, chemotherapy and radiation therapy. We recommend that future development of novel therapies should focus on the cancer-host interactions. This may improve treatment efficacy since the tumor-associated microenvironment may be protective to cancer cells, preventing the regression or apoptosis of treated tumors. Targeting only the cancer cells may not be sufficient since cancer cells and their associated stroma co-evolve. The field of tumor-stroma biology has expanded our understanding of cancer as more than a single cell disease. Rather, cancer development and progression involves reciprocal interaction and co-evolution between cancer cells and host stroma with reactive oxygen species, soluble growth factors, chemokines, cytokines and extracellular matrices serving as the key mediators. We and others have shown that cancer cells and their associated stroma are remarkably plastic and capable of expressing genes mimicking the tumor microenvironment. These new understandings of cancer-stroma interaction raise the possibility of co-targeting not only the cancer cell component but also cancer-associated stroma, and blocking not only autocrine but also paracrine cell signaling. Further expansion of our understanding of tumor-stroma biology could lead to the successful development of more effective animal models to study the mechanisms of prostate cancer metastases. This will be a novel step toward the discovery of more effective therapeutic interventions for prostate cancer metastases through the interruption of cancer-stromal fibroblasts, cancer–bone, cancer-endothelium, cancer-stem cell, cancer-nervous system and cancer-immune system communications (55,66,67).

Targeting the epithelial to mesenchymal transition (EMT) in prostate cancer

EMT is a highly conserved process where polarized immotile epithelial cells transition to motile mesenchymal cells. EMT is commonly associated with cancer migration, invasion and metastasis. The common feature of EMT is the loss of E-cadherin and an increased expression of vimentin and N-cadherin. In cancer, EMT could facilitate cancer aggressive behavior by infiltrating surrounding tissues and metastasize to soft tissues and bone. EMT can be enhanced by the augmentation of specific growth factor/growth factor signaling and hence can be targeted by growth factor receptor signaling or at the level of downstream cadherin-switch such as antibody against N-cadherin to prevent the switch between E-cadherin to N-cadherin (75). In prostate cancer, EMT has been described as a notable feature of the androgen-independent prostate cancer ($ARCaP_E$/$ARCaP_M$, C4-2/C4-2B, and PC-3) cell models and was confirmed in clinical specimens and circulating tumor cells (CTCs) harvested from patients (32,56,66,76,77). RANKL is a potent paracrine factor for osteoclastogenesis and bone resorption. Under physiologic conditions, RANKL, expressed by osteoblasts, stimulates osteoclast maturation and bone resorption through the surface RANK receptor-expressing osteoclasts. We previously demonstrated that mesenchymal metastatic human prostate cancer cells ($ARCaP_M$ cells) express higher levels of functional RANKL, capable of promoting osteoclast maturation (78). Interestingly, RANKL-derived from cancer cells can also promote the transition of $ARCaP_E$ cells, with an epithelial phenotype, to express a mesenchymal phenotype, like those of $ARCaP_M$ cells, thus suggesting autocrine function of RANKL in the induction of EMT. In experimental human xenograft and cell models, RANKL is a biomarker associated with EMT (78). Since RANKL is also expressed by the cells in the tumor microenvironments, such as osteoblasts; B- and T-cells, we observed that RANKL can also promote MET in androgen-sensitive LNCaP cells in both autocrine and paracrine manner, and drive their bone and soft tissue metastases through an activation of downstream c-MET signaling. We confirmed that this action of RANKL in promoting EMT and downstream c-MET is highly relevant in both experimental and human prostate cancer towards their development of CRPC phenotype (79) (and Chu *et al.*, poster presentation at the Cancer-induced bone disease meeting, November 30- December 3, 2011, Chicago, IL, USA). In addition to RANKL, prostate cancer cell lines and clinical samples are shown to secrete soluble factors such as β2-microglobulin (β2-M). This protein is not only responsible for driving EMT and bone metastasis of human prostate cancer cells but also in human breast, renal and lung cancer cells. The resulting $ARCaP_M$ cells had high levels of the mesenchymal markers such as vimentin, N-cadherin and Snail and exhibit 100% incidence of bone metastasis in an intracardiac injection model. β2-M interacts with its receptor, hemochromatosis (HFE) protein, to modulate iron responsive pathways in cancer cells. Inhibition of either β2-M or HFE results in reversion of

EMT (56,80,81). These results demonstrate the role of β2-M in cancer metastasis and lethality. Thus, β2-M and its downstream signaling pathways are promising prognostic markers of cancer metastases and novel therapeutic targets for cancer therapy. Preclinical studies in both immune-compromised and immune-intact mouse models of prostate cancer revealed anti-β2-M monoclonal antibody significantly reduced tumor burden of primary tumors and bone metastasis ((56) and unpublished data). Currently, humanization of anti-β2-M is underway with the goal of initiating phase I clinical trials in prostate cancer patients with bone metastases. As such we propose that the addition to β2-M-targeted therapy to RANKL inhibition may be an effective way to treat skeletal metastasis in human prostate cancer.

Targeting immune microenvironment of prostate cancer

Impairment of immune cell function in the cancer microenvironment is believed to be an important step in tumor progression. It is hypothesized that co-targeting of immune cells in addition to cancer cells will lead to better killing of cancer cells. Recent studies highlight the tumor-promoting role of myeloid and lymphoid cells in the progression of solid tumors, linking inflammation and cancer (82-91). Though studies from the last century reported that mononuclear cells infiltrate solid tumors, it took several years to establish that such cells are causally involved in tumor progression. This became possible due to the discovery, phenotypic and functional characterization of a variety of subsets of T cells, B cells, macrophages and dendritic cells facilitated by discovery of novel markers and use of cutting edge technologies including flow cytometry.

Most of the human solid cancers develop in immune intact human beings. The progression of tumors from low-grade, localized disease to metastasis involves an interaction between the tumor cells and the host immune system. Most of our studies performed with human prostate cancer cell lines in laboratories use immune-deficient athymic nude mice (which lack T cells), SCID mice (lacking B and T cells) or NOD-SCID mice (lacking B, T and NK cells). These immune-deficient mice have allowed human prostate cancer xenografts to grow, greatly facilitating pre-clinical studies of targeted cancer therapies. Given the recent evidence that a vast majority of solid tumors are infiltrated by immune cells that facilitate tumor growth (rather than suppressing the tumor growth), it is imperative to understand the biology of these immune cells in the context of the tumor microenvironment.

Role of T lymphocytes in prostate cancer

Evidence supports a close link between inflammation and prostate cancer have come from epidemiological studies which indicate that prostate cancer is more common in populations with more baseline inflammation (92). Both CD4+ and CD8+ T cells are present in prostate glands. CD4+ T cells include both T helper 17 (T_H17) and regulatory T (T_{Reg}) cell populations. Prostate-infiltrating CD8+ T cells in humans are non-functional and do not upregulate activation markers. T cells surrounding cancer cells may upregulate negative inhibitory molecules that suppress their anti-tumor activity (93). T cells may become anergic or undergo apoptosis due to reactive oxygen species generated by cancer cells. Though T cells surround prostate cancer, increasing evidence suggests that either they exhibit suppressive properties (Tregs) or they become non-functional (CD8+ T cell), thus allowing prostate cancer to grow. Overall, research from human and mouse models supports a model where evolving tumors generate T cells with an anti-cancer potential but, in the absence of some intervention, such T cells exist in a non-functional or anergic state (86,94,95).

Role of macrophages in prostate cancer

Tumor associated macrophages (TAM) influence diverse processes such as angiogenesis, tumor cell proliferation, and metastasis during tumor progression and thus play a pro-tumorigenic role (96). TAMs have been shown to play a key role in tumor growth and spread. Macrophages secrete growth factors, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and transforming growth factor (TGF)-beta, as well as cytokines such as TNF-alpha and IL-1 that have been shown to promote metastatic spread in several animal models of tumors. In a variety of tumor types including prostate cancer, the amount of TAM has been associated with poor prognosis (97). One of the mechanisms involved in TAM-enhancement of cancer cell invasion involves a paracrine loop in which epidermal growth factor (EGF) produced by TAMs increases the invasiveness and migration of neighbouring breast cancer cells that express the EGF receptor (EGFR). Cancer cells in turn express CSF1, which acts as a potent chemoattractant

524

Gururajan et al. Future perspectives of prostate cancer therapy

and chemokine for CSF1R-expressing TAMs. This reciprocal cross-talk can be blocked by either EGFR or CSF1R antagonists, resulting in a decrease in migration and invasion of both cancer cells and macrophages (98).

Myeloid-derived suppressor cells

Another bone marrow-derived myeloid cell type (BMDC), which may share a common progenitor with TAMs, is the Myeloid-derived suppressor cells (MDSCs). MDSCs suppress the adaptive immune response by blocking the functions of $CD4^+$ and $CD8^+$ T cells, in part through arginase and nitric oxide production, by expanding the regulatory T cell pool, and by inhibiting NK cell activation (99). MDSC levels are increased in the bone marrow, blood and spleen of cancer patients and tumor-bearing mice, and their accumulation is associated with tumor growth and malignant progression. Disruption of transforming growth factor-β (TGF-β) signalling, through *Tgfbr2* deletion, was also shown to increase MDSC homing to tumors in a spontaneous mammary cancer model, an effect that was mediated through the SDF1-CXCR4 and CXCL5-CXCR2 (also known as IL8RB) chemokine axes (100-102). In prostate cancer, MDSCs are recruited to the bone and at the primary sites (103). Clinical trials are being planned to target CXCL12 chemokine in CRPC (57). Another cytokine secreted by prostate cancer cells which recruit myeloid suppressor cells include CCL2 against which blocking antibodies are being tested for its therapeutic utility in solid tumors (58,59).

Mesenchymal stem cells

Another cell type that resides predominantly in the bone marrow, although is not of haematopoietic origin, is the mesenchymal stem cells (MSCs). MSCs are multipotent cells that differentiate into osteoblasts, chondrocytes and adipocytes. MSCs are found in large numbers in primary tumors and, MSCs have been proposed as a cellular vehicle to deliver anti-cancer drugs into the tumor.

Immunotherapy for prostate cancer: Current success and future challenges

Recently, the FDA approved the first immunotherapy-based approach (sipulucel-T, Provenge; Dendreon Inc) for treat patients with asymptomatic metastatic prostate cancer (60-62). In general, immunotherapy approaches seek to utilize the host immune cells to attack the underlying cancer. Despite a long history of negative clinical trials, in a definitive trial powered for overall survival, sipulecel-T was associated with a 23% decrease in prostate cancer mortality despite the absence of alternation of progression free survival. This finding has created significant interest in this advancing area of cancer research. Due to a variety of mechanisms by which cancer cells evade immune surveillance, cancer therapy has for years centered on chemotherapy. These toxic chemicals are designed to be more lethal to the rapidly dividing cancer cells than on normal tissue. Unfortunately, normal cells often are killed along with the malignant cells. Professor Ralph Steinman of Rockefeller University, a leading mind in cancer immunotherapy, identified the dentritic cell- a unique and important part of the immune cascade. For this Prof. Steinman was posthumously awarded the 2011 Nobel Prize in Medicine (63,104-107). The dendritic cell is one of the initial workhorses or sentinels of the immune system, processing foreign materials such as viruses and then presenting them to cytotoxic T cells that are activated in turn to attack the foreign antigens. Dr. Steinman isolated his dendritic cells, exposed them to his pancreatic cancer cells, and thus instructed his T cells to recognize those tumor antigens. The prognosis for the type of pancreatic cancer Dr. Steinman is only 4 months but Dr. Steinman survived for more than 4 years since he was first diagnosed with the disease (107). This immunotherapy regimen while not curative may have prolonged his life.

Prostate cancer immunotherapy seems promising and extends the mean survival of metastatic patients by an average of 4 months. Sipuleucel-T (Provenge), the first immunotherapeutic agent approved for the treatment of CRPC, is a dendritic-cell vaccine that is produced *ex vivo* from dendritic cells harvested from the patient in the clinic, which then are transported to a local GLP facility where the dendritic cells are loaded with a recombinant granulocyte macrophage colony-stimulating factor/prostatic acid-phosphatase fusion protein (61). These *in vitro* activated cells are reinfused into a patient. Side effects are modest, including fatigue, fevers, and chills at the time of infusion. Three randomized phase III trials comparing sipuleucel-T to placebo have been performed in patients with metastatic CRPC. In all three studies, those patients who were randomized to the placebo arm received a frozen dendritic-cell product at progression. Although the primary end point of progression-free survival was not met in either of the first two randomized trials, a survival benefit of 3-4 months was

observed. The third randomized trial evaluating sipuleucel-T, involved patients (n=512) randomly assigned on a 2:1 basis to receive sipuleucel-T or placebo. A median survival benefit of 4 months was observed in favor of the patients receiving sipuleucel-T. At 3 years after study entry, 32% of patients treated with sipuleucel-T are alive compared to 23% of patients treated with placebo (60,62,64,108).

Vaccine based therapies are being currently under trials in CRPC. Two randomized trials using the allogeneic vaccine G-VAX viral vectors have failed to demonstrate a survival benefit (60,64,65,108). These viral vectors can mimic natural infection and, thus, boost the immune response. The viral vectors of poxvirus family have been used to deliver tumor (PSA) antigens as well as other immunomodulatory factors. A clinical trial was performed in 125 asymptomatic minimal CRPC patients who received either PROSTVAC-VF (Bavarian Nordic, Kvistgaard, Denmark) or control viral vectors. Although progression-free survival was similar in both groups, patients treated with PROSTVAC-VF had an 8.5-month improvement in median survival (24.1 *vs.* 16.6 months in control patients) (109). A randomized phase III trial is underway evaluating the role of this vaccine in asymptomatic CRPC (64).

The clinical trials of sipuleucel-T demonstrated a statistically significant and clinically meaningful improvement in overall survival in patients with mCRPC (64). However, none of these studies showed a concomitant improvement in progression-free survival. When traditional cytotoxic therapies are evaluated, progression-free survival is considered as a critical endpoint to assess the efficacy of therapy. This apparent disconnect between *progression-free survival* and *overall survival* while comparing immunotherapy versus conventional therapy can be explained in many ways. Unlike chemotherapy drugs, the primary target of immunotherapy based drugs is not the tumor itself but the immune system which targets the tumor. It may take few weeks to few months to mount a clinically significant immune response following immunotherapy. However, a vaccine or immunotherapy induces what is called long-lived memory cells which persist in the human body in the lymphoid tissues for years with the potential to continuously generate cytotoxic T cells to act against tumors, resulting in a slowing of the tumor growth. This process is well documented in vaccines that target infectious diseases. For example, vaccines against pox viruses confer lifelong immunity due to persistence of memory B cells and memory T cells. In a tumor, there is a turnover rate of tumor cells which is influenced by tumor cell division,

antitumor immune response, combined with factors introduced into the tumor environment (e.g., conventional therapies). An effective anti-tumor immune response may alter the tumor growth equilibrium so that more tumor cells are killed by the immune system. This effect takes time and may not translate into immediate goals of short-term (within 3-4 months) improvements in progression-free survival, but may be long-lasting and overall survival may ultimately follow. Additional approaches to measure intermediate endpoints are the need of the hour to measure the efficacy of immunotherapy-based drugs.

Checkpoint inhibitors

Cytotoxic T-lymphocyte antigen 4 (CTLA4) is a cell-surface receptor expressed on the surface of helper T cells and interaction of CTLA-4 with its ligand downregulate T-cell responses. Ipilimumab is a fully human monoclonal antibody whichattenuates negative signals provided to T cells through the cell surface molecule CTLA-4, thereby blocking a negative checkpoint. Blocking the negative checkpoint leads to activation of T cells which would then kill cancer cells. This antibody has been evaluated in patients with metastatic melanoma and demonstrated an improvement in survival of 4 months. Ipilimumab may be very effective in CRPC. In contrast to vaccine or dendritic-cell based therapy, decline in PSA levels have been observed with this antibody therapy (110). Recent phase III clinical trial data demonstrate that ipilimumab prolongs survival in patients with melanoma, and 2 phase III overall survival trials are investigating the activity of ipilimumab in patients with mCRPC (111-114). The first trial combines a single 8 Gy dose of radiation with either ipilimumab or placebo in the post-docetaxel space, and the second evaluates the activity of ipilimumab versus placebo in chemotherapy-naïve patients.

Combination therapies

The standard of care for men with mCRPC includes docetaxel and prednisone. It is time we consider combinations of chemotherapy and immunotherapy as well. Frequent administration of doses of docetaxel in combination with immunotherapy may be a rational approach. However, studies that combines vaccine with higher doses of docetaxel (chemotherapy) and prednisone (anti-inflammatory drug) leads to immunosuppression wherein immune-cells are depleted by this approach. One
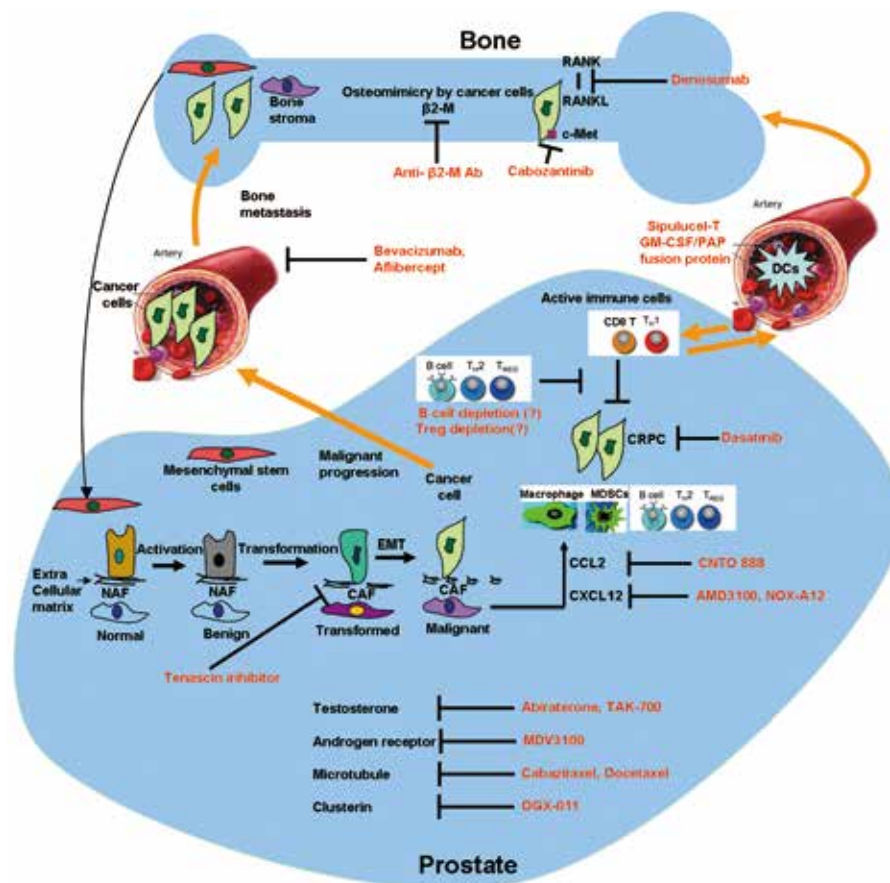
Figure 1 Diagrammatic illustration of therapies that target primary prostate tumor cells, CRPC, cells (stromal/endothelial/immune cells) and soluble factors in tumor microenvironment of the prostate and the bone.

way to overcome this problem might be to administer immunotherapy first followed by chemotherapy to avoid the immunosuppressive effects of chemotherapy. This approach will also facilitate a proinflammatory microenvironment in which tumor-cell killing by chemotherapy can be boosted by cytotoxic T cell mediated tumor killing. Although overall survival has been the only endpoint to demonstrate clinical benefit in clinical trials of vaccine in prostate cancer, it is possible that combination studies of therapeutic vaccines with other modalities may lead to earlier discriminatory endpoints, such as time to progression or PSA response, which could accelerate clinical trials for improved personalized oncology. It is imperative to consider cancer vaccines or immunotherapeutic approaches at the earlier stages of disease in prostate cancer and it can also be considered as an ideal adjuvant therapy post-surgery or -radiation in which presumably the bulk of the tumors have been removed and a smaller cluster of tumor cells may

reside at the metastatic niche.

Summary and Conclusions

We have summarized the current approved treatments, ongoing clinical trials and preclinical studies in *Table 1* and *Figure 1*. Prostate cancer patients with metastatic disease are treated with androgen ablation therapy. These patients respond efficiently with improvement in bone pain, regression of soft-tissue metastases, and decreases in serum PSA levels. After a period of two years, nearly all patients progress to the castrate-resistant state. Until 2004, these patients were treated for symptoms with chemotherapeutic agents, such as mitoxantrone combined with prednisone, as well as isotope therapy or external-beam radiation therapy for painful bone metastases. Two new agents were approved by the FDA in 2010, cabazitaxel (chemotherapy) and sipuleucel-T (immunotherapy), with

abiraterone approved in 2011 all further boost the choice of drugs to treat this deadly bone disseminating disease (1,6-8). The primary endpoints for these drugs are vastly different as we discussed in detail earlier. The need of the hour is to research on exploration of novel biological markers to determine the appropriate drug to use in a given situation. Clearly, future studies, and eventually clinical practice, will need to incorporate newer imaging methods to track cancer cells, biological markers in blood, bone marrow, and circulating tumor cells, to determine the treatment efficacy of individual agent, or combination of hormonal agents, chemotherapy, immunotherapy and/or radiation therapy.

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Footnote

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528

Gururajan et al. Future perspectives of prostate cancer therapy

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530

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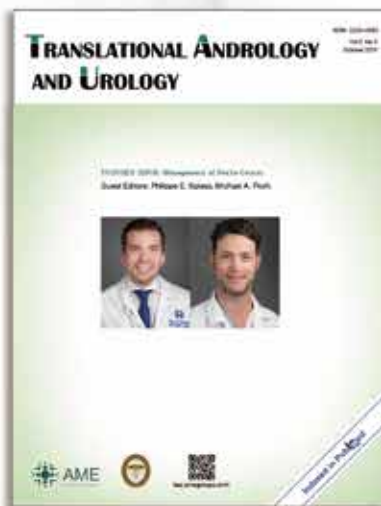
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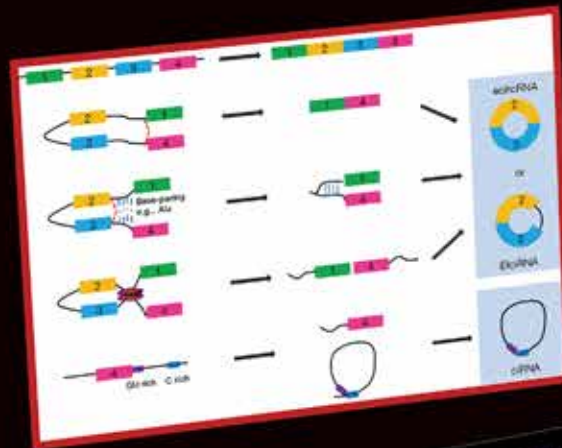
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