

PROSTATE CANCER



Basic Mechanisms and
Therapeutic Approaches

Chawnshang Chang *Editor*



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Editor

Chawnshang Chang

George H. Whipple Professor
University of Rochester, USA

 **World Scientific**

NEW JERSEY • LONDON • SINGAPORE • BEIJING • SHANGHAI • HONG KONG • TAIPEI • CHENNAI

Published by

World Scientific Publishing Co. Pte. Ltd.

5 Toh Tuck Link, Singapore 596224

USA office: 27 Warren Street, Suite 401-402, Hackensack, NJ 07601

UK office: 57 Shelton Street, Covent Garden, London WC2H 9HE

Library of Congress Cataloging-in-Publication Data

Prostate cancer : basic mechanisms and therapeutic approaches / edited by Chawnschang Chang.
p. cm.

Includes bibliographical references and index.

ISBN 981-256-067-X (alk. paper)

1. Prostate--Cancer. I. Chang, Chawnschang, 1955-

RC280.P7P75835 2005

616.99'463--dc22

2004062902

British Library Cataloguing-in-Publication Data

A catalogue record for this book is available from the British Library.

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Typeset by Stallion Press

Email: enquiries@stallionpress.com

Printed in Singapore.

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LIST OF CONTRIBUTORS AND AFFILIATIONS

Bo-Ying Bao, *Departments of Pathology & Laboratory Medicine and Urology, University of Rochester Medical Center, Rochester, New York*

Elie A. Benaim, *Department of Urology, UT Southwestern Medical Center, Dallas, TX*

Ralph A. Brasacchio, *Department of Radiation Oncology and JP Wilmot Cancer Center, University of Rochester Medical Center, Rochester, New York*

Chawnshang Chang, *George H. Whipple Lab for Cancer Research, Departments of Urology, Pathology, Radiation Oncology, and the Cancer Center, University of Rochester, Rochester, New York*

Eugene Chang, *Departments of Urology and Pathology, University of Rochester Medical Center, Rochester, New York*

Philip Chang, *George H. Whipple Lab for Cancer Research, Department of Urology, Pathology, Radiation Oncology, and the Cancer Center, University of Rochester, Rochester, New York*

Ching-Shih Chen, *Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus, Ohio*

Hong Chen, *Department of Urology, UT Southwestern Medical Center, Dallas, TX*

Kuen-Feng Chen, *Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus, Ohio*

Ming Chen, *Departments of Urology and Pathology, University of Rochester Medical Center, Rochester, New York*

Siu-Ju Chen, *Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE*

Anand Chokkalingam, *Celera Diagnostics, LLC, Alameda, California*

Sonal J. Desai, *Department of Biological Chemistry and Cancer Center, University of California at Davis, Sacramento, CA*

Noahiro Fujimoto, *Department of Urology, University of Occupational and Environmental Health, Iseigaoka, Yahatanishiku, Kitakyushu, Japan*

Thomas A. Gardner, *Urology Research Laboratory, Departments of Urology, Microbiology and Immunology, Walther Oncology Center, Indiana University Medical Center, Indianapolis, Indiana*

Kelley M. Harsch, *The Cleveland Clinic Foundation, The Lerner Research Institute, Cleveland, Ohio*

Warren D.W. Heston, *The Cleveland Clinic Foundation, The Lerner Research Institute, Cleveland, Ohio*

Jer-Tsong Hsieh, *Department of Urology, UT Southwestern Medical Center, Dallas, TX*

Ann W. Hsing, *Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, Maryland*

Yueh-Chiang Hu, *George H. Whipple Lab for Cancer Research, Departments of Urology, Pathology, Radiation Oncology, and the Cancer Center, University of Rochester, Rochester, New York*

Juan Antonio Jiménez, *Urology Research Laboratory, Departments of Urology, Microbiology and Immunology, Walther Oncology Center, Indiana University Medical Center, Indianapolis, Indiana*

Chaeyong Jung, *Urology Research Laboratory, Departments of Urology, Microbiology and Immunology, Walther Oncology Center, Indiana University Medical Center, Indianapolis, Indiana*

Chinghai Kao, *Urology Research Laboratory, Departments of Urology, Microbiology, and Immunology, Walther Oncology Center, Indiana University Medical Center, Indianapolis, Indiana*

Jose A. Karam, *Department of Urology, UT Southwestern Medical Center, Dallas, TX*

Ani Khodavirdi, *Department of Pathology, Keck School of Medicine, University of Southern California, Los Angeles, California*

Samuel K. Kulp, *Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus, Ohio*

Hsing-Jien Kung, *Department of Biological Chemistry and Cancer Center, University of California at Davis, Sacramento, CA*

Sang-Jin Lee, *Urology Research Laboratory, Departments of Urology, Microbiology and Immunology, Walther Oncology Center, Indiana University Medical Center, Indianapolis, Indiana*

Yi-Fen Lee, *Departments of Pathology & Laboratory Medicine and Urology, University of Rochester Medical Center, Rochester, New York*

Fen-Fen Lin, *Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE*

Ming-Fong Lin, *Department of Biochemistry and Molecular Biology, Department of Surgery/Urology, College of Medicine and Eppley Institute for Cancer Research, University of Nebraska Medical Center, Omaha, NE*

Hiroshi Miyamoto, *Departments of Pathology & Laboratory Medicine and Urology, University of Rochester Medical Center, Rochester, New York*

Peter S. Nelson, *Fred Hutchinson Cancer Research Center, Seattle, Washington*

Jing Ni, *Departments of Urology and Pathology, University of Rochester Medical Center, Rochester, New York*

Rey-Chen Pong, *Department of Urology, UT Southwestern Medical Center, Dallas, TX*

Pradip Roy-Burman, *Department of Pathology and Department of Biochemistry and Molecular Biology, Keck School of Medicine, University of Southern California, Los Angeles, California*

Jun Shimazaki, *Department of Urology, Graduate School of Medicine, Chiba University, Chiba, Japan*

Jason E. Tasch, *The Cleveland Clinic Foundation, The Lerner Research Institute, Cleveland, Ohio*

Huei-Ju Ting, *Departments of Pathology & Laboratory Medicine and Urology, University of Rochester Medical Center, Rochester, New York*

Clifford G. Tepper, *Department of Biological Chemistry and Cancer Center, University of California at Davis, Sacramento, CA*

Suresh Veeramani, *Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE*

Xingqing Wen, *Departments of Urology and Pathology, University of Rochester Medical Center, Rochester, New York*

Chun-Te Wu, *George H. Whipple Lab for Cancer Research, Departments of Urology, Pathology, Radiation Oncology, and the Cancer Center, University of Rochester, Rochester, New York*

Hong Wu, *Department of Molecular and Medical Pharmacology, University of California, Los Angeles School of Medicine, Los Angeles, California*

Qingquan Xu, *George H. Whipple Lab for Cancer Research, Departments of Urology, Pathology, Radiation Oncology, and the Cancer Center, University of Rochester, Rochester, New York*

Zhiming Yang, *George H. Whipple Lab for Cancer Research, Departments of Urology, Pathology, Radiation Oncology, and the Cancer Center, University of Rochester, Rochester, New York*

Shuyuan Yeh, *Departments of Urology and Pathology, University of Rochester Medical Center, Rochester, New York*

Yi Yin, *Departments of Urology and Pathology, University of Rochester Medical Center, Rochester, New York*

Ta-Chun Yuan, *Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE*

Min Zhang, *Departments of Urology and Pathology, University of Rochester Medical Center, Rochester, New York*

PREFACE

Prostate cancer is the second leading cause of death in men. Charles Huggins first found that metastatic prostate cancer responds to androgen-ablation therapy, which heralded the beginning of a new era of prostate cancer therapy. Later, Andrew Schally and others showed that advanced prostate cancer responded to the LHRH agonist as decreased serum testosterone level to 25% and marked reduction in cancer-associated bone pain. The discovery of androgen receptor (AR) led to the screening of chemical libraries for AR blockers. Since then, antiandrogens, including flutamide and casodex, have been in continual use as therapeutic agents. Yet, with either androgen ablation via surgical or medical castration, with or without additional combination of various antiandrogens, eventually most of, if not all, prostate cancers still progress into the Hormone Refractory stage and the detailed reasons for this remain unclear. Cloning of the AR, generation of AR antibodies, finding of AR coregulators and their applications to prostate cancer progression reveals the essential roles of AR in the prostate cancer progression and opened a new approach for AR ablation therapy by targeting the AR, instead of androgens, to battle the prostate cancer.

Chapters 1 to 6 discuss current effective hormonal therapy, immunotherapy, chemotherapy, radiation therapy, and gene therapy, as well as androgen ablation therapy. Chapters 7 to 15 discuss the recent advances in the field of study of the basic mechanisms behind the growth of prostate cancer and how some of these mechanisms can be used to treat prostate cancer. Chapters 16 and 17 include recent research in the study of prostate cancer in newly developed mouse model systems. Many of these studies have distinct potential advantages as they lead toward advances in the clinical treatments of and drug therapies for these androgen-related diseases. We feel our book should be of interest as both

a study guide and research reference for students, basic scientists, and clinicians.

I would like to dedicate this book to my PhD advisor Dr. Liao, whose research philosophy and Taiwanese dignity deeply influences my continued academic career. I would also like to thank Drs. Carbone, Wilding, Messing, Lardy, and Gorski for their help in establishing my independent academic research career at the University of Wisconsin and the University of Rochester. Finally, I thank my copyeditors, Mrs. Karen Wolf and Dr. Loretta Collins, for their invaluable editorial and proofreading assistance.

Chawnsang Chang, PhD

University of Rochester, New York, USA

EDITOR — CHAWNSHANG CHANG, PH.D.

Chawnschang Chang was born and raised in Taiwan. In 1997 he became the George Hoyt Whipple Distinguished Professor at the University of Rochester in Rochester, New York.

He received his B.S. from the National Taiwan University and Ph.D. from the University of Chicago in 1985. In 1990, at the University of Wisconsin-Madison he began his independent research career and was promoted to full professor in 1996.



Dr. Chang has published more than 250 peer-reviewed articles related to AR and TR2/TR4 orphan receptors. His landmark discovery, the first cloning of the androgen receptor (AR) cDNAs (*Science*, 240: 324–326, 1988), provided the framework for the subsequent studies of basic androgen mechanisms and their clinical applications. Several androgen-related diseases, such as prostate cancer, Testicular Feminization Syndrome, and Kennedy's Neuron Disease were linked to mutations of the AR. In recent years several new androgens or anti-androgens were developed based on the targets of the AR.

In 1996, Dr. Chang's Lab isolated the first AR coregulator and within a few years identified and isolated another 10 AR coregulators (*Endocrine Review*, 23: 155–200, 2000), which modulate AR functions, and enable AR to cross-talk to many signal transduction pathways. Generation of the first floxed AR mouse (*PNAS*, 2002) further provided the first tissue-specific AR knockout mouse, as well as the first female mouse without functional AR. These mice allow the *in vivo* study of androgen actions in selective tissues, including prostate, testis, liver, muscle, bone, and the female breast and ovary.

Many Universities have presented Dr. Chang with various awards and honorary professorships. His generosity is well known in the academic community and Taiwanese society and more than 700 labs throughout the world have benefited by collaborations using his AR reagents.

1

HORMONAL THERAPY FOR PROSTATE CANCER: CLINICAL AND EXPERIMENTAL EVIDENCE

Hiroshi Miyamoto and Chawnshang Chang

*Departments of Pathology & Laboratory Medicine and Urology
University of Rochester Medical Center
Rochester, New York, USA*

Introduction

The role and mechanism of androgen function have been studied in a variety of androgen target organs, including the prostate. As is the case with normal prostate development, the growth of prostatic neoplasms is generally dependent on androgens, especially on 5 α -dihydrotestosterone (DHT). Since 1941 when Huggins and Hodges¹ published their Nobel Prize-winning study on the effects of hormone manipulation in patients with metastatic prostate cancer (PCa), hormonal therapy remains the critical therapeutic option for advanced disease. Multiple strategies have been used to reduce serum levels of androgens or interfere with their function via the androgen receptor (AR) (Fig. 1). However, considerable uncertainty remains as to the appropriate choice/timing and actual benefits of hormonal therapy in various situations. Indeed, PCa is still the second leading cause of cancer-related death among men in the United States.² In this chapter, we systematically review clinical and experimental evidence supporting current strategies of hormonal therapy in PCa.

The AR and Androgens

The AR, a member of the nuclear receptor superfamily, functions as a ligand-inducible transcription factor that regulates the expression of target

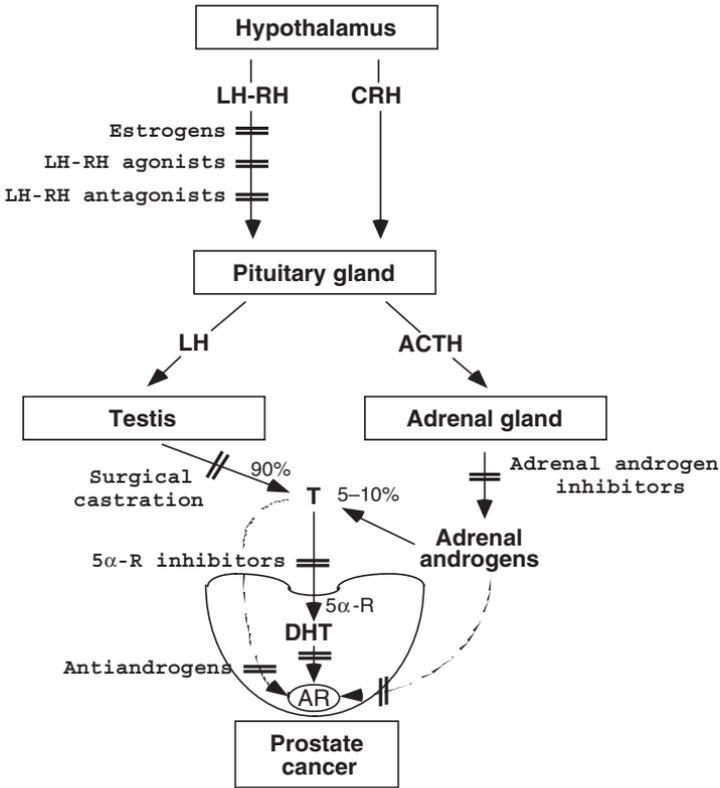


Fig. 1. Strategies for hormonal therapy. LH-RH=Luteinizing hormone-releasing hormone; CRH=corticotropin-releasing hormone; LH=luteinizing hormone; ACTH=adrenocorticotropic hormone; T=testosterone; 5 α -R=5 α -reductase; DHT=5 α -dihydrotestosterone; AR=androgen receptor.

genes in response to ligands in target cells.^{3,4} Recent studies have also revealed that the AR modulates transcription by recruitment of coregulators that influence a number of functional properties of the receptor, including ligand selectivity and DNA binding capacity (reviewed in Ref. 5).

Testosterone is secreted by Leydig cells in the testis and is the major sex hormone circulating within the blood of males. In a variety of androgen-sensitive tissues like the prostate, testosterone is irreversibly converted by 5 α -reductases to the more potent androgen, DHT.^{4,6} Upon binding of androgens, the androgen-AR complexes form homodimers, and they translocate into the nucleus and bind to androgen responsive elements

located on target genes, such as prostate-specific antigen (PSA), which is clinically used for the detection and monitoring of PCa recurrence and progression. Besides testosterone and DHT, several precursors of testosterone mainly secreted by adrenal glands, dehydroepiandrosterone (DHEA), DHEA sulfate, Δ^4 -androstenedione, and Δ^5 -androstenediol, can also stimulate the AR through their conversion to testosterone/DHT in peripheral tissues, including the prostate, or by directly binding to the AR.⁷⁻¹⁰

Strategies of Androgen Deprivation

Multiple approaches at androgen deprivation have been used for the treatment of PCa (Fig. 1). The agents and strategies used for androgen deprivation therapies are listed in Table 1.

Surgical Castration

Surgical castration by bilateral orchiectomy is the most immediate method to reduce circulating testosterone by >90% within 24 hours,¹¹ and there is no risk of a paradoxical flare of the disease. Since the 1960s, the Veterans Administration Cooperative Urological Research Group (VACURG) trials, the earliest large-scale randomized studies of hormonal therapy, demonstrated the clinical effectiveness of surgical castration.^{12,13} Compared to placebo, orchiectomy retarded cancer progression in advanced cases, but no clear survival advantage for castration over placebo was seen. Recent clinical studies (i.e. surgical vs. chemical castration) are discussed later. Although surgical castration may be underused, some studies suggest that many patients prefer this approach for the reasons of convenience and cost.¹⁴ On the other hand, other studies suggest that this treatment approach is unacceptable to many patients, causing considerable psychological problems, with irreversible impairment in libido and erectile function in most cases.^{15,16}

Medical Castration

Diethylstilbestrol (DES)

In the 1940s, the first reversible medical castration method was achieved by administration of DES, a semi-synthetic estrogen compound.¹ The

Table 1. Treatment Options as Hormonal Therapy for Prostate Cancer

Modality	Methodology	Mechanism/Action	Advantages	Disadvantages
Surgical castration	Bilateral orchiectomy	Orchiectomy, ↓T	Rapid ablation of testicular T Relatively simple procedure, lower cost	Definitive castration Associated psychological problems Irreversible loss of libido/sexual potency Reduced muscle mass/energy Hot flashes Anemia/osteoporosis Unaffected adrenal androgens
Medical castration	Estrogens (DES)	Suppresses LH-RH secretion, ↓LH, ↓T Direct effect via ER (?)	Reversible castration	Cardiovascular events (estrogens) Flare phenomenon (LH-RH agonists) Reduced muscle mass/energy
	LH-RH agonists (Leuprolide, Goserelin)	Suppresses LH-RH secretion, ↓LH, ↓T	Ablation of testicular T More acceptable than orchiectomy	Loss of libido/sexual potency Hot flashes Anemia/osteoporosis Unaffected adrenal androgens
	LH-RH antagonists (Abarelix)	Antagonizes LH-RH receptor, ↓LH, ↓T		
CAB	Castration + antiandrogen	Ablation of testicular T + competitive inhibition of adrenal androgens	More effective (?)	Increased side effects Antiandrogen withdrawal response

Table 1. (Continued)

Modality	Methodology	Mechanism/Action	Advantages	Disadvantages
Antiandrogen monotherapy	Non-steroidal antiandrogens (Flutamide, Nilutamide, Bicalutamide)	Antagonizes AR in target tissues, ↑T	Competitive inhibition of testicular/adrenal androgens Retaining sexual potency Less severe side effects Oral administration only	Gynecomastia Less effective (?)
	Steroidal antiandrogens	Antagonizes AR in target tissues,	CAB effect Oral administration only	Cardiovascular events Side effects due to lowering of serum T Gynecomastia
	(CPA, Megestrol acetate)	Suppresses LH-RH secretion, LH, ↓T		
IAB	Intermittent hormonal therapy		Longer androgen-sensitive period(?) Reduced side effects/costs	Investigational May achieve continuous androgen ablation
TrAB	Intermittent CAB + 5 α -R inhibitor (Finasteride, Benzoquinoline)		Superior to IAB or CAB (?)	Investigational
SAB	5 α -R inhibitor + antiandrogen or LH-RH agonist		Superior to monotherapy (?)	Investigational

T = Testosterone; DES = diethylstilbestrol; LH-RH = luteinizing hormone-releasing hormone; LH = luteinizing hormone; ER = estrogen receptor; CAB = combined androgen blockade; AR = androgen receptor; CPA = cyproterone acetate; IAB = intermittent androgen blockade; TrAB = triple androgen blockade; 5 α -R = 5 α -reductase; SAB = sequential androgen blockade.

VACURG studies identified equivalent overall survival rate in the DES group (5 mg/day) to the orchiectomy group, but non-cancer-related deaths, most of which were cardiovascular events, were noted.^{12,13} Subsequent trials have shown that DES at 3 mg/day is equivalent to other treatment options in overall survival rates.^{17–21} However, cardiovascular toxicity with events including myocardial infarction, deep vein thrombosis, edema, and transient ischemic attack was observed in 8%–33% of patients. Gynecomastia was also significantly seen in patients with 3 mg/day DES. A low-dose of DES (1 mg/day) was also evaluated,^{13,22} but whether DES at 1 mg/day is as effective and safe as other treatment options is still controversial. After the development of luteinizing hormone-releasing hormone (LH-RH) agonists, with fewer cardiovascular events and no resulting gynecomastia, DES is now only rarely used as a first-line hormonal treatment in North America. Instead, several studies have evaluated the efficacy of DES as a salvage therapy after failure of first-line androgen deprivation. Recent studies, using 1–3 mg/day DES with or without anti-thrombotic agents, including warfarin and aspirin,^{23,24} identified response rates by PSA measurement to be 43%–79% with median durations of progression of 6–7.5 months and with 2.8%–28% cardiovascular events.

It was generally believed that the primary mechanism of action of DES was to decrease androgen levels through hypothalamic-pituitary suppression, but recent evidence indicates that the mechanism is probably more complex. Kitahara *et al.* reported stronger suppression of testosterone by DES than by surgical castration or other means of chemical castration, such as the administration of a LH-RH agonist.²⁵ The same group also suggested that DES might reduce serum DHEA sulfate.²⁶ A direct cytotoxic effect of estrogens has also been suggested in PCa *in vitro*, presumably through both estrogen receptor (ER)-dependent and ER-independent pathways.^{27–29} This is consistent with the finding that phytoestrogens, which have steroidal structures similar to estrogens and are found in a variety of plant foods, inhibit PCa cell proliferation.⁴⁹ Indeed, ER β has been detected in human PCa cell lines, including LNCaP, PC-3 and DU145, and in normal and malignant prostate tissues, whereas ER α is expressed in PC-3 cells and in stromal (not epithelial) cells of the prostate.^{30–32} Furthermore, it is suggested that loss of ER β in PCa tissues is associated with tumor progression.^{32,33} These findings might be able to

explain the evidence that administration of DES could be more effective than other androgen ablation therapies in suppressing PCa growth if unfavorable side effects of DES are not considered.^{12,13} On the other hand, we previously showed that a natural estrogen, 17 β -estradiol, but not DES, increased AR transcriptional activity in PCa cells.³⁴

LH-RH Agonists and Antagonists

The introduction of LH-RH analogues, obtaining medical castration, has led to a dramatic change in the treatment of advanced PCa.³⁵ In the United States, two LH-RH agonists are commercially available: leuprolide acetate and goserelin acetate.

LH-RH is generally secreted by the hypothalamus in pulses, leading to pulsatile secretion of LH by the pituitary. This in turn promotes testosterone secretion by the Leydig cells of the testes. However, constantly high levels of LH-RH that occur with agonist administration down-regulate the receptors in the pituitary, inhibit LH secretion, and thereby reduce testosterone production. In addition, some studies have suggested a direct inhibitory effect of LH-RH via LH-RH receptors in PCa cells.^{36,37}

Several randomized studies showed the equivalent effectiveness between surgical castration and LH-RH agonist administration.^{38,39} Recently, depot LH-RH agonist preparations have been developed, which last 3 to 4 months and have the same efficacy as classical preparations.⁴⁰ Thus, the depot preparations have now become the most widely used form of androgen deprivation. Side effects of LH-RH agonists include hot flashes, reduced libido, and osteoporosis.⁴¹ In addition, LH-RH agonists often cause an initial surge of LH release, with a corresponding increase in serum testosterone and DHT lasting 1 to 2 weeks. This surge may stimulate PCa growth with a worsening of related symptoms, which is known as the flare phenomenon.⁴² Therefore, administration of an antiandrogen or estrogen for a week before and during the first few weeks of LH-RH agonist therapy is often used in an attempt to limit the clinical sequelae caused by this hormonal surge.^{42,43}

LH-RH receptor antagonists recently have been developed for androgen deprivation.⁴⁴ Since abarelix, the first peptide antagonist, directly blocks the binding of LH-RH to its receptor without agonist activity, there is no initial flare phenomenon as occurs with LH-RH agonists.^{44,45} Recent

clinical studies have demonstrated that abarelix monotherapy achieves medical castration and a reduction of serum PSA levels to the same extent achieved by LH-RH agonists.^{46–48} However, long-term follow-up studies are necessary to determine whether LH-RH antagonists can be routinely used for advanced prostate cancer.

Combined Androgen Blockade (CAB)

Monotherapy with surgical or medical castration results in marginal or no decline of adrenal androgens that not only can be converted to testosterone/DHT but are likely to possess intrinsic androgenic activity.^{9,10,49} Thus, men who undergo castration still have relatively high levels (up to 40%) of DHT and 5%–10% of testosterone.^{7,50} The basis of CAB (also called maximal androgen blockade) is concomitant neutralization of both testicular and adrenal sources of androgens. CAB consists of treatment with a LH-RH agonist or surgical castration combined with a non-steroidal antiandrogen. Antiandrogens include a number of compounds that interfere with the binding of androgens to the AR in the target cell, which ultimately prevents the activation of AR pathways in those cells. CAB has been advocated as the most effective hormonal treatment for patients with advanced PCa. However, this approach implies increased side effects and cost, and there are few supportive data showing a meaningful improvement in survival associated with the addition of antiandrogen.^{51,52}

Several early, randomized trials demonstrated a significant survival advantage of CAB in patients with advanced PCa, compared to castration alone.^{53–56} In 1998, however, Eisenberger *et al.*⁵⁷ reported a trial of 1387 patients with metastatic PCa who were randomized to surgical castration and placebo vs. flutamide. There were no differences in progression-free or overall survival between the two arms. Several factors were hypothesized to explain the discrepancy between the results of this study and earlier reports. First, patients in this study might have had less aggressive disease. Second, castration with a LH-RH agonist, especially a daily regimen of leuprolide injections in the first study,⁵³ might not have been as complete as surgical castration. Third, the LH-RH agonist plus placebo group may have experienced initial flare leading to worsening the disease. In 2000, the Prostate Cancer Trialists' Collaborative Group⁵² published a meta-analysis

of 27 trials of CAB vs. monotherapy involving 8275 patients with advanced PCa. The difference in the 5-year survival rate was not statistically significant (25.4% with CAB vs. 23.6% with castration alone). However, a statistically significant difference ($p < 0.02$) in favor of castration plus a non-steroidal antiandrogen was observed. More recently, another meta-analysis of 20 randomized trials concluded that there was a 5% improvement in 5-year survival (30% vs. 25%) with CAB.⁵⁸ However, only 7 of the 20 studies might be considered as high-quality trials and no significant improvement with CAB was seen in the meta-analysis of these 7 studies. In summary, recent data show that CAB provides a minimal advantage (up to 5% improvement in 5-year survival) over castration monotherapy. It is generally recommended to use an antiandrogen before and during the first several weeks of LH-RH agonist therapy to prevent possible symptoms of the flare. With these data, prolonged treatment beyond 1 month with CAB may not be the first choice of hormonal therapy for advanced PCa.

Antiandrogen Monotherapy

There are two types of antiandrogens, steroidal, such as cyproterone acetate (CPA) and megestrol acetate, and non-steroidal, such as flutamide, nilutamide, and bicalutamide. As noted, antiandrogens are generally used in conjunction with castration as CAB. However, castration based approaches are usually associated with side effects, which have a negative impact on quality of life (QOL). Monotherapy with a (non-steroidal) antiandrogen is becoming an increasingly attractive alternative therapeutic approach. Most of non-steroidal antiandrogens increase within normal physiological range the serum levels of androgens due to the suppression of the pituitary feed-back. Thus, this means of androgen blockade can preserve gonadal function and therefore provide potential QOL benefits, particularly in terms of retained potency and libido, no muscle weakness, and less bone demineralization.

Flutamide

Flutamide was the first non-steroidal antiandrogen that was widely used as a component of CAB. However, the use of flutamide monotherapy for advanced PCa has not been extensively studied in phase III trials.⁵⁹ Initial open studies assessing the clinical efficacy of flutamide as monotherapy

were reviewed by Delaere and Van Thillo.⁶⁰ Among approximately 500 previously untreated patients with advanced PCa, 68% achieved at least a partial response. But most studies were relatively small, and there seemed to be differences in the criteria of response. Several trials have compared the efficacy of flutamide as monotherapy with that of DES, orchiectomy, or CAB. Boccardo reviewed these studies and found no significant differences in response rates/duration among these groups.⁵² In a double-blind randomized study to compare the efficacy of flutamide with 3 mg/day DES,²¹ however, DES produced significantly longer overall survival than flutamide (43.2 months vs. 28.5 months). Because some adverse effects, such as hepatotoxicity, were noted, the rate of treatment withdrawal for any drug-related adverse events was highest with flutamide among 3 non-steroidal antiandrogens.⁵⁹ There have been no comparative studies of the efficacy of different non-steroidal antiandrogens as monotherapy.

Nilutamide

No randomized studies of monotherapy with nilutamide or comparative studies with any other hormonal therapy have been reported. One small study (26 patients) evaluated the efficacy of nilutamide as monotherapy, demonstrating that 21 (91%) of the 23 evaluable previously untreated patients with metastatic PCa had a response, with a median overall survival of 23 months.⁶¹ The survival rate in this study might be less than that achieved by CAB with nilutamide.⁶² In addition, nilutamide was associated with a high incidence (31%) of visual problems (light-dark adaptation disorders).⁶¹ Other unique adverse effects of nilutamide, when used as either monotherapy or a component of CAB, include alcohol intolerance and interstitial pneumonitis.^{61,62} Nilutamide has been reported to cause a higher incidence of nausea and vomiting than the other non-steroidal antiandrogens, whereas the incidence of diarrhea and gynecomastia is lower with nilutamide than flutamide.^{59,62} These results may discourage conducting larger trials with nilutamide monotherapy.

Bicalutamide

Of available non-steroidal antiandrogens, bicalutamide as monotherapy has been most extensively studied. In early comparative trials using

bicalutamide at 50 mg/day, castration was shown to be superior to bicalutamide monotherapy, in terms of survival rate in patients with metastatic disease.⁶³ However, subsequent trials with bicalutamide at 100 or 150 mg/day have revealed equivalent efficacy between bicalutamide monotherapy and surgical or medical castration.^{52,64,65} Other comparative studies also showed no statistically significant differences in survival between bicalutamide at 150 mg/day monotherapy and CAB (castration with flutamide or nilutamide) with better tolerability in the bicalutamide monotherapy group.^{66,67} Bicalutamide at 150 mg/day has been shown to have a more favorable side effect profile than flutamide and nilutamide,⁵⁹ although there was still a high risk of gynecomastia and breast pain. Since bicalutamide has a longer elimination half-life of approximately 6 days than flutamide (6 hours) or nilutamide (56 hours), it can be given once daily vs. flutamide (or nilutamide in many studies) dosed 3 times daily.^{62,68,69} The most recent and largest trials involving 8113 patients confirmed these observations (clinical efficacy, QOL benefit, and tolerability).⁷⁰ Thus, bicalutamide at 150 mg/day is thought to be an appropriate dosage, and this treatment, either alone, referred to as peripheral androgen blockade, or as adjuvant therapy, could be a standard option in patients with localized or locally advanced PCa.

CPA

CPA, a progestational antiandrogen, was the first antiandrogen used for the treatment of advanced PCa in Europe. It acts as an AR antagonist, as well as causes partial suppression of pituitary gonadotropins, which results in a rapid and sustained 70% decrease in testosterone levels.⁷¹ Therefore, CPA, as a single agent, may yield CAB. In clinical studies, there were no significant differences in tumor response rates or disease specific survival between CPA and any other forms of androgen deprivation, such as surgical castration, estrogens, LH-RH agonists, and non-steroidal antiandrogens.^{59,72} Unfortunately, CPA has been reported to induce severe cardiovascular complications in about 10% of patients, although the rate is lower than those of DES (up to 33%).¹⁸ Other complications include gynecomastia, loss of libido, erectile dysfunction, and central nervous system effects such as headache, fatigue, and weakness

that are possibly attributable to the lowering of serum testosterone levels. Therefore, the use of CPA as monotherapy might be limited to those who find surgical castration unacceptable. In addition, CPA can be used to block LH-RH induced flare reactions and to suppress surgical or medical castration-related hot flashes.^{71,72}

Neoadjuvant/Adjuvant Hormonal Therapy with Radical Prostatectomy

Neoadjuvant Hormonal Therapy

Radical prostatectomy is a treatment modality which can offer the possibility of PCa cure if surgical margins are negative. However, surgical attempts for a cure in patients with apparently localized PCa often fail because the cancer is incompletely resected possibly due to clinical understaging before the surgery or micrometastases existing at the time of surgery. The theoretical purposes of neoadjuvant treatment are to lower the pathological stage, reduce the likelihood of positive margins, eliminate micrometastases, and ultimately increase patient survival.

Laboratory experiments using the Shionogi tumor model support this rationale.⁷³ Pathologically positive surgical margins were detected in 66% of mice undergoing wide tumor excision (group 1) and in 33% of mice treated with neoadjuvant castration 10 days before wide excision of progressed tumor (group 2). Subsequent androgen-independent tumor recurrences were seen in 92% of group 1 and in 44% of group 2. There were statistically significant differences in overall tumor-free survival rates (group 1: 20% vs. group 2: 56%, $p < 0.05$).

Several prospective randomized trials have been performed to investigate the significance of neoadjuvant androgen deprivation for 3 months before radical prostatectomy.⁷⁴⁻⁷⁷ Most studies demonstrated a significant reduction in prostate volume and margin-positive rates in the patient groups with neoadjuvant androgen deprivation. Unfortunately, these studies failed to show a significant improvement in seminal vesicle invasion, lymph node involvement, or PSA recurrence. None showed an advantage of neoadjuvant treatment in overall survival. Possible reasons for this discrepancy include an insufficient duration of neoadjuvant hormonal therapy. Gleave *et al.*⁷⁸ observed 547 patients who were randomized to

receive neoadjuvant CAB for 3 or 8 months prior to radical prostatectomy. Positive margin rates were significantly lower in the 8-month than 3-month group (12% vs. 23%, $p = 0.0106$), and the authors concluded that the optimal duration of neoadjuvant androgen deprivation is longer than 3 months. However, rates of local or biochemical recurrence and long-term survival were not reported in this study. In addition, an 8-month delay of surgery might carry a high risk for patients with androgen-independent tumor. Neoadjuvant hormonal therapy should therefore remain under investigation.

Adjuvant Hormonal Therapy

There are a few retrospective studies showing a significantly positive effect of adjuvant hormonal therapy following radical prostatectomy on disease-free survival.^{79,80} In a large retrospective, non-randomized series from the Mayo Clinic, continuous hormonal therapy prolonged overall survival in patients with nodal metastases who underwent radical prostatectomy. However, in earlier analyses, the benefits of this treatment were seen only in men with DNA diploid cancers.⁸⁰ Zincke *et al.* also retrospectively reviewed 707 patients with stage pT3b disease, including 157 patients who received adjuvant hormonal therapy, and found that adjuvant hormonal therapy significantly improved the mean 10-year survival rates.⁸¹ The Eastern Cooperative Oncology Group (ECOG),⁸² in a prospective randomized clinical trial, investigated the effect of adjuvant hormonal therapy in 98 patients with clinically localized PCa and lymph node metastases. Androgen deprivation (goserelin or surgical castration) was initiated within 12 weeks of radical prostatectomy and pelvic lymphadenectomy in the adjuvant group, whereas, in the observation group, androgen deprivation was delayed until disease progression (almost always initiated at diagnosis of metastases). After 7.1 years of median follow-up, immediate treatment was associated with significant advantages in overall (85% vs. 64%; $p = 0.02$) and cause-specific (93% vs. 68%; $p = 0,001$) survival rates. The ECOG study has been criticized because of its relatively small number of patients and lack of central pathological review to determine Gleason grades.⁸³ However, a recent reanalysis of Gleason grades by central pathology review reveals no

significant changes in outcomes, including survival.⁸⁴ With mean follow-up of 10 years highly significant differences in overall (72% vs. 49%; $p = 0.025$) and cause-specific (87% vs. 57%; $p = 0.001$) survival rates were observed.⁸⁴ Adjuvant therapy with antiandrogen, such as flutamide⁸⁵ or bicalutamide,⁷⁰ has also been reported to reduce biochemical recurrence in a broad spectrum of post-prostatectomy patients. However, these studies are too premature to evaluate survival or other meaningful outcomes.

Hormonal Therapy with Radiation Therapy/ Brachytherapy/ Chemotherapy

Radiation Therapy

Zietman *et al.*⁸⁶ demonstrated that prior androgen deprivation enhanced the effect of radiation on eradicating androgen-sensitive Shionogi mouse mammary tumors. An additive effect of androgen deprivation and radiation on apoptosis was also observed in both Dunning rat prostate tumors and LNCaP human PCa cells.^{87,88} Also a recent study using a xenograft model demonstrated a synergistic inhibitory effect of castration and radiotherapy.⁸⁹ LNCaP-bearing mice treated with castration prior to radiation had significantly decreased mean tumor volume and serum PSA levels, compared to those treated with castration or radiation alone, throughout the observation period up to 11 weeks after initiation of treatment. Interestingly, in an androgen-sensitive Dunning rat prostate tumor model, testosterone treatment after castration and radiotherapy failed to stimulate tumor growth, suggesting cancer cells lost their androgen sensitivity through irradiation.^{90,91} Moreover, in this model, castration 14 days prior to radiation was found to be superior in suppressing tumor growth, compared to androgen deprivation alone, radiation alone, or androgen deprivation 3 days after radiation.⁹¹

Three prospective studies revealed statistically significant improvements in overall survival in favor of early hormonal therapy in the radiotherapy setting. The European Organization for Research and Treatment of Cancer Genitourinary Group conducted a randomized phase III trial comparing external irradiation alone with combined therapy, with concomitant plus adjuvant androgen deprivation plus radiation, in locally

advanced PCa patients.⁹² From 1987 to 1995, 415 patients with WHO grade 3, stage T1-2 cancers, or stage T3-4 tumors of any grade were randomized to (1) radiotherapy plus goserelin, starting on the first day of irradiation and continuing monthly for 3 years (CPA was also given during the first month to prevent flare phenomena), *vs.* (2) radiotherapy alone followed by the same hormonal therapy upon clinical progression. With median follow-up of 66 months, 5-year clinical disease-free survival was 74% in the early hormonal therapy group and 40% in the control group ($p = 0.0001$), and 5-year overall survival was 78% and 62%, respectively ($p = 0.0002$).⁹² Five-year local disease control was particularly impressive (although biopsies were not done), with 98% in the combined treatment group *vs.* 74% in the control arm being clinically free of local recurrence.

The Radiation Therapy Oncology Group (RTOG) has conducted several large, prospective randomized trials to assess the potential benefit of early *vs.* late and of short-term *vs.* long-term hormonal therapy in PCa patients treated with radiotherapy. In the RTOG protocol 85-31, 977 patients with T1-2 N1 or T3 non-metastatic disease, including post-prostatectomy cases, were randomized to receive goserelin starting at the last week of radiotherapy, and continuing indefinitely, or radiotherapy with deferred androgen deprivation at relapse. While initial publication of results at a median follow-up of 4.5 years reported that immediate goserelin treatment significantly improved local and distant disease control as well as disease-free survival (all $p < 0.0001$), there was no difference in overall survival.⁹³ However, a recent update of data at a 7.3-year mean follow-up demonstrated significant improvement in overall survival with estimated 10-year survivals being 53% and 38% in the immediate and deferred treatment groups, respectively.⁹⁴

A parallel trial (RTOG 86-10) was performed to evaluate the efficacy of short-term hormonal therapy in PCa patients receiving definitive radiation therapy.⁹⁵ A total of 456 patients with T2-4 tumors were randomized to receive CAB with goserelin and flutamide for 4 months (2 months before and 2 months during radiotherapy) with radiotherapy *vs.* radiotherapy alone, with salvage hormonal therapy with orchiectomy, LH-RH agonist, or antiandrogen to be initiated when clinically indicated for relapse or progression of disease. At median follow-up of 6.7 years, early hormonal therapy was associated with a significant improvement in local and

distant disease control and disease-free survival. Fewer patients in the combination arm (45%) received salvage hormonal therapy than those in the control arm (63%) ($p < 0.001$). However, no significant differences between the two arms were apparent for either overall survival at 5 years (71% vs. 69%) or at 8 years (53% vs. 43%).

Horwitz *et al.*⁹⁶ compared the above two studies and concluded that statistically significant improvements in biochemical disease-free status, distant metastases failure, and cause-specific failure rates were observed for adjuvant long-term hormonal therapy compared with short-term adjuvant hormonal therapy or radiotherapy alone in patients with locally advanced non-metastatic PCa.

Hanks *et al.*⁹⁷ reported the results of another randomized RTOG study (protocol 92-02) comparing short-term and long-term hormonal therapy involving 1554 men with T2c-4 disease and a PSA < 150 ng/ml who received goserelin and flutamide 2 months before and 2 months during radiotherapy plus either no further therapy or 24 months of additional goserelin alone. With median follow-up of 4.8 years, long-term androgen deprivation led to significantly improved local ($p = 0.0001$) and distant ($p = 0.001$) disease control and a trend in longer disease-free survival (92% vs. 87%, $p = 0.07$). However, there was no significant difference in 5-year overall survival between the two arms (78% vs. 79%). A subset analysis comparing the results from centrally reviewed Gleason scores 8–10 patients from the RTOG 85-31 also showed a statistically significant advantage in patients receiving long-term androgen deprivation in estimated 5-year overall survival (80% vs. 69%; $p = 0.02$) and disease-free survival (90% vs. 78%; $p = 0.007$) rates.

Brachytherapy

Brachytherapy is increasingly used in patients with localized, low- to intermediate-grade PCa. Neoadjuvant androgen deprivation therapy is commonly given to patients who have a large prostate, to downsize the prostate, making the brachytherapy procedure easier and more feasible. Indeed, it has been reported that prostate volume was reduced by up to 40% after 3 months of androgen deprivation therapy.⁷⁸ Thus, combining hormonal therapy with prostate brachytherapy may reduce brachytherapy-related

morbidity and improve patient outcome. However, no additional benefits of adjuvant hormonal therapy over the prostate brachytherapy on survival were apparent.⁹⁸ Because no prospective, randomized studies have been published, the impact of hormonal therapy in conjunction with brachytherapy remains unclear.

Chemotherapy

Previous studies have established the role of chemotherapy in the palliation of symptoms in patients with PCa after failure of hormonal therapy,^{99,100} although its clear survival benefit is not reported. Among a variety of drugs, mitoxantrone- and estramustine-based regimens have been extensively studied and shown to have a significant palliative benefit.¹⁰⁰ Estramustine has an estradiol moiety and has been used in PCa patients for several decades. Estramustine, as a single agent, decreases serum testosterone to castration levels, with significant cardiovascular toxicity. Combination regimens of chemotherapy with other hormonal therapies have also been investigated for locally advanced, presumably androgen-sensitive, PCa. In a study by Pettaway *et al.*,¹⁰¹ 33 high-risk patients (either clinical stage T3 or Gleason score >7) were treated with ketoconazole, doxorubicin, vinblastine and estramustine plus a LH-RH agonist and an antiandrogen for 12 weeks before radical prostatectomy. Thirty-three percent of them were found to have prostate-confined disease at the time of surgery. In another multicenter study, 50 locally advanced patients were treated with paclitaxel, estramustine, and carboplatin plus a LH-RH agonist for 4–6 months. Of the 23 patients who underwent radical prostatectomy, 45% of them attained organ-confined disease.¹⁰⁰ There were no comparisons of combination regimen to hormonal therapy alone.

Several *in vitro* studies investigated combinations of chemotherapy and hormonal therapy. Kreis *et al.*¹⁰² showed synergistic effects on growth inhibition of either androgen-sensitive LNCaP, androgen-insensitive DU145 and PC-3, or all cell lines, using combinations of estramustine plus flutamide or PSC833 (Sandoz) plus bicalutamide. Other studies demonstrated that androgen deprivation could trigger apoptosis of androgen-sensitive cancer cells via a transient increase in cytosolic calcium, resulting in activation of Ca²⁺- and Mg²⁺-dependent endonucleases.^{103,104}

Therefore, chemotherapy may become more effective when combined with androgen deprivation. In contrast, androgen deprivation was also shown to promote androgen-dependent cells to enter the G₀ phase of the cell cycle instead of undergoing apoptosis.¹⁰⁵ Therefore, these cells might be more difficult to eradicate with subsequent chemotherapy.

Intermittent Androgen Deprivation

Intermittent androgen blockade (IAB) aims at delaying the onset of androgen-independent growth of PCa, as well as reducing side effects and costs. Laboratory studies have supported the hypothesis that IAB prolongs the initial androgen-sensitive period. Langelier *et al.*¹⁰⁶ showed that intermittent androgen suppression could delay the emergence of androgen-independent clones induced in LNCaP after long-term culture with androgen deprivation. Akakura *et al.*¹⁰⁷ and Sato *et al.*¹⁰⁸ studied IAB in castrated animals bearing androgen-dependent tumors treated with intermittent exposure to androgens. The results suggest that IAB induces multiple apoptotic regressions of androgen-dependent PCa and prolongs the time to androgen-independent progression, compared to continuous androgen deprivation.

The first attempt at IAB was reported in 1986.¹⁰⁹ Twenty patients with advanced PCa were treated with intermittent hormonal therapy (DES in 19 cases and flutamide in one case) until subjective improvement was noted, with a mean initial treatment duration of 10 months (range 2–70 months). The therapy was then stopped, and re-started when tumors relapsed, with a mean interval time of 8 months (range 1–24 months). All relapsed patients responded to re-administration of the drug. Patients had better QOL during the break in the treatment and DES-induced erectile dysfunction was reversed in 9 of 10 patients within 3 months of treatment interruption.

The availability of agents that induce reversible medical castration, such as LH-RH agonists, and serial serum PSA measurements after the mid-1980s, made it easier to introduce IAB and to monitor disease activity. Several clinical studies of IAB have been reported.^{110–113} These intermittent hormonal therapies consist of an initial androgen deprivation period using a LH-RH agonist with or without a non-steroidal antiandrogen of usually between 6 and 9 months, followed by an off-therapy interval

(6–15 months). When PSA values meet threshold criteria (>5 – 10 ng/ml), treatment is resumed. Most of the initial responders (57%–100%) respond to re-treatment. This cyclic treatment continues until the patient develops androgen-independent tumors. While off-treatment, many patients had improvement in libido, erection, and energy, as well as fewer hot flashes. However, retrospective comparison of survival in these patients was similar to those who were treated with continuous androgen blockade. Interestingly, in certain patients, especially in those who received androgen deprivation for longer periods, gonadal function and serum testosterone levels did not recover.¹¹⁴ These findings suggest that intermittent administration of LH-RH agonists may achieve continuous androgen deprivation, resulting in reduction of cost. A recent study also showed that the median duration of castration levels of serum testosterone was 5.5 months (range 3.5–10 months) after a single injection of long-acting (3-month) depot LH-RH agonist and that the method of re-dosing LH-RH agonists based on serum testosterone levels appeared efficacious, safe, and cost-effective.¹¹⁵

The debate continues as to whether IAB improves survival. Large, randomized, phase III clinical trials, comparing intermittent *vs.* continuous androgen deprivation are currently ongoing to assess endpoints including survival, time to androgen-independent progression, and QOL. Furthermore, intermittent triple androgen blockade (TrAB), another form of IAB using a 5α -reductase inhibitor, finasteride, during off-treatment periods, is also being evaluated.¹¹⁶

5 α -Reductase Inhibitors

Two 5α -reductase enzymes have been identified: type 1, the predominant enzyme in extraprostatic tissues, such as skin and liver; and type 2, predominantly expressed in the prostate.⁶ The type 2 5α -reductase has been implicated in, at least partially, the regulation of early prostate growth as well as later hyperplastic growth. Therefore, finasteride, the first 5α -reductase inhibitor specific for the type 2 enzyme, which significantly decreases levels of both serum and intraprostatic DHT by 70%–80%, reduces the total size of the prostate gland.¹¹⁷ Thus, finasteride treatment has been a useful form of androgen deprivation for benign prostatic hyperplasia (BPH), with

fewer adverse effects than antiandrogen treatment. However, the therapeutic activity of finasteride itself on PCa has not been identified. The effect of finasteride in conjunction with other forms of hormonal therapy has been investigated. In addition to TrAB,¹¹⁶ sequential androgen blockade (SAB), a combination therapy with finasteride plus an antiandrogen or an LH-RH agonist, has been evaluated and has been shown to substantially decrease the PSA levels in men with metastatic PCa while maintaining sexual potency in most patients.^{118,119} However, phase III studies, comparing SAB with traditional hormonal therapy, such as CAB, have not been conducted and the survival benefit thus remains unknown.

The benzoquinoline, LY320236, is a newer and dual (type 1/2) 5α -reductase inhibitor currently in phase I trials of PCa.¹²⁰ The antitumor activity of benzoquinoline has been demonstrated in human PCa xenograft models.

Concluding Remarks

Many options involving the AR, androgens, and their antagonists are available for the treatment of PCa (Fig. 1). Numerous clinical studies have shown equivalent effects on therapeutic benefits by different hormonal treatment strategies. Each treatment strategy/hormonal agent has favorable and unfavorable effects (Table 1). Patients with advanced PCa will clearly benefit from androgen deprivation-based treatments for symptom palliation and improvement of their QOL. However, whether these therapies prolong survival when administered before there are symptoms caused by disease progression remains controversial. Thus, despite a number of previous clinical and experimental studies, finding suitable patients, timing of, and options for hormonal therapy remain problematic.¹²¹ Data from recent studies support the premise that an earlier treatment in patients' disease course likely leads to better outcomes,^{82,84,92} but it is not easy to predict the best timing of hormonal therapy for patients with asymptomatic advanced disease. Observation may still be a reasonable choice for these patients.

Currently, available options for hormonal therapy almost never lead to cures in patients with advanced PCa because these patients eventually develop androgen-independent tumors. In addition, another type of failure

of hormonal therapy, antiandrogen withdrawal syndrome, has been observed in a significant number (15%–80%) of patients treated with CAB. Although the exact mechanisms for androgen-independent PCa and antiandrogen withdrawal syndrome are far from being fully understood, possible mechanisms were discussed in recent review papers.^{122,123} To improve overall survival of patients with advanced PCa, novel treatment strategies that prolong the androgen-dependent state, but will not induce antiandrogen withdrawal syndrome, and that are effective against androgen-independent disease, need to be identified. Furthermore, it may be necessary to explore more individualized approaches, such as selectively blocking the activated AR pathway in cancer cells. Finally, second-line hormonal therapy and PCa chemoprevention using hormonal therapy are other interesting topics that are not discussed in this chapter (please refer to Chapter 6 in this volume).

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2

IMMUNOTHERAPIES FOR PROSTATE CANCER

Kelley M. Harsch, Jason E. Tasch and Warren D. W. Heston

*The Cleveland Clinic Foundation, The Lerner Research Institute
Cleveland, Ohio 44195, USA*

Introduction

Prostate cancer is the second leading cause of death in men, with approximately 220,000 new cases and an expected 28,000 deaths in the year 2003.¹ A decrease in prostate cancer related deaths has been attributed to early prostate-specific antigen (PSA) detection, more effective chemotherapy treatments, and immunotherapies. Although tumors can often evade an immune response by modulating their tumor antigens, reducing major histocompatibility complex-1 (MHC-I) expression or inhibiting cytotoxic T-cell activity, the use of immune modulation for prostate cancer is a relatively new concept because the prostate is not generally considered a site where immune processes typically occur. Since tumors arise when cancer cells evade the immune system, the prostate is an ideal target for immunotherapy.²

The four most common types of lesions associated with the prostate are acute/chronic prostatitis (bacterial/abacterial), proliferative inflammatory atrophy (PIA), benign prostatic hyperplasia (BPH), and prostate carcinoma.³ The types of proliferative lesions that occur in the prostate are in different regions of the prostate. Most hyperplasias are prevalent in the transitional and periurethral zones, whereas carcinomas are found mostly in the peripheral zone.⁴

PIA, a newly recognized prostate lesion, is hypothesized to be a precursor to prostatic intraepithelial neoplasia (PIN) and to prostate cancer.

PIA lesions, which contain proliferating epithelial cells that fail to fully differentiate into columnar secretory cells, are typically present in the peripheral zone of the prostate, where prostate cancers arise, and are often directly juxtaposed to PIN and/or prostate cancers.⁵⁻⁹ PIA may link inflammation with prostatic carcinogenesis.⁶

Inflammation

Virtually 100% of prostate specimens contain histological and immunological evidence of chronic inflammation.¹⁰ Inflammation is a physiological response to a variety of stimuli such as infection, tissue injury, growth factors, or chemokines.^{2,11} The distribution, location and histology of leukocytes determine the type of inflammation.¹² Persistent immune activation resulting in chronic inflammation often has pathological consequences. Acute/chronic inflammation is characterized by distinct cellular changes whereas precancerous lesions are associated with the change in the balance in the angiogenic and apoptotic cell cycle process.¹¹

During the inflammation process, activated macrophages release various hydrolytic enzymes and reactive oxygen and nitrogen species that may contribute to tissue damage. Chemokines, plasma enzyme mediators (bradykinin, fibrinopeptidases), opsonins, leukotrienes, and prostaglandins are all mediators that have some role in the inflammatory process.² The normal prostate is populated by $\alpha\beta$ T-cells, B-cells and macrophages, with the T-cells being evenly distributed throughout the interstitium and between the epithelial cells.^{13,14} There is some indication that the number of T-cells increases with age,¹² which correlates with the incidence of prostate inflammation during the aging process.

The proliferation and differentiation of prostate tissue are modulated by growth factors^{15,16} as well as hormonal androgen therapy.^{17,18} Evidence of hormonal impact on the prostate is seen in atrophy of the prostate following castration,⁴ as well as treatment of BPH samples with a 5α -reductase inhibitor. When this inhibitor is given to patients, there is a regression of dihydrotestosterone (DHT) levels and a reduction in prostate volume.^{19,20}

Recent data suggests that mutations in RNase L predispose men to an increased incidence of prostate cancer, which in some cases reflect more aggressive disease and/or decreased age of onset compared with

non-RNase L linked cases. It is proposed that RNase L functions in counteracting prostate cancer by virtue of its ability to degrade RNA, thus initiating a cellular stress response that leads to apoptosis.²¹ RNase L is a uniquely regulated endoribonuclease that requires 5'-triphosphorylated, 2',5'-linked oligoadenylates (2-5A) for its activity. The presence of both germline mutations in RNase L segregating with disease within HPC-affected families, and loss of heterozygosity (LOH) in tumor tissues suggest a novel role in the pathogenesis of prostate cancer. The association of mutations in RNase L with prostate cancer cases further suggests a relationship between innate immunity and tumor suppression.

Microbial activators may contribute to acute/chronic inflammatory processes, which could then lead to malignancy.¹¹ Differentiating between acute/chronic bacterial prostatitis and chronic abacterial prostatitis is done by quantification of bacterial cultures and microscopic examination of urine.⁴ Depending on the severity and duration of the inflammation, acute bacterial prostatitis displays histological evidence of stromal leukocytic infiltration accompanied by increased elaboration of prostatic secretion or leukocytic infiltration within the glandular spaces.⁴

In contrast, bacterial/abacterial chronic prostatitis shows histological evidence of aggregation of numerous lymphocytes, plasma cells, macrophages, and neutrophils within the prostatic substance. Chemokines act as chemoattractants, which activate lymphocytes and other immune modulators into neighboring tissues via extravasation.² Since antibodies penetrate the prostate with poor efficiency, this type of inflammation is difficult to treat.⁴ The normal aging process in the prostate results in aggregations of lymphocytes, which are prone to appear in the fibromuscular stroma of the gland. Frequently, this histology of the aging prostate is diagnosed as chronic prostatitis even though the macrophages and neutrophils are absent.⁴

Stromal-epithelial interactions are crucial for normal growth and homeostasis within the prostate.²² These interactions are thought to influence the rate of development of BPH and prostate carcinoma.²² BPH is characterized by diffuse infiltrates of activated T-lymphocytes in fibroblastic, fibromuscular, and stromal nodules.^{14,23} Histological evidence of nodular hyperplasia or BPH is present in 20% of men 40 years of age, 70% by age 60, and 90% in men 70 years of age.²⁴ The usual BPH nodule

weighs between 60–100 grams with some nodules weighing over 200 grams.⁴ Studies indicate that BPH samples also display chronic mononuclear inflammation, which contain CD3⁺ T-lymphocytes and express the T-cell receptor.²⁵ The epithelial cells associated with the inflammatory infiltrate were observed in the periglandular stroma²⁵ and were almost exclusively activated T-cells expressing CD45RO, and producing IL-2 and IFN γ .^{3,24} Expression of IFN- γ , IL-2, and IL-4 mRNA in BPH suggests that the disease is associated with Th₁ and Th₂ response.²⁶

The Immune System

The goal for cancer immunotherapy is to induce antibody and/or T-lymphocyte immune response targeted to the cancer cells. There are several branches of the immune system that can be targets for immunotherapy. They include antibody producing B-cells, CD8⁺ cytotoxic T-cells, CD4⁺ T-helper cells, natural killer (NK) cells, natural killer T (NKT)-cells, and monocytes. B-cells produce antibodies that kill antigen presenting cells via complement, antibody dependent cellular cytotoxicity (ADCC), or apoptosis.²⁷ Cell mediated response appears to play a major role in a tumor immune response. Many tumors induce a specific cytotoxic T-cell response that recognizes antigens presented by MHC-I, which can elicit a higher response.²

NKT-cells share several features with NK cells, such as CD161 and CD122 expression. These cells display intermediate levels of T-cell receptor (TCR) and are CD4⁺ or CD4⁻/CD8⁻. NKT-cells produce IL-4, a pro-inflammatory cytokine, in response to engagement of the T-cell receptor.²⁸ In the presence of IL-18 and IL-12, NKT-cells will produce IFN- γ and kill target cells in a Fas ligand (FasL) dependent manner without engagement of the TCR.^{29,30}

NK as well as NKT-cells recognize tumor cells through cell-cell contact and mediate killing with Fas/FasL or with the induction of cytokines or lytic enzymes.²⁷ NK cells recognize target cells based on expression of activating or inhibitory receptors. Since NK cells do not recognize target cells based on MHC expression, a decrease in MHC expression does not limit their activity. Also, some Fc receptors on NK cells can bind to antibody coated tumor cells leading to ADCC.²

Naïve T-cells require more than one signal for activation and subsequent proliferation into an effector cell. This activation is triggered by recognition of MHC-peptide complex and a co-stimulatory signal. Frequently, tumor cells give little or no co-stimulatory signals that can inhibit the activation of cytotoxic T-cells. The co-stimulatory signal occurs by interaction of B7 on antigen presenting cells and CD28 on the T-cells. CTLA-4 and CD28 are T-surface antigens, which bind to B7-1 or B7-2 ligands on antigen presenting cells to activate a T-cell response and control proliferation.² CD28 is expressed on resting and active cells while CTLA-4 is virtually undetectable on resting cells. Their ligands, B7-1 and B7-2, are two related forms of immunoglobulin superfamily members with similar extracellular domains but with different cytosolic domains. These ligands are constitutively expressed on dendritic cells and can be induced on macrophage and B-cells. Signaling through CD28 produces a positive co-stimulatory signal and increases CTLA-4 levels on the T-cells. Although CTLA-4 and CD28 are structurally similar, they act antagonistically. Surface levels of CTLA-4 are lower than CD28, but it competes favorably for B7 binding sites due to its high avidity.²

Targets of Immunotherapy

The complexity of the immune system presents many legitimate targets for the induction of an immune response. One aspect of the innate immune system present throughout the body, including the prostate epithelium and stroma, is the presence of toll-like receptors (TLR). TLRs are capable of recognizing foreign antigens and act as molecules with pattern recognition capabilities and may be soluble or cell-associated receptors. Pattern recognition receptors (PRR) are extracellular or present on cell membranes and target microbes or components in tissue fluids and blood. Typically, signals transduced through a TLR result in transcriptional activation, synthesis, and secretion of cytokines. This signaling process results in the activation of antigen presenting cells, all of which are involved in or promote inflammation.² For instance, TLR-5 mRNA is found in the prostate, testis, ovaries, and leukocytes. TLR-5 interacts with microbial lipoproteins leading to nuclear factor-kappa B (NF- κ B) activation, cytokine secretion, and inflammation.^{31,32} Other TLR activation induces secretion of cytokines,

such as IFN- γ , MAPK pathways,³³ or acts as a target for CpG islands (found in bacterial DNA) or double stranded (ds)RNA.¹¹

Cytokines

A second potential target for immunotherapy lies in the world of cytokines. Cytokines are low molecular weight regulatory proteins or glycoproteins that regulate the immune response, hematopoiesis, control of cellular proliferation and differentiation, and are involved in wound healing. Cytokines share many properties with hormones and growth factors in that they are secreted soluble factors that elicit biological effects. As cytokines are discovered, many can be grouped into families based on protein structural homology. Several examples of cytokine families are: interferons, tumor necrosis factors, and interleukins. These molecules are redundant and have overlapping functions. Once a cytokine encounters the appropriate receptor, it acts in an antigen non-specific manner and can induce a series of protein tyrosine phosphorylations. The two main cell types responsible for cytokine secretion are the T-helper cell and macrophage.²

Interferons are one of the major groups of cytokines that have been used for clinical cancer studies. IFN- α is produced by macrophages and increases MHC-I expression, activates NK cells, induces an anti-viral state, and inhibits cell division of normal or malignant transformed cells *in vitro*. IFN- β , produced by fibroblasts, increases MHC-I expression and activates NK cells. IFN- γ is produced by CD8⁺ T-cells and NK cells and activates macrophages, increasing both MHC-I and MHC-II expression when foreign antigen is present. Data suggest that malignant tumors display a decrease in MHC-I expression and that the interferons may be responsible for restoring MHC-I expression, thereby increasing cytotoxic T-cell activity towards the tumor.² Daily injections of recombinant INF- α have been shown to induce partial or complete regression in hematological cancers (i.e. leukemia and lymphoma), as well as some solid tumors (i.e. breast and renal cancer).

Tumor necrosis factors TNF- α and TNF- β have been shown to display anti-tumor activity by direct killing of the tumor cells, reducing proliferation rate (while sparing the normal cells), and inhibiting angiogenesis by damaging vascular endothelial cells. Frequently, when treated with either factor, the

tumor undergoes hemorrhagic necrosis and regression. Macrophages, monocytes, and other cell types including fibroblasts and T-cells secrete TNF- α . However, TNF- β is only produced by activated T-cells and B-cells and is a mediator of immune function and involved in wound healing. Both INF- γ and TNF- α are associated with chronic inflammation.² The complexity of cytokines and how they may potentially interact with each other has been one major obstacle of this type of therapy. Many cytokines have short half-lives and depending on the circumstances can act as either a pro-inflammatory or anti-inflammatory agent (i.e. IL-7, IL-9).^{34,35} Systemic administration of a large amount of cytokines has led to serious consequences and has even been fatal, therefore these immunotherapies can be limiting.²

Growth Factors

Many tumors display high levels of growth factor receptors on their membranes making growth factor receptors a likely target for immunotherapy. Inappropriate expression of either a growth factor or its receptor can result in uncontrolled proliferation.² Vascular endothelial growth factor (VEGF) is a potent mitogen for cells and is one of the most well studied growth factors. VEGF mRNA expression is seen in breast cancer and is associated with poor prognosis of colon cancer and non-small cell lung carcinoma.^{36,37} VEGF can be activated by *ras* oncogene causing inactivation of p53 and Von Hippel Landau (VHL), as well as cause activation of PKC.³⁸ VEGF is expressed in the epithelial and stromal areas of the human prostate, however, hyperplastic glands stain very poorly for the growth factor.^{38,39} Several studies have shown that prostate cancer specimens display 32% staining in the stroma and 56% staining in the epithelium.^{40,41} In contrast, staining for VEGF in BPH displayed 73% staining of the stroma and 50% staining in the epithelium.^{42,43} Of note is the use of the 5 α -reductase inhibitor, Finasteride[®], which has been shown to decrease expression of VEGF in prostatic tissue.⁴⁴

Tumor Antigens

Tumor specific antigens may result from mutations that cause altered cellular proteins or may be normally expressed at certain stages of

differentiation encoded by a variant form of the normal gene or may be exclusively expressed by the tumor.² Tumor associated glycoprotein-72 (TAG-72) is a mucin found on many adenocarcinomas including colorectal, pancreatic, gastric, ovarian, endometrial, and mammary, as well as some prostate cancers.^{45,46}

Tumor antigens, while being specific for tumor tissue, can vary widely from tumor to tumor. The use of tissue specific antigens is usually undesirable, as normal tissue would also be targeted with the tumor. The case of prostate cancer is unique in that the prostate is not a vital organ and could be targeted without serious harm to the patient. This allows for the targeting of tissue specific antigens in the prostate. Several prostate specific antigens have been discovered and are targets of immunotherapy. These tissue specific antigens include PSA, prostate alkaline phosphatase (PAP) and prostate specific membrane antigen (PSMA). Although these self-proteins are not always immunogenic they do provide a basis for further development and testing.²⁷

Monoclonal Antibody Therapy

Antigenic modulation in the treatment of many diverse cancers has been used for a number of years. In fact, the Food and Drug Administration has approved several monoclonal antibodies for treatment of various cancers and non-malignant diseases.⁴⁷ Ideally, by treating tumor cells with an antibody, one would hope for complete destruction of the tumor without recurrences. However, tumors seem to regenerate once the antibody treatment has ceased.² Passive administration of antibodies or active vaccination to induce antibodies can target cells that express antigenic proteins on their cell membranes.²⁷ Monoclonal antibodies are often conjugated to chemotherapeutic agents, biological toxins, radioactive compounds, or immunotoxins.^{2,48,49} These immunoconjugates target the neoplastic cells expressing tumor specific or tumor-associated markers.⁵⁰ Problems with antibody specificity, delivery, and cost are often hurdles for therapy.^{2,27} Unlike antibodies to Her2-*neu* for breast cancer or antibodies to CD20 for non-Hodgkin's lymphoma, there are limiting numbers of antibody targets for the treatment of prostate cancer.²⁷

CC49, a murine IgG₁ antibody, recognizes TAG-72 and shows disease response when coupled to a radioisotope in ovarian cancer⁴⁵ and has been shown to be expressed in prostate cancer cells.⁴⁶ A clinical trial utilizing ¹³¹I-CC49 failed to show any clinically relevant data.⁵¹ However, when ¹³¹I-CC49 was used in conjunction with INF- γ , up-regulation of TAG-72 and enhancement of the response was seen. The trial included 16 patients with androgen independent prostate cancer (AIPC), of which 12 patients had antibody localization to the tumor. None of the patients had a $\geq 50\%$ decline in their PSA or a radiologic response, however several had moderate pain relief from bone metastases. Rapid production of anti-mouse antibodies and development of thrombocytopenia precluded further dosing.⁵² In a subsequent clinical study, 14 patients were treated with IFN- α prior to the administration of ¹³¹I-CC49.⁵³ Two patients had a minor radiographic response while 3 had a $\geq 50\%$ reduction of their serum PSA levels. Therefore, IFN- α may be acting as an adjuvant yielding a greater response in comparison to just ¹³¹I-CC49 therapy.

Since PSA is found in the serum, many researchers have used the antigen for a potential target for immunotherapy. *In vitro* data show that the generation of antibodies that recognize PSA and CD3 on T-cells would direct non-specific CD3⁺ T-cells to PSA,⁵⁴ and in turn, this would re-direct preactivated peripheral mononuclear cells to lyse PSA expressing cells. Although demonstrated *in vivo*, a human trial is necessary. Since PSA is in the serum, directing antibodies to the prostate tissue would be difficult.⁵⁴

PSMA is an ideal target for monoclonal antibody therapy since the target cell is always internalizing the protein and its internalization is augmented by monoclonal antibody contact⁵⁵ and is strongly expressed on nearly 100% of prostate tumors.^{56,57} Prostacint[®] (7E11 from Cytogen) is an anti-PSMA antibody used to image the prostate. Prostacint[®] has been found to bind an intracellular epitope of PSMA and, therefore, likely binds areas of tumor necrosis. Second-generation anti-PSMA antibodies have been developed to target the extracellular domain of PSMA due to the fact that internal domain binding antibodies, such as 7E11 and PM2J0004.5 (Hybritech) do not bind viable cells.^{58,59}

Most antibody therapies to PSMA have used J591, a mouse monoclonal antibody that is immunogenic. J591 has been genetically modified to eliminate the mouse antigens and is now fully "humanized," allowing

repeated dosing without generating anti-mouse antibodies. The unmodified antibody could focus the immune system on tumor sites to complement activation, however, dramatic responses to naked antibodies are infrequent.^{47,60} J591 binding to PSMA is rapidly internalized into the cell and has been quite useful for imaging of known sites of metastasis.^{47,60,61}

Antibodies to the extracellular domain of PSMA and coupled to toxins or radioisotopes have been shown to have some effect in prostate cancer cell lines and murine models.^{50,62–64} In one study, J591, PEQ226.5, and PM2P079.1 were conjugated with ricin A chain (RTA), a holotoxin containing an α subunit that inactivates protein synthesis and facilitates intracellular trafficking of RTA.^{48,49} Since J591 and PEQ226.5 recognize the same epitopes that are related to PSMA, a lower cytotoxic effect was observed of the antibodies in cell monolayers in comparison to treatment with RTA alone, while the specificity of PSMA expression of the tumors was increased.^{48,49}

A study performed by Dr. Bander and colleagues at the College of Medicine of Cornell University enrolled 53 patients into a phase-I study to assess disease staging, metastatic or recurrent disease. Twenty-nine patients received ¹¹¹In/⁹⁰Y-DOTA-J591 while 24 patients received ¹⁷⁷Lu/DOTA-J591. The results indicated 98% of the patients had successful targeting of J591 to the bone and soft tissue with 87% having radiographic evidence of metastasis and 13% had zero visible lesions. Remarkably, 16 of 18 patients with no evidence of metastasis showed positive J591 staining.⁴⁷ Other biotechnology companies have developed external domain specific anti-PSMA antibodies using mice genetically engineered to express human antibodies, resulting in the development of monoclonal antibodies that are non-immunogenic. These anti-PSMA antibodies have demonstrated significant activity in clinical trials.⁶⁵

Modulation of T-Cells

Modulation of the co-stimulatory signals required for T-cell activation has been shown to be an effective therapy through blocking cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) with antibodies and prolonging a T-cell response.²⁷ Anti-CTLA-4 blocks B7 binding to CD28, preventing stimulation and decreasing expression of T-cells.^{66,67}

Anti-CTLA-4 antibody treatment in animal models can induce tumor rejection in immunogenic tumors.⁶⁸ Coupled with an anti-tumor vaccination, it can induce rejection of minimally immunogenic tumors in the TRAMP animal model.⁴⁷ In a study of CTLA-4 blockade administered immediately after primary tumor resection, a reduction of metastatic relapse from 97.4% to 44% was observed. Consistent with this, lymph nodes obtained 2 weeks after treatment reveal marked destruction or complete elimination of C2 metastases in 60% of mice receiving adjunctive anti-CTLA-4 whereas 100% of control antibody-treated mice demonstrated progressive C2 lymph node replacement.⁶⁹

Adjuvants are often used with various immunotherapies because they can increase B7 co-stimulation and activate macrophages. Activated macrophages can then cluster around tumors and are better T-helper activators. These increase both humoral and cell-mediated responses and correlate with tumor regression.² The Fc portion of human IgG has been fused to the B7 binding domain of CTLA-4 to produce a chimeric molecule, CTLA-Ig.² The human Fc portion gives the molecule a longer half-life and the B7 binding domain allows binding to CD28. A humanized antibody for CTLA-4, designated MDX-101 (Medarex, Inc.) has recently been tested in a Phase-I trial, which included 14 patients with AIPC. The study showed successful blocking of the co-stimulatory signaling with no T-cell activation occurring. The therapy was well tolerated and 2 patients had a $\geq 50\%$ decline in PSA levels.⁷⁰

Vaccines

Tumor vaccines cause induction of a cell-mediated response to antigens and are often composed of tumor-associated proteins mixed with a non-specific antigen.^{2,27} Demonstrating that antigen specific T-cells are up-regulated by a particular vaccine strategy is important for immunologic therapies.²⁷ Antigen presentation is critical for any immunization technique and its enhancement can modulate tumor immunity. Anti-tumor vaccines that activate cytotoxic lymphocytes (CTLs) and human tumor infiltrating lymphocytes (HTLs) would be most desirable since HTLs induce CTLs. HTLs produce both IFN- γ and granulocyte macrophage colony stimulating factor (GM-CSF) and kill tumor cells. Therefore,

vaccines that induce anti-tumor CTLs include MHC-II restricted epitopes, which would trigger a HTL response to tumor-associated antigens.⁷¹ A method of activating these T-cells may be to use the antigen presenting dendritic cells to initiate the immune response.

Dendritic cells are the most potent antigen-presenting cells, capable of presenting antigen to CD8⁺ (MHC-I restricted) and CD4⁺ (MHC-II restricted) T-cells.⁷² Dendritic cell-based vaccines use a patient's bone marrow derived antigen presenting cells that are able to sensitize naïve T-cells to new antigens. By combining dendritic cells with tumor antigens, the therapy supposes that the dendritic cells will then activate T-cells with tumor antigen.²⁷

Mouse dendritic cells, pulsed with tumor fragments and incubated with GM-CSF, were re-infused into the mice to activate the T_H and CTL response to the tumor antigen.⁷³ Mouse tumor cells are immunogenic, therefore animals injected with killed tumor cells do not grow tumors when challenged with live tissue, a term designated protective immunity.² The same is true for humans. When tumor cells were transfected with GM-CSF and given back to the patient, they were able to secrete more GM-CSF and enhance the differentiation and activation of the host antigen presenting cells. As the dendritic cells surround the tumor cells, GM-CSF is secreted by the tumor and enhances presentation of antigen to the T_H and CTL cells.²

Denedron Corporation has developed Provenge[®], a recombinant fusion protein with GM-CSF fused to prostate acid phosphatase (PAP), a prostate specific isozyme of acid phosphatase that is secreted by prostate cells.^{27,74} This strategy uses autologous dendritic cells combined with human GM-CSF. Thirty-one patients with prostate cancer were enrolled in the clinical study and received three monthly infusions and one final boost at 24 months if the disease had not progressed. Results showed 38% of the patients had a T-cell response against native PAP while some had a decline in their PSA levels. T-cells collected after the treatment revealed the presence of IFN- γ , a reflection of successful activation.

The use of PAP as a vaccine has also been studied, since serum PAP levels increase with prostate cancer progression, from 33% up to 92%, making it a more important marker for advanced disease.⁷⁵ One study used a xenogenic homologue of PAP (mPAP), which was given to patients

with metastatic prostate cancer. The homologous mPAP possessed sufficient differences from self-antigen to render it immunogenic, but similar enough that they would cross-react with human PAP. Seven out of 21 patients had stable disease following the vaccination beyond one year while three patients had stable disease beyond three years. All of the patients had T-cell immunity to mPAP and 11 out of 21 had induced immunity to human PAP.^{76,77}

A PAP peptide (termed PAP-5) capable of binding the HLA-A2 molecule was used to pulse an antigen presenting cell fraction containing dendritic cells isolated from a healthy HLA-A2 donor. The cells were expanded and employed to elicit a CD8⁺ CTL response. The peptide lysed prostate tumors in an antigen specific manner. CTLs were evaluated for peptide specific activity and potency in an *in vitro* chromium release assay. The assay revealed that the CTLs generated after stimulation of PAP-5 peptide loaded dendritic cells were able to endogenously process the PAP-5 antigen.⁷⁴

Human prostate cancer cells were removed at the time of surgery and expanded in culture. They were transfected to secrete a high amount of GM-CSF via *ex vivo* retroviral transduction with GM-CSF cDNA.⁷⁸ Eight of 11 patients were then irradiated and given a subcutaneous injection of their corresponding vaccine every 21 days (3–6 doses). Biopsies showed the presence of macrophages, dendritic cells, T-cells, and eosinophils. Delayed type hypersensitivity (DTH) *vs.* irradiated, unmodified, autologous tumor cells and recall antigen were tested pre/post treatment to assess specific tumor cells and recall antigens to determine if a tumor specific response was achieved. Two of eight patients had a DTH response prior to the vaccination while seven out of eight patients had a DTH response post vaccination. Biopsies of the DTH sites showed that 80% of the T-cells expressed CD45RO with the presence of Th₁ and Th₂ cells.⁷⁸ Expression of CD45RO indicates that the T-cell has switched isoforms and is now acting as an effector cell.⁷⁹

A vaccine study targeting PSMA enrolled twenty-six patients with various stages of prostate cancer.⁸⁰ Patients were given either a cDNA plasmid encoding the extracellular domain of PSMA (with or without CD86), an adenoviral vector expressing PSMA, or both in a prime-and-boost strategy trial. Some of the patients received GM-CSF in addition to their

treatment. A DTH response to the PSMA expressing plasmid was seen in some of the patients including all 10 patients receiving the adenoviral vector. PSA decline was seen in some patients receiving vaccination only. Due to the various stages of disease and GM-CSF combination treatments, the results of this study are difficult to interpret.²⁷

T-Bodies

A T-cell receptor that has been modified so the intra/extracellular part of the domain is the same but the most distal part of the receptor is replaced with a single chain antibody, is known as a T-body. The distal portion of the receptor being modified is the portion that would normally recognize the peptide antigen complex in the MHC cleft. A T-cell could then be activated to attach to a tumor using a specific antibody to a tumor specific antigen.⁸¹ Sadelain *et al.* have created an artificial T-cell receptor (Pz-1) that is composed of an external PSMA-specific single chain antibody, linked to the CD8 hinge and transmembrane domain, followed by the cytoplasmic T-cell receptor signal transduction domain.⁵⁷ The receptor is capable of redirecting the specificity of the T-cell to target PSMA expressing cells, independent of MHC. *In vitro* data shows successful lysis of PSMA expressing prostate cancer cells lines and no effect on the non-PSMA expressing cells. These results indicate proliferation of modified T-cells in response to the presence of PSMA expression.⁸²

Summary

More than 80% of prostate carcinoma tissue consists of tumor cells at advanced stages, with minor infiltration of inflammatory cells.¹¹ This indicates that the immune system is not involved. As a result, researchers have the opportunity to tap into a powerful natural defense system that can be augmented to involve prostate cancers. Immunotherapy can focus the immune system on a particular cancer with a wide range of alternatives that can be used singly or in concert to provide a tremendous benefit to the patient. By combining therapies involving biological response modifiers (i.e. cytokines and growth factors), conjugated monoclonal antibodies (including toxins and radiolabels), and cancer

vaccines (tumor marker proteins with or without dendritic cell augmentation), the future of immunotherapeutic treatment of prostate cancer looks very promising.

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RADIATION THERAPY AND HORMONAL THERAPY FOR PROSTATE CANCER

Ralph A. Brasacchio

*Department of Radiation Oncology and JP Wilmot Cancer Center
University of Rochester Medical Center
Rochester, New York, USA*

Introduction

Prostate cancer is the most common cancer among American men (33% of the estimated 699,560 new cancer cases in males in 2004) and is second only to lung cancer in male cancer-related deaths (10% of the estimated 290,890 cancer deaths in males in 2004).¹ Because the incidence of prostate cancer increases more rapidly with age, and the average age of the American male is increasing, the number of patients with prostate cancer is expected to steadily increase over the next decade. Therefore, prostate cancer has become a major health concern.

Several treatment options exist for prostate cancer depending on the stage of disease and other prognostic factors. These include orchiectomy, prostatectomy, radiation therapy (RT) [external beam (EBRT) and brachytherapy], hormonal therapy, and chemotherapy. Clinicians frequently combine these options in treating the disease in order to optimize results.

This review will focus on the use of RT in the treatment of localized prostate cancer and the role and mechanisms of hormonal therapy in combination with RT.

Conventional and Conformal Radiation Therapy

Localized Prostate Cancer

External beam radiation therapy (EBRT), conventional and more recently three-dimensional conformal radiation therapy (3D-CRT) with dose escalation, including intensity modulated radiation therapy (IMRT), has proven to be a highly effective treatment for men with localized prostate cancer. Treatment results from several series for patients with favorable prognostic factors of prostate-specific antigen (PSA) ≤ 10 ng/ml, Gleason score < 7 (i.e. well-moderately differentiated), and T₁–T_{2a} (i.e. locally-confined, low volume) disease, have been excellent. In a large series from MD Anderson Cancer Center, patients with favorable risk factors experienced a freedom from biochemical failure rate of 88% at 5 years and 84% at 10 years.² Similar results have been seen in a number of other series across multiple institutions.^{3–5}

Despite these reports showing the clinical benefit of RT in early-stage, low-risk prostate cancer, patients with intermediate risk prognostic factors and with high risk/locally advanced disease [i.e. clinical stage T_{2B}, extensive unilateral disease, or Gleason score 7 (i.e. moderately–poorly differentiated), PSA 10–20 ng/ml; and clinical stage \geq T_{2C}, bilateral often bulky disease, Gleason score ≥ 8 (i.e. poorly differentiated) and PSA ≥ 20 ng/ml] have a 25%–50% and a greater than 50% risk of biochemical recurrence in 5 years.⁶ Ten-year survival rates of only 40% have been observed with standard RT alone for patients with locally advanced disease.^{7,8} The rates of survival free of biochemical failure at 5 years were 69% for intermediate risk patients and 47% for high risk patients in a multi-institutional review.³

These relatively poor results of RT in patients with intermediate and high risk disease have led to trials investigating radiation dose-escalation using conformal RT methods, such as 3D-CRT and IMRT, and external beam combined with hormonal therapy, which is the main subject of this review.

In one trial of dose escalation performed at Fox Chase Cancer Center, an improvement in PSA-relapse free survival (RFS) was observed among the subgroup of patients with PSA > 10 when the RT dose was increased from 68 Gy to 79 Gy.⁹ The 5-year biochemical disease-free survival (DFS) was

significantly improved for patients with pre-treatment PSA > 10 who received greater than 76 Gy. Patients with an initial PSA of 10.0–19.9 had a 5-year biochemical DFS of 29%, 57%, and 73% if they received <71.5 Gy, 71.5–75.6 Gy, and ≥ 75.6 Gy respectively ($p = 0.02$). Patients with an initial PSA > 20 had a 5-year biochemical DFS rate of 8%, 28% and 30% if they received <71.5 Gy, 71.5–75.6 Gy and ≥ 75.6 Gy, respectively ($p = 0.02$).¹⁰

Similar dose-response results have been observed at Memorial Sloan Kettering Cancer Center, indicating improvement in biochemical DFS for patients with intermediate and high-risk disease who received a dose of ≥ 75.6 Gy ($p \leq 0.05$).¹¹ The study from Memorial Sloan Kettering showed that clinical response was dose dependent, with 90% of the patients receiving 75.6 Gy or 81.0 Gy achieving a PSA nadir < 1.0 ng/ml compared with 76% and 56% for those treated with 70.2 Gy and 64.8 Gy ($p < 0.001$). Five-year actuarial PSA-RFS for patients with favorable prognostic indicators (stage T_{1–2}, PSA < 10.0, Gleason score < 6/10) was 85% compared with 65% for those with intermediate prognosis and 35% for the group with unfavorable prognosis (≥ 2 indicators with high risk features) ($p < 0.001$).¹¹ Positive biopsy rates also were significantly less in those receiving the higher doses.

In a large series of patients, analyzed at the University of Michigan,¹² 3D-CRT with dose escalation reduced the risk of biochemical failure among intermediate risk patients. Hormonal therapy was not associated with reduced rate of failure in patients with intermediate risk features, although for patients with high-risk features, adjuvant and neoadjuvant hormonal therapy significantly increased failure-free survival in radiation treated patients ($p < 0.05$), as is also reported in the randomized trial discussed below.

A French study accrued 164 patients to a dose escalation trial. The patients had clinical T_{1b–T₃} disease and were prescribed doses ranging from 66 Gy to 80 Gy, with group 1 patients receiving a standard dose of 66–70 Gy, and group 2 patients receiving a dose of 74–80 Gy. Although mean follow-up was short for both groups (32 months for group 1, $n = 46$; 17.5 months for group 2, $n = 118$) the probability of achieving nadir PSA ≤ 1 ng/ml was significantly higher for group 2 patients.¹³

The results of a randomized study from MD Anderson Cancer Center comparing 70 Gy vs. 78 Gy showed an improvement in 5-year freedom

from PSA failure, most significant in patients with pre-treatment PSA > 10 ng/ml.^{2,14} The 5-year freedom from failure (FFF) rate was 75% for the patients in the high risk arm vs. 48% for those in the control group ($p = 0.011$). Updated results at 60 months showed an improvement in crude biochemical failure rate for the experimental group (21% vs. 32%, $p = 0.03$).¹⁵ Kaplan-Meier analysis¹⁶ showed that although FFF rates were improved in the experimental group the differences narrowed, with longer follow-up, to 6% at 6 years. Improvements in FFF were most significant for patients with intermediate to high risk disease (6-year FFF rate of 43% vs. 62% for the control and experimental arms, respectively). There also appeared to be a reduction in freedom from distant metastasis at 6 years for patients with pre-treatment PSA > 10 ng/ml (88% for the 70 Gy arm vs. 98% for the 78 Gy arm [$p = 0.056$]). There was no overall survival difference seen at 6 years.

Therefore, both radiation dose escalation and randomized series show a more pronounced effect of dose escalation in patients with intermediate risk prostate cancer. Patients with more favorable disease may not benefit from higher doses because conventional doses are adequate to eradicate these tumors. Patients with high risk disease may have a local control benefit from high dose RT; however, due to the high likelihood of occult metastases, long term biochemical no evidence of disease (NED) and DFS may not be affected. For this high-risk group of patients, adjuvant hormonal therapy and possibly chemotherapy may be necessary in addition to dose escalation. It is also not clear that dose escalation is without potential toxicity with longer follow-up¹⁵ and particularly when higher doses are used, although studies in general report an improvement in late sequelae of 3D-CRT and IMRT dose escalation.¹⁷⁻¹⁹

Radiation Therapy With or Without Androgen Ablation Therapy

As discussed in the previous section, several retrospective and prospective dose escalation trials have suggested that the use of higher radiation doses, particularly in intermediate risk groups (clinical stage T_{2B} or Gleason score 7, PSA 10–20 ng/ml) of prostate cancer patients, may lead to improvements in biochemical control and DFS. However, the optimum radiation dose has yet to be determined, and in especially high-risk

patients, RT dose escalation alone may not be adequate. In addition, the more long-term results of dose-escalation have yet to be assessed. Therefore, investigators have attempted to improve the outcome for patients with localized but locally-advanced prostate cancer by the addition of hormonal therapy to EBRT. Green *et al.*²⁰ was one of the early workers in this area. In 1975, he reported a study including 80 patients treated with RT for prostate cancer, 35 of whom also received estrogen during the radiation and for the subsequent 3 months. The majority of the patients had locally advanced but non-metastatic disease. The author noted that the most favorable tumor response was seen in those who received estrogen along with radiation compared with those who received radiation or estrogens alone.

This same investigator later reported on a group of 35 patients with pelvic or pelvic and para-aortic lymph node metastasis. These patients were treated with 3 mg of diethylstilbesterol (DES) for 8 weeks prior to and continuing during RT, then discontinued after 6 months in the majority of patients. The study suggested a benefit of DES regarding cytoreduction and the role of estrogen therapy in allowing RT to improve control of disease.²¹ Green *et al.* also reported on 36 patients with bulky prostate cancer of whom 25 received 3 mg DES before RT and were compared with a similar group of 11 patients treated with RT alone. With a median follow-up of 4 years, the group reported a local control rate of 72% for the combined group compared to 53% local control for the RT alone group.²²

Other nonrandomized studies have investigated the role of hormonal therapy (estrogens) with RT. van der Werf-Messing *et al.*²³ retrospectively analyzed patients with T₃-T₄N_xM₀ prostate cancer, 26 of whom received orchiectomy and hormones (1 mg DES) alone, 30 were treated with external RT alone, and 30 received hormones and external RT. Patients in the RT alone group had a significantly greater overall survival at 4 years than the other groups. There were 10 cardiovascular deaths in the groups treated with hormones that likely accounted for the decreased survival. The combined therapy group also had a greater percentage of patients with poorly-differentiated tumors.

The first randomized series comparing hormonal therapy plus radiation to radiation alone was initiated by Del Regato in 1967.²⁴ This was a multi-institutional trial including patients with locally-advanced

(stage T₃/T₄) prostate cancer. DES was started at the end of RT and continued indefinitely, most patients continuing for several years and many permanently. The long-term results of the trial encompassed patients treated at MD Anderson. Seventy-eight patients were randomized to RT plus DES at 2 mg or 5 mg daily, or RT alone. There was an improvement in disease freedom, although a survival difference due to adjuvant DES was not observed, despite a median follow-up of 14.5 years.²⁵ This may have been related to small patient numbers since large numbers of patients must be studied because of the large number of intercurrent deaths in the elderly prostate cancer population; and to the association of DES with increased cardiac mortality, which may have counterbalanced any potential survival benefit from the hormonal therapy.

Pilepich *et al.* have reported a Radiation Therapy Oncology Group (RTOG) Phase II trial investigating potential benefit of hormonal cytoreduction in locally advanced prostate cancer. This trial compared the efficacy and toxicity of megastrol versus DES as cytoreductive agents before and during RT. Patients had locally-advanced adenocarcinoma of the prostate, stage B₂ and C with or without pelvic lymph node involvement, and were stratified by stage, grade and nodal status. They were randomized to receive either 40 mg megastrol 3 times daily (tid) or 1 mg DES orally, tid. The drugs were started 2 months before RT, continued during RT, and then discontinued. This study accrued 203 patients, of which 197 were evaluable. With a median follow-up of 32 months, only 6.5% of all evaluable patients manifested evidence of local failure. There was no significant difference between DES and megastrol regarding tumor clearance, although the DES appeared to be more effective in suppressing testosterone. This came with an increased rate of toxicity, especially gynecomastia and fluid retention. Patients with medical conditions predisposing them to thromboembolic phenomenon were excluded from this trial because of the risk of DES contributing to cardiovascular events.²⁶

These preliminary investigations evaluating the role of neoadjuvant and/or adjuvant androgen deprivation therapy plus RT in the treatment of locally advanced prostate cancer have led to a number of randomized trials.

RTOG 85-31 randomized 977 patients with clinical stage T₃ disease (57%), post-prostatectomy patients with seminal vesicle or extracapsular disease (15%), and T₁/T₂ patients with node positive disease (28%), to

either indefinite androgen deprivation *vs.* no androgen deprivation therapy with goserelin (LHRH analog therapy) following EBRT and goserelin continued indefinitely. Improved outcome was observed for those treated with the combination with regard to local, biochemical, and distant control ($p < 0.0001$). At median follow-up of 5.6 years there was numerical, although not statistically significant ($p = 0.52$), improvement in overall survival at 5 and 8 years for all patients. In a subgroup of patients with centrally reviewed biopsy (Gleason score 8–10), and who did not undergo prostatectomy, there was a significant improvement in the estimated 8-year overall and cause-specific survival ($p = 0.036$ and 0.019 , respectively) with immediate hormonal therapy. However, results of subset analysis should be reviewed with caution and require confirmation in an appropriately randomized and stratified study.^{27,28}

The EORTC studied 415 men with T₁–T₄ prostate cancer, the majority of whom had T₃–T₄ disease without evidence of lymph node involvement. Men with clinically localized disease had a Gleason score of 8–10. Patients were randomized to immediate hormonal therapy with LHRH agonist goserelin monthly starting on the first day of radiotherapy and continuing for 3 years, also receiving an antiandrogen for 1 week before and for 2–4 weeks after the first LHRH agonist injection in order to ameliorate possible LHRH agonist flare reaction. Those in the control arm received the same hormonal therapy at clinical progression. The initial analysis was reported in 1997, and showed overall survival at 5 years for the RT plus adjuvant hormonal therapy of 79% *vs.* 62% for the RT alone group. PSA-determined DFS at 5 years was significantly improved with the addition of hormonal manipulation, 85% *vs.* 48%. Local control was 97% for the combined group *vs.* 77% for the RT alone group. An update with median follow-up of 66 months continued to show a statistically significant survival benefit (78% *vs.* 62%; $p = 0.0002$) and clinical DFS benefit (74% *vs.* 40%; $p = 0.0001$) for the patients treated with combined therapy *vs.* RT alone respectively.^{29,30}

The RTOG has conducted a number of randomized trials addressing the potential benefit of cytoreduction given neoadjuvantly to RT. In the RTOG 86-10 trial, patients with locally advanced prostate cancer, *i.e.* bulky T₂–T₄ disease with palpable tumor >25 cc, with or without pelvic lymph node involvement, were randomized to receive goserelin 3.6 mg

subcutaneously every 4 weeks and the antiandrogen flutamide, 250 mg tid, for 2 months before and 2 months during RT (arm I) or RT alone (arm II). Of the 456 patients determined to be eligible at median follow-up of 4.5 years (471 patients accrued), arm I showed a significant improvement in local progression compared with arm II, 46% vs. 75% ($p < 0.001$) and progression-free survival 36% vs. 15% ($p < 0.001$). There was a trend toward improvement in distant metastasis-free survival ($p = 0.09$) but not improvement in overall survival.³¹ Their data indicated that the combined treatment results in an additive effect over RT alone in freedom from relapse or rising PSA.

In an update of this trial³² with median follow-up of 6.7 years and 8.6 years for surviving patients, there was a statistical benefit for neoadjuvant hormonal therapy plus RT in local failure, NED survival, biochemical NED survival with PSA < 4 ng/ml, biochemical survival with PSA < 1.5 ng/ml, and cause-specific failure. Patients who seemed to benefit the most from cytoreduction therapy were patients with Gleason score 2–6, who also had an improvement in overall survival. The survival improvement was not seen in the higher Gleason score groups who only showed an improvement in biochemical NED survival.

Subset analysis of RTOG 85-71 and 86-10 supported that the use of long-term hormonal therapy for locally-advanced but non-metastatic prostate cancer, was beneficial for patients with Gleason score 7 and 8–10. These patients showed an improvement in both cause-specific and overall survival with long-term hormonal therapy.³³

In order to attempt to answer the question of long vs. short-term hormonal therapy with RT, RTOG protocol 92-02 randomized 1554 men with clinical stage T₂–T₄ disease and PSA < 150 ng/ml, to goserelin given for 2 years after neoadjuvant total androgen blockade (goserelin and flutamide for 2 months before and 2 months during radiotherapy) and radiotherapy, or to neoadjuvant total androgen blockade and radiation followed by no further therapy. At a median follow-up of 4.8 years, the group receiving long term hormonal therapy showed a statistically significant improvement in estimated 5-year DFS, 46% vs. 28% ($p < 0.001$); clinical local progression, 6% vs. 12% ($p = 0.001$); distant metastasis, 12% vs. 17% ($p = 0.0035$); and biochemical failure 28% vs. 56% ($p = 0.001$). The 5-year survival rate for both groups was comparable

at this early endpoint. Patients in the study were stratified according to pretreatment Gleason score. In the subset of patients with Gleason score 8–10, there was an improvement in estimated 5-year overall survival (80% vs. 69%; $p = 0.02$) and disease-specific survival (90% vs. 75%; $p = 0.0007$) favoring the long term arm.³⁴

RTOG 94-13 was a 4-arm randomized trial which accrued 1327 patients with clinical stage T_{2c}–T₄ disease with pretreatment PSA < 100 ng/ml and estimated >15% risk of lymph node involvement based on the equation: % + LN = 2/3PSA + [(GS – 6) × 10].³⁵ The randomization was to neoadjuvant hormonal manipulation with LHRH agonist plus antiandrogen for 2 months before and during RT or the same hormonal manipulation for 4 months after RT. Patients were also randomized between whole-pelvis RT plus prostate boost versus radiation to the prostate only. With a median follow-up of nearly 5 years, patients receiving neoadjuvant hormonal therapy and RT had a 4-year progression-free survival of 53% vs. 48% for the adjuvant hormonal arm ($p = 0.33$). Patients treated with whole-pelvis RT plus a boost had a 4-year progression-free survival rate of 56% vs. 46% for the prostate only RT ($p = 0.014$). In the whole pelvis RT plus neoadjuvant hormonal manipulation arm, the progression-free survival rate was 61% at 4.5 years vs. 45% for the prostate only plus neoadjuvant hormonal manipulation, 49% for the whole-pelvis plus adjuvant hormonal therapy arm, and 47% for the prostate only without adjuvant hormonal therapy ($p = 0.005$). Overall survival was not statistically significant for any of the arms ($p = 0.15$), being 88% for the whole-pelvis plus neoadjuvant hormonal therapy vs. 81–83% for each of the remaining three arms.³⁶

Two additional randomized studies have been reported: Laverdiere accrued 120 patients to a 3-arm trial of RT alone, vs. RT plus 3 months of neoadjuvant hormonal manipulation with LHRH-agonist, vs. RT plus neoadjuvant LHRH-agonist and an additional 6 months of adjuvant LHRH-agonist. Patients were eligible if they had clinically measurable disease by digital-rectal exam and no distant metastasis. At 12 and 24 months there was a statistically significant decrease in the positive biopsy rate in favor of the radiation plus hormonal therapy arms and an increase in biochemical control at 12 months in the hormonal and radiation arms, although this was not apparent at 24 months.³⁷

Finally, Granfors *et al.* randomized men to hormonal therapy and radiation versus RT alone, with hormonal therapy deferred until clinical disease progression. All patients underwent pelvic lymph node staging prior to randomization and both node-positive and node-negative patients were included. The patient population in this study was small since the study was closed early due to a significant difference in the two arms. The hormonal therapy was orchiectomy performed approximately one month before radiotherapy. Pelvic radiation fields were given. At a median follow-up of 9.3 years, immediate orchiectomy was associated with an increase in median survival of more than 3 years. Overall survival was 62% in the immediate therapy group vs. 39% in the deferred therapy group ($p = 0.02$). When the results were analyzed by node status at study entry, the survival difference favoring immediate therapy was only seen in the node positive patients ($p = 0.007$), which may reflect the lower number of events in node negative, i.e. more favorable patients.³⁸

In summary, the available data from trials comparing RT plus androgen ablation to RT alone have shown significant differences based on endpoints such as biochemical failure and biopsy positivity. Two studies have demonstrated an improved overall survival for immediate hormonal therapy.^{29,30,38} Two have shown no significant difference in survival between immediate and deferred therapy^{8,25,28} and it is too early to draw definitive conclusions from the RTOG 92-02 and RTOG 94-13 studies. A difference in the studies, which may partially explain these differences in survival, may be that the hormonal therapy was commenced before the patients underwent the first RT treatment, whereas in those studies in which no survival benefit was seen, the hormonal therapy was commenced at the end of the course of RT. Patients with clinical T₃ tumor with favorable Gleason score (≤ 6) seem to benefit from short course neoadjuvant total androgen blockade with LHRH agonist and antiandrogen (flutamide) for 4 months (2 months before RT and during RT). Patients with any T stage with Gleason score 8–10 seem to benefit from LHRH treatment (≥ 2 years). Based on a metaanalysis of RTOG protocols, patients with T₃ Gleason score 7 tumors also appear to benefit from long term hormonal manipulation. This may more likely reflect the control of micrometastatic disease.³⁹ Finally, a study recently completed by the RTOG (RTOG 99-10) is investigating, in a randomized fashion, 2 months vs. 7 months total

androgen blockade prior to definitive RT in intermediate risk prostate cancer patients.

It appears therefore, that there are benefits to the combination of hormonal therapy and RT for patients with adenocarcinoma of the prostate, likely as a result of its potential for cytoreduction, as well as for control of metastatic disease.

Hormone Therapy and Brachytherapy

No randomized trial has been completed analyzing the role of hormone suppression in patients treated with brachytherapy, i.e. the placement of radioactive seeds within the prostate. A retrospective matched-pair analysis failed to identify an improvement in survival for low risk patients treated with hormone therapy and brachytherapy *vs.* brachytherapy alone. Stratification by Gleason score and pretreatment PSA also failed to reveal a subpopulation that benefited from hormonal therapy.⁴⁰

In a series from Seattle, the addition of hormone suppression did not significantly increase freedom from PSA failure in patients treated with the combination of EBRT and brachytherapy.⁴¹ However, Stone and Stock⁴² reported improved freedom from biochemical failure at 4 years for intermediate-risk patients treated with 6 months of hormonal therapy and brachytherapy compared to brachytherapy alone. High risk patients appeared to have a similar benefit in another report.⁴³ However, at this time the primary benefit of short-term use of hormonal therapy with brachytherapy is limited to reduction of prostate volume. It is possible that intermediate-risk patients may benefit from hormonal therapy in association with brachytherapy, although the majority of studies have short follow-up and therefore the effect of hormonal therapy is difficult to interpret, as it may be masking PSA failure in the combination arm.

Potential Mechanisms of Androgen Ablation and Radiation Therapy

Androgen dependence of the prostate was described more than 100 years ago when the effect of castration on prostate hypertrophy was demonstrated.⁴⁴ Higgins and Hodges in 1941 showed the androgen dependence

of prostate adenocarcinoma through orchiectomy and measuring the levels of acid phosphatase in patients with metastatic disease.⁴⁵ This work ultimately led to that of Walsh, who summarized the mechanism of action of androgens on prostate growth.⁴⁶ Histologic changes resulting from androgen deprivation in malignant prostate cancer cells include glandular shrinkage, cytoplasmic vacuolization, nuclear pyknosis, and degeneration of the tumor cells.⁴⁷

The mechanism by which malignant and non-malignant tissues balance cell numbers are being better elucidated. Various factors are capable of tilting the balance in one direction or another. Active prostate cell cycling, for example, may be promoted by androgens through stimulation of growth factors and through bcl-2-mediated gene products. Programmed cell death (apoptosis) may conversely be triggered by androgen deprivation, influencing expression of cathepsin-D by abnormal p53 gene expression and by radiation. The role of apoptosis is supported by the presence of pyknosis and nuclear fragments.⁴⁸

The mechanism of radiation cell kill in prostate cancer remains uncertain. DNA double-strand breaks are responsible, as well as induction of apoptosis. A number of investigators have shown radiation-induced apoptosis in a variety of cell systems.^{49,50} Pollack *et al.* demonstrated apoptosis following radiation of prostate adenocarcinoma in the Dunning R3327 rat model.⁵¹

The rationale for androgen ablation (AA) plus RT may be divided by the treatment strategies of neoadjuvant *vs.* adjuvant AA. Neoadjuvant AA decreases clonogen numbers, leaving fewer cells for radiation to eradicate. The addition of RT leads to an additive cell kill, which is more likely to be curative to patients who otherwise might have failed single modality treatment. Neoadjuvant AA plus RT may also enhance cell death by a supra-additive effect, *i.e.* cells “weakened” (but not eradicated) by the hormonal therapy become more susceptible to cell killing by RT. This latter mechanism may also be that by which adjuvant AA, *i.e.* hormonal therapy started during or immediately after RT has its beneficial effect. This may occur via apoptosis, with radiation and hormonal therapies shunting more cells down the apoptotic pathway than either therapy alone. Additive cell killing may also be a mechanism for the effect of adjuvant AA.

Work by Joon *et al.* using the Dunning R3327 rat model showed that radiation alone or castration alone induced apoptotic indices of about 2% or less, primarily through propagation of the cell cycle, but when AA preceded RT, approximately 10% apoptotic indices were noted, representing a 5-fold increase. In a protracted course of fractionated RT this may total a significant degree of additional cell kill. The maximum effect was sequence and timing dependent, since apoptosis was significantly enhanced when AA was given 3 days before 7 Gy radiation and no supra-additive effect was achieved when AA was initiated at the same time as radiation or when radiation was given 3–4 weeks after AA.⁵² These data suggest that neoadjuvant AA may be preferable to adjuvant AA although fractionation studies are needed to investigate the impact of fractionation on cell killing. It should also be noted, somewhat paradoxically, that it has been reported that some patients with abnormal p53 expression may fare more poorly if androgen deprivation is part of their therapy, than if it is not.⁵³

The Shionogi tumor system has been an important model for prostate cancer as it is androgen-sensitive and progresses in an androgen-independent manner following androgen deprivation. In a series of studies, the radiation dose required to control 50% of the tumors (TCD₅₀) was reduced 50% by both neoadjuvant and adjuvant androgen deprivation; however, the effect of androgen deprivation was greatest when it preceded radiation and if radiation was deferred until maximal tumor regression. If the period of neoadjuvant deprivation is extended too far, however, the advantage is largely lost. The degree of response to neoadjuvant androgen deprivation can also be used in the system as a predictor of response to subsequent radiation, since some tumors which showed a slow response to androgen deprivation had a significantly higher TCD₅₀, than those that had a rapid response to androgen deprivation. Androgen deprivation given after RT also reduces TCD₅₀, but to a lesser degree than neoadjuvant therapy. It is possible that the neoadjuvant approach leads to a synergistic effect with RT, while the adjuvant approach reflects the effect of independent cell killing.^{54,55}

Other mechanisms may also be involved in the favorable results of hormonal therapy and RT, including volume reduction leading to improved oxygenation of tumor, which through the production of free oxygen radicals, leads to increased radiation sensitivity.

Future Directions

The use of hormonal therapy in the treatment of prostate cancer has moved from castration, to the use of estrogen and similar compounds, and now to the use of LHRH agonists. The use of GnRH antagonists, which inhibit LH production and cause suppression of testosterone and dihydrotestosterone, is also being investigated.^{56–58} GnRH antagonists do not stimulate LH and testosterone production, which may cause a temporary worsening of cancer or symptoms, as is the case when LHRH agonists are initiated. The optimal combinations of hormonal therapy, whether LHRH alone, or with antiandrogen, or the use of other new agents to be used in conjunction with RT, have yet to be fully defined. In addition, the optimal timing and duration of hormonal therapy and which group of patients stand to benefit the most, taking into account potential for disease control, side effects, and possible survival benefit, still need to be elucidated. The integration of chemotherapy and other therapies, e.g. gene therapy⁵⁹ and AA, with RT in the treatment of prostate cancer also represents important challenges to improving the results of treatment. It is hoped that such improvements will lead to a positive impact on the lives of the many patients with this disease.

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4

GENE THERAPY FOR PROSTATE CANCER

Juan Antonio Jiménez, Chinghai Kao, Sang-Jin Lee,
Chaeyong Jung and Thomas A. Gardner

*Urology Research Laboratory, Departments of Urology, Microbiology
and Immunology, Walther Oncology Center
Indiana University Medical Center
Indianapolis, Indiana, USA*

Introduction

In 2003, it was estimated that prostate cancer would account for the most new cancer diagnoses, aside from skin cancer, at 220,900 men in the United States and was the second most common cause of cancer deaths at 28,900 men. A steady decline in the annual age-adjusted prostate cancer death rates over the past five years as recorded in the SEER Cancer Statistics Review would suggest improvements in early detection and treatment of locally confined prostate cancer.¹ While this may be true, at the present time, treatment options prolong life; however, most patients will eventually experience local recurrence or develop advanced disease. Androgen ablation therapy slows the dissemination of the disease but once the cancer changes its androgen status, tumors become refractory to hormonal treatment. A greater understanding of the molecular events underlying cancer and the subsequent development of metastatic disease allows gene therapy approaches targeted against these molecular events to be developed. Our laboratory has focused its energy on the development and application of tissue-specific promoters such as *osteocalcin (OC)* and *prostate-specific enhancer sequence (PSES)* as well as the development of molecular therapy for androgen-independent primary and metastatic prostate cancer.²⁻⁶

Gene Therapy Strategy

The ultimate goals of research in this area are to develop treatment modalities that increase survival, enhance quality of life, and cure men afflicted with prostate cancer. In order to do so, four factors must be considered when developing strategies. These include the selection of the disease to be targeted, the genetic material to transfer, the method of delivery, and the route of administration. The safety and efficacy of the trial underlie each of these factors.

The discussion begins with the selection of the disease and patient to target. The progression of prostate cancer from benign dysplasia to malignancy to metastatic disease is well known and results from the accumulation of multiple genetic defects. Several stages of prostate cancer are susceptible to treatment with gene therapy. Currently, most of the stages in the progression from localized prostate cancer to metastatic disease have been targeted; however, many more can be targeted as vector development and understanding of the molecular events continue. Lack of conventional therapies for locally advanced, locally recurrent, or metastatic prostate cancer makes these patients excellent candidates to target. Of course, as with most therapies, the ability to treat men with low volume disease should enhance the success of the therapy. This is a critical point for the therapeutic approaches discussed below.

With further research into the genetic determinants of prostate cancer progression, a protective approach could be used to decrease the morbidity and mortality associated with conditions such as benign prostatic hyperplasia (BPH) and prostatic intraepithelial neoplasia (PIN). Current studies have demonstrated a prophylactic effect against the development of prostate cancer with conventional therapies for BPH.⁷ As prostate-specific targeting and tumor killing efficacy continue to increase, the laboratory vision of a molecular prostatectomy may soon translate into a clinical reality. Because the prostate is a non-essential organ post-fertility and the side effects from chemotherapy, radiation, and surgical intervention are so great, molecular ablation has vast appeal.

The genetic material for transfer is determined by the objective of the therapy. For example, gene therapy for prostate cancer can provide corrective therapy for genetic alterations that give the cancer a survival

advantage such as those affecting tumor suppressors or growth-promoting oncogenes; however, because multiple mutations occur in the pathogenesis and progression of cancer, correction of one genetic insult may not be sufficient to change the cancer cell phenotype. Nevertheless, *in vivo* correction of single gene defects has been successful in several preclinical studies.⁸⁻¹¹ Targets for corrective therapy include the proto-oncogenes *p53*, *p21*, *p16*, and *retinoblastoma (Rb)*, and certain cell adhesion molecules (CAMs), as well as oncogenes *ras*, *myc*, and *bcl-2*. A second objective of molecular therapy for prostate cancer is to deliver genes that are capable of destroying tumor cells either directly or indirectly. Such cytoreductive therapies include the direct killing of prostate cancer cells by replication-competent oncolytic viruses and the indirect killing of cancer cells through the delivery of suicide genes such as pro-drug enzyme and apoptosis-inducing genes. The final objective of gene therapy for prostate cancer is to enhance the body's antitumor immune response. Current approaches involve *ex vivo* gene therapy of autologous tumor cells and subsequent vaccination with the irradiated cells now expressing cytokines such as interleukin-2 (IL-2) and granulocyte-macrophage colony-stimulating factor (GM-CSF), *ex vivo* gene transfer of genes encoding tumor antigens and subsequent vaccination leading to enhanced induction of T-cell immunity, *in vivo* intratumoral gene transfer of cytokine genes, and delivery of naked tumor DNA or RNA and the subsequent uptake and expression by antigen presenting cells such as dendrites.¹² Table 1 summarizes these objectives as they appear in clinical trials registered with the Office of Biologic Activities (OBA).

The currently available methods of delivery of genetic information are listed in Table 2. The ideal method of delivery would transfer the genetic material efficiently and specifically to the targeted organ, not be harmful to the patient, and be inexpensive to produce and administer. Each vector has advantages and disadvantages as couriers of genetic information. Adenovirus, perhaps the most commonly administered vector, clearly has its advantages and disadvantages. For example, adenovirus can deliver large amounts of genetic information with high efficiency regardless of the cell cycle status; however, it is highly immunogenic. To further compound this issue, it is believed that up to 75% of the population have humoral immunity to several serotypes of adenovirus due to prior infection.

Table 1. Current Approaches for Prostate Cancer Gene Therapy

Strategy	Vector(s)	DNA Transferred	
Corrective	Retrovirus	p53	
	Adenovirus	p16 c-myc	
Cytoreductive (suicide)	Adenovirus	TK CD TK/CD NIS OC promoter	
		(oncolytic)	PB/PSA promoter PB/PSE promoter
Immunotherapy	Retrovirus	GM-CSF	
	Vaccinia/fowl	MUC-1/IL-2 PSA	
	Liposome	PSA hTERT PSMA IL-2	
		RNA	Tumor RNA
		AAV	GM-CSF
		Adenovirus	IL-12 Inf- α

Efforts have been made to create less immunogenic forms of the virus as well as gutless forms of the virus that are incapable of replicating in immunocompromised patients. Such replication-deficient adenoviruses are achieved by deleting the early genes responsible for the control of viral replication.¹³

Finally, the route of administration is determined by a combination of the previous three factors. At the present time, most prostate cancer gene therapy trials involve the intratumoral injection of the vector which is quite suitable given the ability to visualize the prostate using ultrasound and its convenient transrectal access. Ultimately, the desired route of administration is systemic intravenous transfusion of the vector. Limiting factors include vector half-life, hematologic inactivation of the vector, and

Table 2. Comparison of Vector Systems in Gene Therapy

Attribute	Retrovirus/ Lentivirus	Adenovirus (Ad)/‘gutless’	Adeno- associated Virus (AAV)	Vaccinia/ Fowlpox Virus	Non-viral (Liposome)
<i>In vivo</i> gene transfer rate	Low/High	High	High	High	Low
Size limit of DNA transfer	8 kb	10/35 kb	<5 kb	>30 kb	? Limit
Cell cycling dependent	Yes/No	No	No	No	No
Genome integrating	Yes	No	Yes	No	No
DNA expression stability	Stable	Transient/ stable	Stable	Transient	Transient
Immunoreactive	No	Yes/No	No	Yes	No
Use in previous clinical trial	Yes/No	Yes/No	Yes	Yes	Yes

This table lists the important attributes of the currently used gene therapy vectors for prostate cancer. The bold items are felt to be the particular vector.

infection of non-target organs. As prostate-specific promoter systems become more effective, gene therapy may become the silver-bullet that seeks and destroys cancer cells in the prostate and distant metastatic sites.

Tissue-Specific Promoters

Recently, much effort has been made to develop tissue-specific delivery systems that eliminate the threat of harm to the patient. Several studies have demonstrated the importance of tissue-specific vectors, revealing systemic toxicity with the administration of high doses of nonspecific vectors.^{14,15} Essentially, viral vectors can transfer their therapeutic genes to any cell in the body, provided that it expresses the correct receptors for the virus. Through the use of prostate-specific promoters and enhancers, the expression of a therapeutic gene can be limited to cells that contain the

appropriate activators and transcription factors. One limitation to this technology is low level leaky activation of prostate-specific promoters in non-prostatic tissues; however, the development of chimeric promoters promises greater prostate-specificity.

Osteocalcin Promoter

OC is a highly conserved bone gamma-carboxyglutamic acid protein (BGP) that has been shown to be transcriptionally regulated by 1,25-dihydroxyvitamin D₃.¹⁶ This noncollagenous bone protein constitutes 1–2% of the total protein in bone, and its expression is limited to differentiated osteoblasts and osteotropic tumors.¹⁷ Figure 1 depicts expression of *OC* RNA and protein in primary and metastatic prostate cancers. The osteoblastic nature of osseous prostate cancer metastases is well characterized,¹⁸ and the mechanism is believed to be via its osteomimetic properties, specifically its ability to express bone-related proteins such as OC.¹⁹ The human *OC* promoter contains numerous regulatory elements including a vitamin D-responsive element (VDRE), making it inducible by vitamin D₃ administration,^{20,21} a glucocorticoid response element (GRE), an AP-1 binding site,²² and an AML-1 binding site which has been shown to be responsible for 75% of *OC* expression.²³ The *OC* promoter retains its tissue specificity in a recombinant *OC* promoter-driven thymidine kinase (TK)-expressing adenoviral vector (Ad-OC-TK). Following infection with Ad-OC-TK, only cells of osteoblastic lineage expressed TK; furthermore, Ko *et al.* demonstrated that the addition of acyclovir (ACV) resulted in osteoblast-specific cell toxicity.²⁴ A similar strategy has been developed for the intralesional injection of Ad-OC-TK to osseous prostate cancer metastases followed by administration of valcyclovir (VAL). In phase I clinical trials, this therapy induced apoptosis in every lesion treated without serious adverse effects to the patients.^{3,6}

Prostate-Specific Enhancer Sequence

Prostate-specific proteins such as prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA) are released into the bloodstream when the prostatic basement membrane is compromised, such as

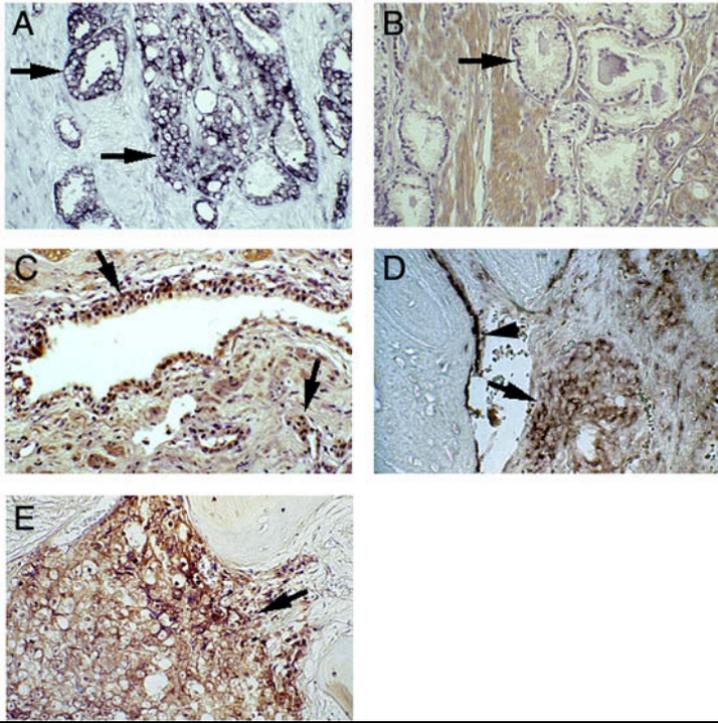


Fig. 1. Expression of OC RNA and protein in primary and metastatic prostate cancers. Nine primary tumors and ten osseous metastases stained positive for OC protein. RNA *in situ* hybridization was to characterize the expression of OC in prostate cancer cells. OC RNA transcripts were detected in the majority of primary (A) and metastatic (D) prostate cancers. OC protein was detected by immunohistochemical staining with OC4-30 anti-OC antibody (Takara Shuzo Co., LTD, Shiga, Japan). In three primary tumors, OC-positive staining was homogeneous (C); however, the other six had more OC-negative glands than positive glands (B). On the other hand, all ten osseous metastases stained OC-positive (E). Arrows indicate prostate cancer. The arrowhead indicates an osteoblast.

occurs in prostate cancer, and therefore is used as a sensitive marker for diagnosis and progression of prostate cancer.²⁵ PSA expression is androgen receptor (AR)-dependent, and its transcript levels are significantly reduced in the absence of androgen.²⁶ AR regulates *PSA* expression by binding to an androgen-responsive enhancer core (AREc) in the upstream 5' flanking region of the *PSA* gene.^{27,28} This promoter confers high tissue specificity and has been used in several gene therapy studies;²⁹⁻³¹

however, its utility in men undergoing androgen ablation therapy is limited. On the other hand, PSMA expression is upregulated under androgen-depleted conditions.³² Its expression is elevated higher in prostate cancer than in benign hyperplasia or normal prostate.³³ In addition, serum PSMA levels are highest in patients with metastatic disease, suggesting enhanced PSMA expression as prostate cancer progresses.³⁴ Recently, the PSMA enhancer (PSME) was discovered within the third intron of the PSMA gene, *FOLH1*³⁵ and has been used for prostate-specific gene delivery under low androgen levels.³⁶ Our laboratory hypothesized that AREc and PSME could function synergistically and developed a novel chimeric promoter, prostate-specific enhancer sequence (PSES), with high transcriptional activity and strong prostate specificity.

Through deletion and linker scan mutagenesis, PSES was developed by locating the minimal sequences, AREc3 and PSME(del2) in AREc and PSME, respectively and placing AREc3 upstream from PSME(del2). AREc3 contains six GATA transcription factor binding sites and three AR binding sites leading to high enhancer activity once surrounding silencer regions are deleted. PSME(del2) contains eight AP-1 and three AP-3³⁷ binding sites acting as positive regulators in the absence of androgen and a downstream deletion of an *Alu* repeat that functions as a transcriptional silencer. As depicted in Fig. 2, PSES drives luciferase activity five-fold higher than universal promoter RSV and slightly higher than CMV promoter, and luciferase expression was detected in several PSA- and PSMA-positive prostate cancer cell lines, but not in PSA- and PSMA-negative prostate cells or non-prostate cell lines. PSES retains its prostate-specific nature in recombinant adenoviral vectors as well. Figure 3 shows the results after injection of *BALB/c* nude mice with Ad-CMV-luc and Ad-PSES-luc.⁴ Due to its small size, high level of tissue specificity, and strong promoter activity in the presence or absence of androgen, PSES is an ideal promoter for use in prostate cancer gene therapy.

Human Telomerase Promoter

Telomeres are tandem repeat structures found at the termini of chromosomes that maintain chromosomal integrity by preventing DNA rearrangements, degradation, and end-to-end fusions. In most normal

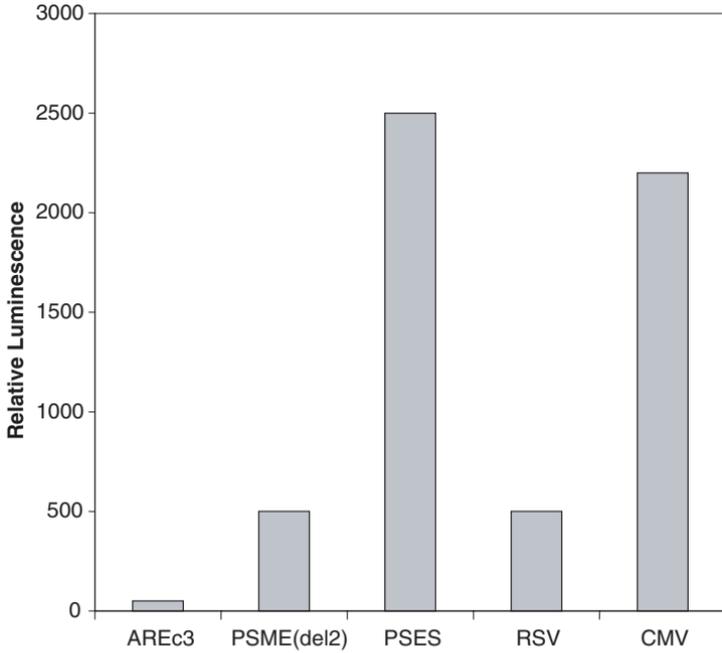


Fig. 2. Comparison of strength of promoter activities. 0.5 μ g of pGL3/TATA containing AREc3, PSME(del2), PSES, RSV, or CMV promoters along with pRL-SV40 (*Renilla* luciferase) were transfected. Cells were grown in the absence of androgen. PSES demonstrates strong promoter activity at 5-fold the activity of RSV and slightly higher than that of CMV promoter. Furthermore, the activity of PSES is greater than the activity of AREc3 and PSME(del2) combined, suggesting a synergistic effect.

somatic cells, the telomeric cap is shortened with each cycle of DNA replication and cell division. When telomeres shorten to a critical length, cells progress toward irreversible arrest of growth and cellular senescence.³⁸ In contrast, tumor cells have evolved a means to prevent telomere shortening through the activation of the catalytic component of human telomerase reverse transcriptase (hTERT).³⁹ The *hTERT* promoter region has been cloned and characterized, and has high GC content. Unlike most promoters, it does not contain TATA or CAAT boxes.⁴⁰ Importantly, the *hTERT* promoter is active in most cancer cells including prostate cancer⁴¹ and inactive in normal cells, thereby providing a unique tool to target cancer cells. Promising results have been reported using the *hTERT* promoter

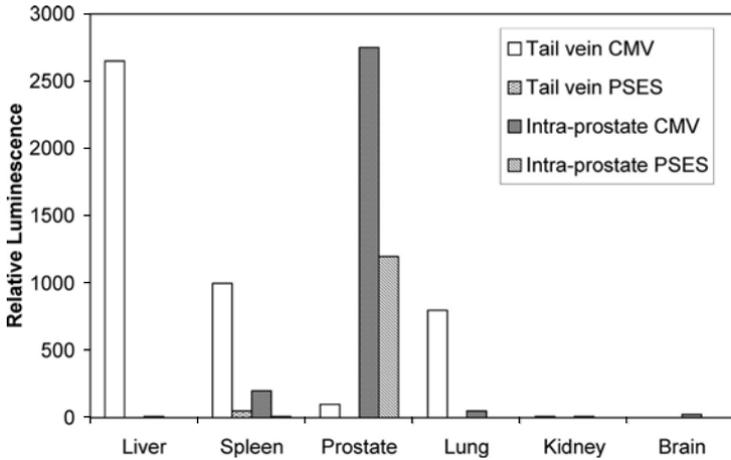


Fig. 3. *In vivo* PSES prostate-specificity comparison. Recombinant adenoviruses Ad-CMV-luc and Ad-PSES-luc were injected into male athymic mice, 7×10^{10} virus particles by tail vein injection or 1.4×10^{10} virus particles by intra-prostate injection. Mice were sacrificed and organs were harvested for luciferase activity 2 days post-injection. Following systemic injection, negligible levels of PSES-driven luciferase activity were detected in organs expected to take up adenovirus, mainly liver, spleen, and lung. To overcome the low infectivity of the prostate, intraprostatic injections were performed, revealing high PSES activity in the prostate.

to deliver TRAIL, an inducer of apoptosis⁴² and to control the replication of an oncolytic adenovirus.⁴³ Our laboratory is investigating the use of the *hTERT* promoter in conjunction with a prostate-specific promoter to control adenoviral replication in a prostate cancer-specific manner.

Past Approaches

Over the past ten years, three categories of gene therapy approaches for prostate cancer have emerged, corrective gene therapy, cytoreductive gene therapy, and immunotherapy. Each molecular therapy has a strong foundation of preclinical data allowing for the approval of several clinical studies. Currently, 56 gene transfer protocols registered with the OBA are targeted against prostate cancer. This accounts for 15% of all cancer protocols listed to date.⁴⁴ Table 3 highlights the details of the prostate cancer

Table 3. Prostate Cancer Gene Therapy Trials (OBA Protocol List, 17 December 2003)⁴⁴

Principal Investigator	Institution	Vector	Genetic Material	Year Reviewed
1. Simons, JW	Johns Hopkins, MD	Retrovirus	GM-CSF	1994
2. Steiner, MS	Vanderbilt Univ., TN	Retrovirus	c-myc	1995
3. Chen, AP	Nat. Naval Med, MD	Vaccinia/ fowlpox	PSA	1995
4. Paulson, DF	Duke Univ., NC	Liposome	IL-2	1995
5. Scardino, PT	MSKCC, NY	Adenovirus	HSV-TK	1996
6. Eder, JP	Dana-Farber, MD	Vaccinia/ fowlpox	PSA	1996
7. Sanda, MG	Univ. of Michigan, MI	Vaccinia/ fowlpox	PSA	1997
8. Belldegrun, AS	UCLA, CA	Liposome	IL-2	1997
9. Hall, SJ	Mt. Sinai, NY	Adenovirus	HSV-TK	1997
10. Belldegrun, AS	UCLA, CA	Adenovirus	p53	1997
11. Simons, JW	Johns Hopkins, MD	Retrovirus	GM-CSF	1997
12. Logothetis, CJ	MD Anderson, TX	Adenovirus	p53	1997
13. Kadmon, D	Baylor College, TX	Adenovirus	HSV-TK	1998
14. Simons, JW	Johns Hopkins, MD	Adenovirus	PSA	1998
15. Figlin, RA	UCLA, CA	Vaccinia/ fowlpox	MUC-1/IL-2	1998
16. Gardner, TA	Univ. of Virginia, VA	Adenovirus	OC-HSV-TK	1998
17. Eder, JP	Dana-Farber, MD	Vaccinia/ fowlpox	PSA	1999
18. Small, EJ	UCSF, CA	Retrovirus	GM-CSF	1999
19. Kaufman, HL	Albert Einstein, NY	Vaccinia/ fowlpox	PSA	1999
20. Vieweg, J	Duke Univ., NC	RNA	PSA	1999
21. Belldegrun, AS	UCLA, CA	Liposome	IL-2	1999
22. Small, EJ	UCSF, CA	Retrovirus	GM-SCF	1999

Table 3 (Continued)

Principal Investigator	Institution	Vector	Genetic Material	Year Reviewed
23. Kim, JH	Henry Ford Hosp., MI	Adenovirus	CD/TK	1999
24. Aguilar-Cordova, E	Harvard Univ., MA	Adenovirus	TK	1999
25. Gingrich, JR	Univ. of Tenn., TN	Adenovirus	p16	1999
26. Terris, MK	Stanford Univ., CA	Adenovirus	PSA	1999
27. Wilding, G	Univ. Of Wisc., WI	Adenovirus	PSA	1999
28. Belldegrun, AS	UCLA, CA	Liposome	IL-2	1999
29. Dahut, WL	NIH/NCI	Vaccinia/ fowlpox	PSA	1999
30. Arlen, PM	NIH/NCI	Vaccinia/ fowlpox	PSA	2000
31. Vieweg, J	Duke Univ., NC	RNA	Tumor RNA	2000
32. Pollack, A	Univ. of Texas, TX	Adenovirus	p53	2000
33. Gardner, TA	Indiana Univ., IN	Adenovirus	OC promoter	2000
34. Freytag, SO	Henry Ford Hosp., MI	Adenovirus	CD/TK	2000
35. Lubaroff, DM	Univ. of Iowa, IA	Adenovirus	PSA	2001
36. Miles, BJ	Baylor College, TX	Adenovirus	IL-12	2001
37. DeWeese, TL	Johns Hopkins, MD	Adenovirus	PB/PSE promoter	2001
38. Small, EJ	UCSF, CA	Adenovirus	PB/PSE promoter	2001
39. Dula, E	West Coast Clin. Res.	AAV	GM-CSF	2001
40. Freytag, SO	Henry Ford Hosp., MI	Adenovirus	CD/TK	2001
41. Scher, H	MSKCC, NY	Liposome	PSMA	2001
42. Corman, J	VA Puget Sound, VA	AAV	GM-CSF	2001
43. Pantuck, AJ	UCLA, LA	Vaccinia/ fowlpox	MUC-1/IL-2	2001
44. Vieweg, J	Duke, NC	RNA	hTERT	2001
45. Corman, J	VA Puget Sound, VA	Adenovirus	PSE promoter	2001
46. Vieweg, J	Duke, NC	RNA	PSA	2001
47. Dinney, CP	MD Anderson, TX	Adenovirus	Inf- α	2002
48. Dahut, W	NIH/NCI	Vaccinia/ fowlpox	PSA	2002

Table 3 (Continued)

Principal Investigator	Institution	Vector	Genetic Material	Year Reviewed
49. Morris, J	Mayo Clinic, MN	Adenovirus	NIS	2002
50. Kaufman, HL	Columbia, NY	Vaccinia/ fowlpox	PSA	2002
51. Zanetti, M	Univ. of Calif., CA	Liposome	hTERT	2002
52. Malkowicz, SB	Univ. of Penn., PA	Liposome	PSA	2002
53. Arlen, PM	NIH/NCI	Vaccinia/ fowlpox	PSA	2002
54. Corman, J	Virginia Mason, WI	Adenovirus	PSA	2003
55. Freytag, S	Henry Ford Hosp., MI	Adenovirus	HSV-TK	2003
56. Kantoff, P	Dana-Farber, MD	Vaccinia/ fowlpox	PSA	2003

trials registered with the OBA. Prostate cancer will remain a focus for laboratories due to the limited availability of treatments for advanced disease and the large population that is affected.

Corrective Gene Therapy

This approach repairs inherited or acquired genetic defects affecting the regulation of the cell growth cycle. A single prostate cancer cell may harbor several such mutations, giving it a survival advantage. The replacement of a damaged gene with the wild-type is often sufficient to suppress the growth of the cancer or lead to apoptosis, as evidenced in the pre-clinical and clinical trials outlined below.

p53

Tumor suppressor p53 is referred to as the molecular gatekeeper, protecting the integrity of the genome.⁴⁵ When cellular DNA damage occurs, wild type p53 is activated and stimulates the expression of *GADD45* (growth arrest and DNA damage) and the cyclin-dependent kinase (CDK) inhibitor, *p21*. p21 inhibits the CDK-cyclin D complex required to phosphorylate Rb,

thereby halting the cell at the G₁/S checkpoint to allow for DNA repair. If GADD45-mediated DNA repair is unsuccessful, p53 activates *bax* which mediates apoptosis.⁴⁶ p53 mutations occur in approximately one third of early prostate cancers,⁴⁷ and this increases in patients with advanced and metastatic disease.⁴⁸ Replacement of wild-type p53 with recombinant adenoviral vectors (Ad-p53) has resulted in growth inhibition and induction of apoptosis in prostate cancer both *in vitro*^{9,49} and *in vivo*.⁵⁰ In addition, intratumoral administration of Ad-p53 has been shown to slow the progression of prostate cancer to metastatic disease.⁵¹ Perhaps the most powerful use of p53 replacement is in combination with conventional therapies. Ad-p53 has been shown to sensitize prostate cancer cells *in vitro* and *in vivo* to chemotherapeutic agents⁵² and *in vitro* to radiation therapy.⁵³ Clinical trials are ongoing to determine the safety of such therapies.^{54,55}

p16

Similar to p53, tumor suppressor *p16* is a negative regulator of the cell growth cycle, preventing the phosphorylation of Rb by sequestering CDK4 of the CDK-cyclin D complex. Hypophosphorylated Rb arrests the cell at G₁, and loss of normal *p16* function is common in prostate cancer.⁴⁶ Small homozygous deletions have been identified as the major mechanism of inactivation of *p16*, which occurs in 40% of primary prostate cancers and 71% of advanced androgen-independent prostate cancers.^{56–58} Replacement of *p16* using adenoviral vectors suppressed cell growth and induced senescence in several prostate cancer cell lines including LNCaP (androgen-dependent) and C4-2, DU-145, PPC-1 and PC-3 (androgen-independent).^{10,59,60} Furthermore, intratumoral injection of Ad-CMV-p16 inhibited the growth of PC-3 tumor xenografts in experimental animal models and prolonged the animals' survival.⁶⁰ Currently, one clinical trial utilizing *p16* is in progress.

c-myc

The oncogene *c-myc* plays an important role in the progression of the cell cycle. As a transcription factor, it regulates the expression of CDC25 phosphatases that control CDK activity.⁶¹ *c-myc* amplification is a

common mutation in prostate cancer and its levels correlate with increasing tumor grade.⁶² The approach developed to suppress the overexpression of oncogenes delivers antisense RNA complimentary to the sense strand for that gene. This approach not only inhibits cancer cell growth, but also induces apoptosis through the down-regulation of *bcl-2* resulting from *c-myc* suppression. Steiner *et al.* developed a replication-incompetent retroviral vector to deliver antisense *c-myc* transcripts intratumorally in DU-145 nude mice xenografts. Reduction in tumor size and even complete tumor obliteration were observed.¹¹ One *c-myc* clinical trial is in progress.

Cytoreductive Gene Therapy

This approach to the molecular therapy of prostate cancer results in the direct or indirect killing of prostate cancer cells by replication-competent oncolytic viruses such as Ad-OC-E1a, pro-drug enzyme genes such as thymidine kinase, and apoptosis-inducing genes such as TRAIL. Perhaps one of the most successful approaches to the molecular therapy of prostate cancer, several clinical trials are ongoing to explore cytoreductive treatments.

Oncolytic Virus Therapy

Previous studies have shown that a replication-competent adenovirus injected intra-organ is sufficient to kill prostate cancer cells.⁶³ Although this therapy alone might be effective, adenovirus is taken up by the liver and lungs. This has the potential to cause hepatic and respiratory distress in immune-compromised cancer patients.⁶⁴ Controlling the replication of adenovirus solely to prostate cancer cells increases the safety of this tumor-eliminating therapy. The replication of adenovirus can be controlled by placing the early gene, *E1a*, a transcriptional activator of adenoviral late genes, under the control of a tissue-specific promoter. Without the expression of the essential late genes, the virus cannot reassemble and propagate in the host cell.⁶⁵ Recently, Henderson *et al.* demonstrated the ability to conditionally drive the replication of adenovirus by a prostate-specific enhancer (PSE) resulting in regression of *in vivo* androgen-independent LNCaP tumors.⁶⁶

Our laboratory has developed a conditional replication-competent adenoviral vector (Ad-OC-E1a) using the mouse *OC* promoter to restrict the expression of *E1a* to prostate epithelia and its supporting bone stroma in osseous metastases of prostate cancer. This virus appears to be more effective than the PSE-controlled virus at killing a broader spectrum of prostate cancer cells including LNCaP, C4-2, and ARCaP (PSA-positive), as well as PC-3 and DU-145 (PSA-negative). Intratumoral injection of Ad-OC-E1a was effective at obliterating subcutaneous androgen-independent PC-3 tumors in athymic mice. In addition, intraosseous C4-2 prostate cancer xenografts responded very well to the systemic administration of Ad-OC-E1a. One hundred percent of the treated mice responded with a drop in the serum PSA below detectable levels. At the conclusion of the study, 40% of the treated mice were cured of prostate cancer as no PSA rebound or prostate cancer cells in the skeleton were detected.⁵ Figure 4 shows the X-ray of a mouse with a C4-2 bone tumor before and after treatment with Ad-OC-E1a.

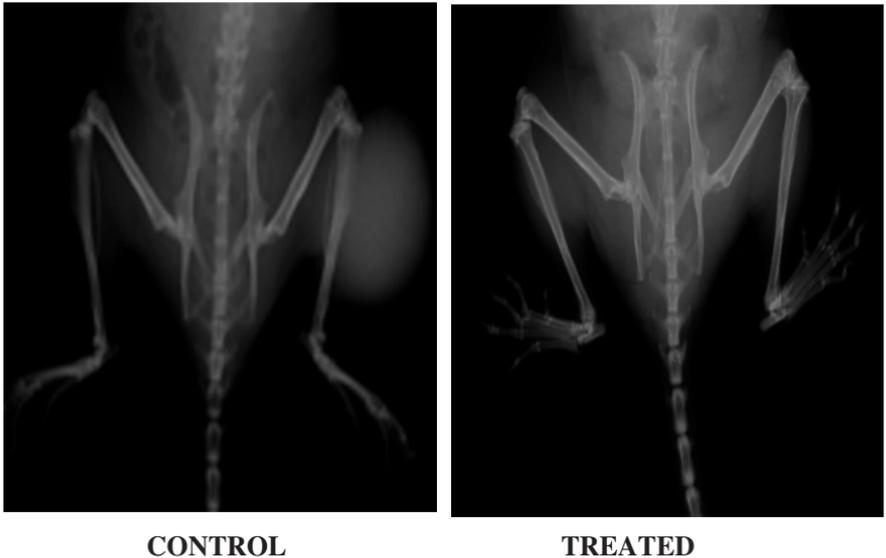


Fig. 4. Therapeutic effect of Ad-OC-E1a on prostate bone tumors. C4-2 prostate cancer cells were injected into the bone marrow space of the right tibia in SCID mice. The left panel shows the X-ray of an untreated mouse with a large mass and deformed tibia. The right panel shows the X-ray of a mouse following intravenous administration of Ad-OC-E1a.

It has been shown that controlling the expression of the early gene *E1b*, in addition to *E1a*, results in better viral replication control.⁶⁷ For this reason, we developed a second replication-competent adenoviral vector, Ad-hOC-E1, containing a single bidirectional human *OC* promoter to control the expression of both *E1a* and *E1b*. Under the control of this VDRE-containing promoter, viral replication is induced 10-fold higher than wild-type viral replication and cytotoxicity is enhanced with the administration of vitamin D.⁶⁸ Although still controversial,⁶⁹ some preclinical studies indicate that vitamin D has an antiproliferative effect on androgen independent prostate cancer.^{70,71} In our preclinical studies, administration of vitamin D₃ in nude mice with subcutaneous DU-145 xenografts demonstrated a therapeutic effect; however, the systemic administration of Ad-hOC-E1 in combination with vitamin D showed marked repression of the tumors, indicating the potential for clinical use.⁶⁸ The previously outlined preclinical findings have translated into a phase I clinical trial of *OC*-driven oncolytic adenoviral intratumoral therapy for androgen-independent prostate cancer.

Pro-drug Enzyme Gene Therapy

The efficacy of this approach depends on the conversion of a non-toxic pro-drug to an active cytotoxic drug by the enzymatic product of a delivered gene not normally expressed in human cells. Following systemic administration of the pro-drug, high concentrations of the lethal metabolite are only found locally at the tumor site, avoiding systemic toxicity. Fortunately, the toxic effect is not limited to the cells that produced the cytotoxic drug, but extends to neighboring cells via the bystander effect. This bystander effect is mediated by intercellular gap junctions and phagocytosis of debris from dying cells. By these means, the cytotoxic effect is amplified, compensating for low gene transfer efficiencies. The most widely applied pro-drug gene therapy in prostate cancer utilizes thymidine kinase from the herpes simplex virus (HSV-TK) and any one of several anti-herpetic agents such as ganciclovir (GCV), acyclovir (ACV), or valacyclovir (VAL). These nucleoside analogues are phosphorylated specifically by HSV-TK. The phosphorylated forms of the drugs are incorporated into cellular DNA during DNA replication resulting in chain termination and ultimately cell death.

The TK/pro-drug system is widely favored because it is a safe approach for cancer gene therapy for a number of reasons. First, apoptosis is induced in the transduced cell only when it divides, allowing the gene therapist to target cancer cells that divide more rapidly than non-cancerous cells. Second, the toxic effect only occurs when the pro-drug is administered allowing the cessation of treatment in the event of adverse effects. Third, several anti-herpetic nucleoside analogue drugs are clinically available, simplifying the approval process for the use of the pro-drug in clinical trials. Finally, the bystander effect greatly increases the killing efficiency of the therapy.

Previously, Eastham *et al.* demonstrated the sensitivity of human prostate cancer cells PC-3 and DU-145 to GCV cytotoxicity following the *in vitro* transduction of the cells with *HSV-TK* using a recombinant replication-deficient adenoviral vector. Similar results were obtained *in vivo* in subcutaneous xenografts of murine and human cancer models following the intralesional injection of Ad-RSV-TK and Ad-CMV-TK.⁷²⁻⁷⁴ Placing *TK* under the control of universal promoters such as RSV or CMV allows for the potential of *TK* activation in any cell without discrimination between normal and cancer cells. Therefore, intratumoral injection of the vector is required to prevent systemic dissemination of the virus. Scardino *et al.* developed the initial TK clinical trial in which a replication-deficient adenoviral vector was injected intralesionally to deliver *HSV-TK*, preceding the administration of GCV, in men with locally recurrent prostate cancer one or more years after definitive external beam radiotherapy. This trial realized the potential of this therapy by demonstrating the tumoricidal activity of this TK/GCV therapy as evidenced by sustained decreases in serum PSA and improved biopsies. As a result of adenoviral leakage from the injection site through the bloodstream and tracking to the liver, several of the patients experienced a self-limiting toxicity and one patient experienced moderate but reversible hepatic dysfunction and thrombocytopenia.¹⁵ To circumvent toxicity to the patient, Chung *et al.* developed a replication-deficient adenovirus to deliver *HSV-TK* driven by the *PSA* promoter.²⁹

Our laboratory developed a clinical protocol to test the hypothesis that the *OC* promoter can regulate *HSV-TK* expression specifically within a prostate cancer cell and the supportive stroma of a metastasis. We performed a phase I clinical trial enrolling 11 patients with locally recurrent

or metastatic prostate cancer. Two post-surgical local recurrences and nine metastatic lesions (five osseous and 4 lymph node) were injected with replication-defective Ad-OC-TK vector followed by the administration of oral valacyclovir.³ All patients tolerated this therapy with no severe adverse effects. Of the eleven men, local cancer cell death was observed in seven patients; however, the treated lesions of all eleven men showed histological changes as a result of the treatment. One patient demonstrated regression and stabilization of the treated lesion for 317 days post-treatment without alternative treatments, as demonstrated in Fig. 5.⁶ This trial opened the door to the development of future adenoviral vectors for the systemic treatment of osseous and visceral prostate cancer metastases.

Suicide Gene Therapy

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), also known as Apo-2 ligand, is a member of the tumor necrosis factor (TNF)

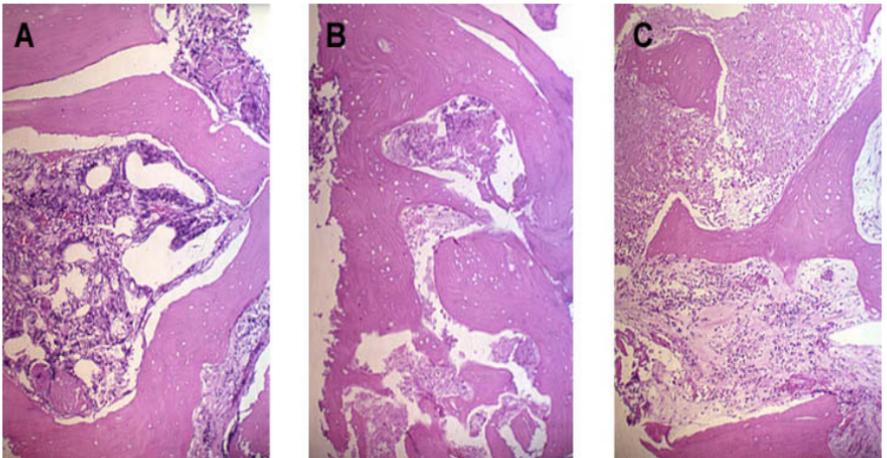


Fig. 5. Histomorphologic study of prostate cancer bone metastases treated with Ad-OC-TK. Tissue biopsies were collected from one patient and H&E-stained before (A), during (day 8) (B), and after (day 30) (C) treatment with Ad-OC-TK and VAL. Malignant prostate cancer cells surround healthy bone tissue before treatment (A). During treatment, cancer cells are replaced and fibrosis occurs (B). By day 30, malignant cells disappear and are replaced completely by fibrosis and inflammatory cells (C).

family and has been shown to preferentially kill tumor cells. Originally discovered because of its similarity to Fas-ligand, TRAIL is a 32 kDa type II transmembrane protein, whose C-terminal extracellular domain (amino acids 114–281) is homologous with other members of the TNF family.^{75,76} TRAIL induces apoptosis by binding to the death domain-containing receptors DR4 and DR5; however, the death signal is not transduced via the adaptor molecule FADD. Instead, the death protease FLICE2 is believed to be engaged, cleaving the initiating caspase 8 and beginning the caspase cascade.⁷⁷

The selectivity of TRAIL for cancer cells over normal cells makes it a prime candidate for anticancer therapy. TRAIL expression has been detected in several normal human tissues which suggests that TRAIL is not toxic to those cells *in vivo*.⁷⁸ In essence, these cells are protected from the apoptotic effects of TRAIL by an antagonistic decoy receptor, TRID, which lacks an intracellular domain and is found on the surface membrane of TRAIL-resistant cells.⁷⁷ Many prostate cancer cell lines including ALVA-31, DU-145, and PC-3 are extremely sensitive to TRAIL and undergo apoptosis when exposed; however, other cell lines such as LNCaP are highly resistant.⁷⁹ This resistance has been shown to be reversed by simultaneous administration of the chemotherapeutic agents doxorubicin, cisplatin, or etoposide⁸⁰ or by infection of those cells with adenovirus.⁸¹ For this reason, TRAIL is a promising suicide gene to consider for prostate cancer gene therapy; however, recent studies suggest that cultured human hepatocytes may be sensitive to TRAIL.⁸² Recombinant forms of TRAIL with reduced hepatotoxicity are being investigated⁸³ in addition to monoclonal antibodies that antagonize the TRAIL receptor in hepatocytes.⁸⁴

Immunotherapy

Prostate cancer, like most cancers, has developed mechanisms to evade the host immune system. Such mechanisms include the down-regulation of class I major histocompatibility complex (MHC) molecules on the tumor cell surface⁸⁵ as well as the down-regulation of the co-stimulatory B7 molecules.⁸⁶ These means of evasion result in decreased presentation of tumor antigens to cytotoxic T lymphocytes (CTLs). The goal of immunotherapy is to enhance the host immune response to prostate cancer

cells. Although several clinical trials have been initiated, only limited results have been published.

GM-CSF

After demonstrating that immunostimulatory molecules could transform cancer cells to activate a tumor-directed immune response,⁸⁷ Simons *et al.* developed the first gene therapy clinical trial protocol for prostate cancer. In this study, eight men with metastatic prostate cancer discovered during radical prostatectomy were administered autologous, *ex vivo* *GM-CSF* transduced, irradiated cancer vaccines. Unfortunately, due to the large number of *ex vivo* manipulations, the vaccination rate decreased, from the original enrollment number of eleven men to eight. Side effects were limited to pruritis, erythema, and swelling at the site of vaccination. Delayed-type hypersensitivity (DTH) reactions to untransduced autologous prostate cancer cells were positive in seven of the eight men, demonstrating the ability to mount a T cell response. Furthermore, new antibodies against prostate cancer polypeptides were discovered in the sera of three of the eight men, suggesting a B cell response to vaccination.⁸⁸ A subsequent clinical trial demonstrated no therapeutic value of systemic administration of recombinant *GM-CSF*, thereby suggesting the importance of local secretion of *GM-CSF* by the cancer vaccines.⁸⁹ Multiple clinical trials are in progress that employ a simple vaccine composed of irradiated prostate cancer cell line allografts expressing *GM-CSF*, which allows for multiple dosing.

IL-2

More recently, *in vivo* immunotherapy approaches have been used. Belldegrün *et al.* developed a gene therapy protocol in which 24 men with locally advanced prostate cancer were administered a DNA-liposome complex encoding the *IL-2* gene intraprostatically. This therapy was well tolerated, with side effects including mild hematuria, transient rectal bleeding, and perineal discomfort. Immunohistochemical analysis of the tumor site demonstrated T cell infiltration. Decreased serum PSA levels were observed in 16 of the 24 men on day 1 and 14 of the 24 by day 8.⁹⁰ Similar clinical trials are still in progress.

Future Directions

Among clinical trials that have been launched to demonstrate the safety and efficacy of adenoviral-based gene therapy on prostate cancer in the past few years, the most exciting results have come from the study of tumor/tissue-restricted replicative adenovirus (TRRA). This strategy allows the viral vector to propagate from a limited number of infected cells to the whole tumor mass, overcoming the problem of inadequate *in vivo* infectivity or biodistribution of the vector; however, TRRA-based therapy is not without its limitations. One of the major limitations of this therapy is the induction of the host immune system, targeted to eliminate the adenovirus from the body.

It will become critical to temporarily suppress the host's immune system or enhance the killing activity of the virus so that it can eliminate tumors within a shorter period of time, allowing it to escape from host immune attack. Among immune regulators, TGF- β and Fas-ligand are likely the best candidates for incorporation into TRRA for cancer gene therapy. There are five members reported in the TGF- β family; three of them (TGF- β 1, TGF- β 2, and TGF- β 3) are expressed in mammals. These three isoforms share a high degree of sequence homology in the mature domain, and have similar actions on cells in tissue culture. Suppression of the immune response includes the inhibition of T and B cell proliferation, the down-regulation of natural killer cell activity and CTL response, and the regulation of macrophage activation.⁹¹ TGF- β is a mediator of immune suppression that allows tumors to escape from immune surveillance, and its use has been explored to suppress the inflammatory and alloreactive immune responses in liver transplantation.⁹² Besides TGF- β , Fas-ligand is another immune modulator that has been explored for use in kidney transplant patients to suppress alloreactive lymphocytes.⁹³ Fas-ligand, also known as CD95 or APO-1, is a membrane-bound protein of the TNF family, and it is expressed in several cell types including tumors, T cells, and B cells. Cells expressing the Fas receptor undergo apoptosis when they encounter Fas-ligand.⁹⁴ Besides their immune modulation function, TGF- β and Fas-ligand are also strong growth inhibitors and induce apoptosis in a variety of cancers, including prostate cancer.⁹⁵ Therefore, incorporating TGF- β or Fas-ligand into a TRRA will potentially enhance

the tumoricidal activity of the vector and blanket the tumor site from the immune system, allowing the TRRA to complete its mission.

Once the TRRA is armed with immune suppressors, the need for tight regulation of viral replication increases. In addition to controlling *E1a* and *E1b* with prostate-specific promoters, *E4* should be considered. The *E4* transcription unit encodes regulatory genes that are critical for viral replication, involving the shut-down of host gene expression and the facilitation of late viral gene expression.⁹⁶ Mutant adenoviral vectors lacking the *E4* region are severely replication defective and can only propagate in *E4*-expressing cells.⁹⁷ Placing *E4* under the control of a prostate-specific promoter should provide additional safety. TK should also be considered for incorporation into a TRRA. In addition to enhancing the tumor-killing activity of a TRRA, TK will allow a gene therapist to monitor adenoviral replication *in vivo* via positron emission tomography (PET) imaging.⁹⁸

Finally, because all tissue-specific promoters have basal activity levels in non-target tissues, the potential exists for accumulation of adenovirus in the liver, resulting in undesirable toxic effects caused by significant expression of therapeutic genes in the liver. Modification of the adenoviral fiber knob can increase viral tropism toward cancers and away from the liver and other vital organs.^{99,100} This is also beneficial because it is well known that expression of the coxsackievirus-adenovirus receptor (CAR) is frequently down-regulated in cancers. Our laboratory is currently investigating an Ad-5/35 hybrid adenovirus for use in prostate cancer gene therapy whose infectivity is CAR-independent and lacks liver tropism.

Conclusion

The safety and efficacy of gene therapy for prostate cancer has been demonstrated through various preclinical and clinical trials. In recent years, interest in this field has expanded and will continue to do so. It is conceivable that, in the near future, a safe gene therapy modality will be developed to replace hormone ablation therapy which causes unpleasant side effects, decreases the quality of life of the patient, and only temporarily controls the disease. Due to its convenient administration through ultrasound-guided transrectal injection, it is also conceivable that gene therapy applied in an outpatient clinic may someday replace radical

prostatectomy and radiation therapy to treat early prostate cancer. Factors that limit the disseminated use of gene therapy as a standard of care include time, funding, and fear from the general public; however, these should diminish as the number of successful clinical trials increase.

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5

CHEMOTHERAPY FOR PROSTATE CANCER

Samuel K. Kulp, Kuen-Feng Chen
and Ching-Shih Chen

*Division of Medicinal Chemistry and Pharmacognosy
College of Pharmacy, The Ohio State University
Columbus, OH, USA*

Introduction

For several decades, androgen deprivation has been the mainstay of therapy for metastatic prostate cancer. The high response rates to this approach, which near 90% by PSA criteria,¹ underscore the activity of this treatment modality in prostate cancer patients. However, satisfaction with the efficacy of androgen deprivation therapy is tempered by the transience of its effects on disease progression. The median time to progression has been reported to range from 9 to 30 months.^{1,2} Although secondary hormonal maneuvers will further benefit a subset of these patients, they are characterized by even shorter durations of response (<6 months).^{3,4} Continued progression in the face of castrate levels of androgen and the lack of responsiveness to hormonal therapies defines the emergence of hormone-refractory prostate cancer (HRPC) for which the prognosis is bleak. The treatment of hormone-refractory tumors constitutes a significant challenge in the management of patients with prostate cancer.

Although HRPC has historically been considered a chemorefractory disease, chemotherapy has an established role in the management of patients with HRPC. Evidence obtained over the last decade has shown some chemotherapeutic regimens to be unquestionably active in these patients.⁵ Indeed, guidelines set forth by the National Comprehensive

Cancer Network (NCCN) currently identify six chemotherapeutic regimens as options for the management of HRPC.⁶ These options, however, are solely palliative. The oft-cited caveat that applies to these active chemotherapeutic regimens is that none have been shown definitively to extend the survival of HRPC patients beyond the typically reported median of 12–16 months.^{3,7} Certainly, palliation and improvement or maintenance of quality of life are important and valuable clinical goals in the management of HRPC patients, but the short duration of response and the lack of clear survival benefit of current chemotherapeutic options emphasize the need for new agents and strategies for the treatment of HRPC.

Opportunities for the development of therapies that achieve, not only palliation, but also prolonged survival, emerge from the elucidation of the cellular and genetic abnormalities that underlie prostate carcinogenesis, malignant progression, prostate cancer cell survival, and resistance to therapy. Such findings from the “bench” have revealed promising targets that can guide the rational development of novel therapeutic agents that make sense in the context of prostate cancer biology.

This chapter is broadly organized into two parts. The first is a brief overview of the current combinatorial chemotherapeutic regimens identified by the NCCN as options for treatment of HRPC. Thorough reviews of the clinical findings of trials utilizing these regimens, as well as their component agents as monotherapy have been presented elsewhere.^{3,8–11} The second section discusses investigational agents designed to target specific molecules within pathways involved in cancer cell survival with a specific focus on signaling through phosphoinositide-3-kinase (PI3K)/Akt.

Present Chemotherapies for HRPC

The NCCN recommends combination chemotherapies as a treatment option in the management of patients with HRPC.⁶ It is understood that these regimens are solely palliative and yield no definitive survival benefit. Nonetheless, their inclusion in these guidelines illustrates the diminished pessimism associated with chemotherapy for HRPC. These regimens include ketoconazole/doxorubicin, mitoxantrone/prednisone, and various estramustine-based combinations that include etoposide, vinblastine, paclitaxel and docetaxel. Among these combinations are two

major chemotherapeutic approaches that represent the most frequently used regimens in the management of HRPC: mitoxantrone/prednisone and estramustine/antimicrotubule agents.^{3,12}

Mitoxantrone-Based Regimens

Mitoxantrone is a semisynthetic anthracenedione. Two large, randomized trials of corticosteroids alone vs. in combination with mitoxantrone demonstrated that the combination regimens (mitoxantrone plus prednisone or hydrocortisone) achieved greater palliation and better quality of life in symptomatic HRPC patients. Despite these important clinical benefits, overall survival was not affected.^{3,12} Nonetheless, the improved palliation and improvements in quality of life led to the FDA approval of this combination for patients with HRPC.

Estramustine Phosphate/Antimicrotubule Agent Combinations

Estramustine phosphate (EMP) is a conjugate of 17β -estradiol and nitrogen mustard. As such, it possesses both estrogenic and alkylating activities; however, its cytotoxic effects on cancer cells are apparently unrelated to its alkylating actions. EMP induces cell death through its binding to microtubule-associated proteins resulting in microtubule disassembly.^{13,14} In combination with cytotoxic antimicrotubule agents, such as the vinca alkaloids and taxanes, antitumor effects were potentiated preclinically and translated to clinical trials in which enhanced palliation and antitumor activity were documented.^{3,15} No significant effects on overall survival, however, were observed. However, tantalizing findings from Phase II trials of combinations containing EMP and paclitaxel or docetaxel reveal median overall survival durations as high as 20 months.³ These promising results have yet to be confirmed in larger, randomized clinical trials.

Future Therapies for HRPC

Despite the valuable palliative role of current chemotherapeutic regimens for HRPC patients, improvement in the survival rates of these patients will

require novel approaches. Efforts to develop new, more selective, and potentially less toxic, molecularly targeted agents have benefited from recent experimental advances identifying genetic and cellular aberrations implicated in prostate cancer cell survival and progression against which these agents can be directed. Signal transduction pathways and networks are frequently altered in cancer cells and represent promising targets for novel therapeutic interventions. The recent clinical successes of trastuzumab (Herceptin, Genentech) and imatinib mesylate (STI571; Gleevec; Novartis) in patients with defined abnormalities in HER2 and bcr-abl or c-kit, respectively,¹⁶⁻¹⁸ has spurred enthusiasm for the discovery and development of novel therapeutic agents targeting aberrant signaling molecules in cancer cells. The PI3K/Akt pathway is upregulated in multiple human cancers, including that of the prostate. Substantial evidence points to a prominent role for abnormal PI3K/Akt signaling in prostatic malignancy and establishes it as a relevant and attractive target for therapeutic intervention.

This section of the review focuses on components of the PI3K/Akt signaling pathway. It is organized by molecular target and includes overviews of the corresponding pathways, their relevance to prostate cancer, and descriptions of new agents directed at these molecules that are currently being developed and evaluated. Agents that have been evaluated clinically in prostate cancer patients are emphasized. Information on clinical trials was gathered from the NCI clinical trials website (http://www.cancer.gov/search/clinical_trials/), unless otherwise indicated.

PI3K/Akt Signaling Pathway

The PI3K/Akt signaling cascade occupies a well-documented, central role in promoting cell survival and proliferation downstream of various extracellular stimuli,^{19,20} commonly those transduced by receptor tyrosine kinases (RTKs). Signaling through this pathway is initiated by RTK-mediated activation of PI3K, which catalyzes the phosphorylation of plasma membrane lipids to form phosphatidylinositol-3,4-bisphosphate [PI(3,4)P₂] and PI-3,4,5-triphosphate (PIP₃). Signaling proteins containing pleckstrin homology (PH) domains, such as Akt and phosphoinositide-dependent kinase-1 (PDK-1), directly bind to these lipid products resulting

in their co-localization at the plasma membrane, where Akt is phosphorylated at Thr308 by PDK-1 and at Ser473 by an undefined mechanism.^{21–23} Phosphorylation at both sites fully stimulates Akt's kinase activity. Activated Akt conveys pro-survival signals by phosphorylating multiple substrates involved in the regulation of apoptosis and cell proliferation.^{20,24,25} Proapoptotic and growth inhibitory substrates that are inactivated by Akt include the proapoptotic proteins, BAD and procaspase 9, transcriptional factors of the forkhead family, which support transcription of Fas ligand and the cyclin-dependent kinase inhibitor p27^{kip1}, and glycogen synthase kinase 3 (GSK3), which inhibits cyclin D1. Substrates activated by Akt include I κ B-kinase- α (IKK α), which frees NF- κ B to induce gene expression.²⁶ Negative regulation of PI3K/Akt signaling can be achieved by two phosphatases, phosphatase and tensin homologue deleted on chromosome ten (PTEN) and protein phosphatase 2A (PP2A). Through its lipid phosphatase activity, PTEN dephosphorylates PIP₃ at the 3-position, thereby countering the action of PI3K and inhibiting activation of Akt. PP2A opposes the activity of PDK-1 by directly dephosphorylating activated Akt.

Substantial evidence indicates that the PI3K/Akt signaling pathway is accentuated in cancer cells. Activation of the pathway occurs indirectly via inactivation of PTEN, the product of one of the most commonly mutated tumor suppressor genes in human cancers.^{27,28} The functional loss of PTEN leading to overactive phosphoinositide signaling and subsequent Akt phosphorylation is a feature of prostate cancer, particularly in advanced disease.^{29–34} Functional activation of this pathway also occurs as PI3K/Akt propagates signals from many of the RTKs involved in growth factor signaling known to be aberrantly upregulated in prostate cancer.³⁵ Indeed, elevated levels of phosphorylated Akt and Akt activity have been demonstrated in prostate cancer cell lines and human tumor specimens in association with corresponding changes in downstream signaling components, such as p27^{kip1} and BAD.^{36–39} Recently, in patient-matched normal epithelium, PIN, and invasive carcinoma, Paweletz *et al.*³⁸ combined laser capture microdissection and protein microarray analysis to show that disease progression was marked by significant elevations in phospho-Akt levels, which were directly correlated with decreased apoptosis; thereby linking suppression of apoptosis with activated Akt. Evidence of an

oncogenic role for dysregulated signaling through PI3K/Akt in prostate cancer is derived from transgenic mouse models. *Pten* heterozygous mice (*Pten*+/-) and mice with prostate-specific overexpression of Akt exhibit phenotypic overlap in the development of PIN.^{40,41} While a *Pten*+/- background accelerates prostate tumorigenesis in TRAMP mice and mice with inactivating mutations of the homeobox gene *Nkx3.1* or the gene for p27^{kip1},^{40,42,43} prostate-specific biallelic inactivation of *Pten* alone is sufficient to initiate early onset prostate tumorigenesis and metastasis.^{44,45} In addition to oncogenesis, Akt is important for endothelial cell survival and angiogenesis,⁴⁶ and is a determinant of chemotherapeutic resistance in multiple cancers.^{36,47-57}

Together, these clinical and experimental findings underscore the prominent role of aberrant PI3K/Akt signaling in prostatic malignancy. This, along with its position as a convergence point for multiple signaling pathways, establishes PI3K/Akt signaling as a relevant and attractive target for therapeutic intervention.

Receptor Tyrosine Kinases

Receptor tyrosine kinases are transmembrane receptors that are activated by a wide variety of ligands. High affinity ligand binding triggers dimerization of receptors resulting in the close apposition of the intracellular tyrosine kinase domains and subsequent tyrosine autophosphorylation. The activated receptors provide docking sites for other intracellular signaling molecules, which are in turn activated by phosphorylation or conformational changes thereby initiating signaling cascades that ultimately alter the transcription of genes regulating proliferation, apoptosis, and differentiation.⁵⁸ Aberrant up-regulation of signaling through RTKs and its subsequent activation of the PI3K/Akt pathway is a frequent defect in many cancers, including prostate cancer.^{35,59} Mechanisms that have been shown to up-regulate RTK activity include receptor overexpression, activating mutations or chromosomal rearrangements, and overproduction of ligands.⁶⁰ The frequent implication of dysregulated growth factor-RTK pathways in prostate carcinogenesis and androgen-independent progression represents a rationale for directing therapeutic approaches against RTK signaling in this disease. Thus far, two approaches for the

direct inhibition of RTKs have achieved degrees of clinical success: (a) Targeting the extracellular ligand binding domains with monoclonal antibodies (Fig. 1, site 1), and (b) targeting the intracellular ATP-binding sites with small molecules (Fig. 1, site 2).

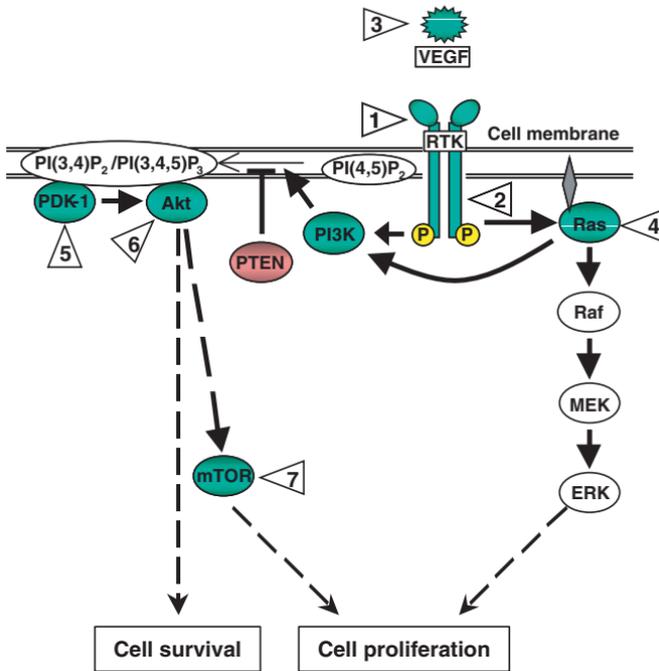


Fig. 1. Therapeutic targets within the PI3K/Akt pathway. The figure shows a schematic representation of the PI3K/Akt signaling pathway and its components that are targeted by the investigational agents discussed in this chapter. The numbered triangles identify sites of action of these agents which are described in the text: (1) ectodomain of receptor tyrosine kinases (targeted by monoclonal antibodies), (2) ATP-binding site of receptor tyrosine kinases (small molecule inhibitors), (3) vascular endothelial growth factor (monoclonal antibody), (4) Ras (farnesyl transferase inhibitors and antisense agents), (5) PDK-1 (small molecule inhibitors), (6) Akt (phospholipid analogs), and (7) mTOR (rapamycin analogs). mTOR, mammalian target of rapamycin; PDK-1, phosphoinositide-dependent kinase-1; PI3K, phosphatidylinositol-3-kinase; PI(4,5)P₂, phosphatidylinositol-4, 5-bisphosphate; PI(3,4,5)P₃, phosphatidylinositol-3,4,5-triphosphate; PTEN, phosphatase and tensin homologue deleted on chromosome 10; RTK, receptor tyrosine kinase; VEGF, vascular endothelial growth factor.

Epidermal Growth Factor Receptor (EGFR) Family

The EGFR superfamily is composed of four structurally related RTKs: HER1 (erbB1, EGFR), HER2 (neu, erbB2), HER3 (erbB3) and HER4 (erbB4). Of these, both HER1 and HER2 have been implicated in prostate cancer progression to androgen-independence. HER1 was expressed in nearly 90% of the androgen-independent metastases examined, in addition to evidence of a shift to an autocrine mechanism of receptor activation in these refractory metastatic tumor specimens.⁶¹ In addition, a mutant HER1 carrying a deletion in the extracellular domain was shown to be constitutively active in the absence of ligand and to be highly expressed in malignant prostate tissue. Moreover, the level of expression of this variant increased in parallel with progression to malignancy.⁶²

The case for HER2's role in prostate cancer biology has been considered less definitive. This uncertainty stems from highly variable data on expression levels of the receptor in HRPC tissues, and the absence of HER2 gene amplification as occurs in a subset of breast cancer patients,^{3,63} in whom dramatic clinical success was achieved with HER2 inhibition. On the other hand, two independent groups reported nearly identical findings showing that the frequency of HER2 overexpression increased with progression from androgen-dependent to androgen-independent cancer,^{64,65} suggesting a role for HER2 in the acquisition of hormone-independence. Supporting this contention is data from experimental systems which show that signaling through HER2 activates two parallel cascades, the Akt and MAPK pathways, which converge to phosphorylate and activate AR in a ligand-independent manner.^{66,67}

In light of the likely importance of HER1 and HER2 in prostate cancer progression, therapeutic strategies for the direct suppression of these RTKs have been developed. Cetuximab (C225, Erbitux; ImClone) and ABX-EGF (Abgenix) are monoclonal antibodies directed against the extracellular ligand-binding domain of HER1. These antibodies block native ligand binding, HER1 autophosphorylation, and activation of the RTK.⁶⁸ Cetuximab has completed Phase I/II evaluation in which disease stabilization was achieved in combination with doxorubicin for androgen-independent prostate cancer.⁵⁹ Phase II evaluation of the fully humanized ABX-EGF in patients with HER1-overexpressing, hormone-resistant

prostate tumors is underway. Trastuzumab (Herceptin, Genentech), a humanized monoclonal antibody directed against HER2, is clinically effective in women with HER2-overexpressing breast tumors. Against prostate cancer, it displayed antitumor activity in both androgen-dependent and -independent human prostate cancer xenograft models in combination with paclitaxel. As a single agent, however, androgen-independent tumors were unaffected by treatment.⁶⁹ The results of a recent Phase II study revealed a lack of efficacy in prostate cancer patients that did not overexpress HER2. In addition, difficulties in collecting metastatic tumor tissue thwarted identification of potentially responsive, HER2-overexpressing patients.⁷⁰ Another humanized monoclonal antibody directed against the HER2 ectodomain is 2C4 (pertuzumab; Genentech), which hinders the recruitment of HER2 into heterodimers with other HER receptors.⁷¹ 2C4 inhibited androgen-independent prostate tumor growth in the 22Rv1 xenograft model⁷² and will be evaluated in HRPC patients in a recently initiated Phase II trial.

A second strategy for therapeutic targeting of HER1 and HER2 signaling is the inhibition of tyrosine kinase activity using membrane-permeable small molecules. These compounds interfere with the ATP binding site in the intracellular domain thereby preventing ligand-induced receptor activation. Gefitinib (ZD1839; Iressa; AstraZeneca) is a highly selective HER1 tyrosine kinase inhibitor with a broad spectrum of antitumor activity against multiple types of human tumor xenografts, including prostate tumors. Combinations of gefitinib with conventional cytotoxic agents displayed additive and supra-additive effects in suppressing tumor growth.⁷³ Promising Phase I results in patients with a variety of solid tumors that express HER1, including advanced prostate cancer, has led to current Phase II studies in patients with HRPC and other malignancies.⁷⁴ Other small molecule inhibitors include the HER1/HER2 dual tyrosine kinase inhibitor, PKI-166 (Novartis), which exhibited antitumor activity against both androgen-dependent and androgen-independent prostate cancer xenografts.⁷⁵

Platelet-Derived Growth Factor Receptor (PDGFR) Family

PDGFRs are RTKs that occur as α -homodimers (PDGFR α) or β -homodimers (PDGFR β). Their ligands are PDGFs, which compose a

family of disulfide-bonded dimeric isoforms. Four different PDGF chains have been identified: PDGF-A, -B, -C and -D, which exist as AA, BB, AB, CC and DD dimers. Ligand-induced receptor activation results in signaling through phosphoinositide-3-kinase (PI3K), phospholipase C and Ras/Raf/mitogen-activated protein kinase (MAPK) pathways.⁷⁶⁻⁷⁸ PDGFs typically target connective tissue cell types, like fibroblasts and smooth muscle cells, to influence their growth, survival and function.⁷⁸

The co-expression of PDGF and PDGFRs has been shown in numerous human malignancies, including prostate cancer,⁷⁹ suggesting a role in autocrine/paracrine growth stimulation. Furthermore, the PDGF-A chain and PDGFR α were preferentially expressed over the B chain and β -receptor in prostate tumors cells as determined by immunohistochemistry, but could not be detected in the normal cells surrounding the lesion.⁷⁹ Similarly, these two proteins were expressed in prostatic intraepithelial neoplasia (PIN) and in the tumor cells of prostate cancer bone metastases, but not in the adjacent normal bone stroma.^{80,81} More recently, PDGFR was detected immunohistochemically in $\geq 80\%$ of primary and metastatic prostate tumors.⁸² These observations suggest that PDGFR expression is a feature of prostate cancer cells, occurs early in carcinogenesis, and is maintained throughout progression.

Small molecule inhibitors of PDGFR kinase activity that interfere with the ATP-binding pocket have been evaluated preclinically and in early clinical trials for efficacy against prostate cancer. Leflunomide (SU101; SUGEN Pharmaceuticals) is a potent inhibitor of PDGFR kinase activity *in vitro* and possesses antitumor activity against PDGFR-driven growth of xenograft tumors, including those derived from prostate cancer.⁸³ Phase II evaluation of leflunomide in pretreated patients with HRPC exhibited only modest clinical benefits, despite the immunohistochemical detection of PDGFR in $\geq 80\%$ of primary and metastatic prostate tumors. This modest activity was attributed in part to the lability of the drug *in vivo*.⁸² Imatinib mesylate (Gleevec, Novartis), the well known inhibitor of the Bcr-abl and c-kit tyrosine kinases, is an equally potent inhibitor of PDGFR kinase activity *in vitro*.⁸⁴ In a nude mouse xenograft model of bone metastasis, systemic treatment with imatinib mesylate reduced bone destruction caused by growth of prostate cancer cells after intratibial implantation. This effect was enhanced by co-treatment with paclitaxel

and correlated with decreased levels of phosphorylated PDGFR. Notably, in untreated animals, strong expression of PDGFs and PDGFRs were detected in tumor cells within bone, but not in those growing outside the bone.⁸⁵ The findings support the clinical utility of PDGFR inhibition in the context of metastatic prostate cancer, and identify differences in tumor microenvironment as a factor that could influence the efficacy of RTK inhibitors.

Tropomyosin Receptor Kinase (trk) Family

Members of the trk family of RTKs, a subtype of the neurotrophin receptor family, are activated by the neurotrophin ligands: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophins (NT)-3 and -4. Activation of trk receptors leads to downstream signaling through the Ras/Raf/MEK/MAPK and PI3K/Akt pathways.⁸⁶ The functional relevance of the trk receptor-neurotrophin axis to prostate carcinogenesis and progression is evident in the changes in the expression patterns of trk receptor types and neurotrophin ligands that occur during these processes. Specifically, in the normal prostate epithelium, a stromal-to-epithelial directed NGF-trkA paracrine axis exists, upon which the epithelial cells are not critically dependent for survival. During progression to adenocarcinoma, however, cancer cells acquire the ectopic expression of neurotrophins BDNF and NT-3, and their cognate receptors, trkB and trkC, thereby establishing a functional autocrine pathway. Also, cancer cells lose expression of p75NTR, which is a low affinity NT receptor that, in the appropriate context, can suppress trkA activity and induce apoptosis. Notably, tumor cells acquire a unique dependence on these acquired neurotrophin-trk pathways for survival.⁸⁷ Thus, neurotrophin-trk signaling represents a rational target for therapeutic intervention in HRPC.

Small molecule indolocarbazole analogs, designated CEP-751 and CEP-701 (Cephalon), are potent inhibitors of the tyrosine kinase activity of trk receptors. In a series of very careful studies, these compounds exhibited significant antitumor and antimetastatic activities in Dunning rat prostate cancer models, as well as human tumor xenografts through induction of apoptosis. By inhibiting trk activities, these agents blocked signaling

through both pathways, Ras/Raf/MEK/MAPK and PI3K/Akt, with the latter pathway identified as the critical survival pathway emanating from ligand-activated trk receptors.⁸⁸⁻⁹⁰ Currently, oral CEP-701 is being evaluated in a Phase II trial for patients with HRPC (www.hopkinskimmel-cancercenter.org/clinicaltrials/).

Vascular Endothelial Growth Factor Receptors (VEGFR)

Tumor growth requires new blood vessel formation, i.e. angiogenesis, which is dependent upon the paracrine action of proangiogenic molecules that are produced by tumor cells and act on the vascular endothelium. Among the proangiogenic factors that have been identified, VEGF (VEGF-A) plays a central role in tumor angiogenesis.⁹¹ Other members of the VEGF family include VEGF-B, -C, -D and -E. VEGF induces angiogenesis by stimulating the proliferation, differentiation, migration and survival of endothelial cells through activation of the RTK, VEGFR-2 (Flk-1, KDR).^{92,93} In prostate cancer, tumor growth and metastasis are dependent upon angiogenesis, and intratumoral vascular density is positively correlated with invasiveness and poor prognosis.^{91,94} Moreover, established human prostate cancer cells lines, as well as human prostate cancer tissue specimens, can express functional VEGFRs.^{95,96} These latter findings suggest the existence of an autocrine pathway through which prostate cancer cells themselves serve as a target for the stimulatory actions of VEGF. Thus, VEGF-induced signaling through VEGFRs is a logical therapeutic target in prostate cancer.

In the preclinical setting, the immunoneutralization of VEGF with monoclonal antibodies (Fig. 1, site 3) has been a successful strategy for the inhibition of angiogenesis and prostate tumor growth in animal models.⁹⁷⁻⁹⁹ Bevacizumab (Avastin; Genentech), the recombinant humanized anti-VEGF monoclonal antibody, completed initial Phase I trials¹⁰⁰ and is currently being evaluated in numerous Phase II/III clinical trials for a variety of malignancies. Updated findings of a Phase III trial for patients with HRPC who received bevacizumab in combination with docetaxel and estramustine revealed high rates for PSA response (79%), measurable response (42%) and stable disease for 6 weeks or more (32%). Survival data from this study is not yet mature.¹⁰¹

ZD6474 (AstraZeneca), an orally available small molecule designed to be an inhibitor of the VEGFR family tyrosine kinase domain, caused profound regression of established PC-3 xenograft tumors in mice.¹⁰² Interestingly, this compound also inhibits HER1 signaling which may add to its therapeutic efficacy.¹⁰³ It is currently being evaluated in a series of active Phase II trials for non-prostate cancers. SU5416 (semaxanib; Sugen) is another small molecule antiangiogenic agent that is a selective inhibitor of VEGFR-2 tyrosine kinase activity. Despite promising results from preclinical *in vivo* studies in prostate cancer models¹⁰⁴ and Phase I/II trials,¹⁰⁵ development of the agent was halted at Phase III.¹⁰⁶

Ras

The Ras family of small GTP-binding proteins mediates signal transduction between the membrane and the nucleus. Its activation is a downstream component of signaling pathways emanating largely from a variety of RTKs which ultimately affect cell proliferation, differentiation, and survival. In order for Ras to function in signal transduction, it must be localized to the cell membrane. This is accomplished by the covalent addition of a farnesyl moiety to the C-terminal Cys of Ras by the enzyme farnesyl transferase.¹⁰⁷ Ras is inactive when bound to GDP. Ras activation occurs through its association with son of sevenless (SOS), which is bound to the activated RTK via the adaptor protein, Grb2. The subsequent conformational change in Ras permits the exchange of GTP for the GDP. Active, GTP-bound Ras then engages a variety of effectors that initiate a multitude of downstream signaling cascades of which the best characterized are the PI3K/Akt and Raf/MEK/MAPK pathways.¹⁰⁸ In the latter pathway, active Ras recruits Raf to the membrane, and then activates it. Raf in turn phosphorylates and activates MAPK kinases (MEK1/2), which then phosphorylate and activate MAPKs (extracellular signal-regulated kinases [ERK1/2]). ERK1/2 phosphorylate a number of substrates, including transcription factors, which can result in mitogenesis.

Activating mutations of Ras are common defects in human cancers;¹⁰⁹ however, such mutations are infrequent in prostate cancers.^{110–112} Nonetheless, accumulating evidence supports the contention that wild-type Ras can be chronically activated by the aberrant autocrine/paracrine

growth factor signaling that is characteristic of advanced prostate cancer³⁵ and implicates signaling through Ras/Raf/MAPK in prostate cancer progression to androgen-independence.^{113,114} Thus, multiple components of this pathway appear to be potential targets for therapeutic intervention in this disease. For the purposes of this chapter, however, cross-talk between the PI3K/Akt and Ras/Raf/MEK/MAPK pathways at the level of Ras-induced activation of PI3K¹⁰⁸ identifies Ras as a therapeutic target for inhibiting PI3K/Akt signaling (Fig. 1, site 4).

The antisense compound, ISIS 2503 (Isis Pharmaceuticals), selectively inhibits the expression of H-Ras, and has been reported to inhibit cancer cell growth and to suppress H-Ras protein levels both *in vitro* and in xenograft models.¹¹⁵ Notably, ISIS 2503 was shown to be effective even in cell lines with mutated K-Ras, as well as those without *ras* mutations, indicating a spectrum of activity not limited to cancers with mutated H-*ras*.¹¹⁶ Although the results of Phase I trials were promising, patients with prostate cancer were not included in these studies,^{116,117} or in any current Phase II trial evaluating this drug. Interestingly, ISIS 5132 (Isis Pharmaceuticals), an antisense drug targeting the downstream kinase, c-Raf, exhibited significant antitumor activity against a broad range of cancer cell types, including prostate cancer, in preclinical models,^{118,119} leading to recent Phase I and II trials in patients with HRPC.¹²⁰ The farnesylation and subsequent localization of Ras to the cell membrane is a prerequisite for its ability to engage its partners and participate in signaling. Thus, the development of small molecule farnesyl transferase inhibitors (FTI) has been a focus for the therapeutic targeting of this signaling molecule.¹²¹ Preclinically, FT inhibition has proven to be an effective strategy for growth suppression of prostate cancer cells *in vitro* and in prostate xenograft tumors *in vivo*.^{122–126} The nonpeptidomimetic FTI, tipifarnib (R115777; Zarnestra; Johnson & Johnson) is undergoing evaluation in a number of clinical trials including a Phase II trial in HRPC patients. However, preliminary findings indicated no significant antitumor activity in these HRPC patients with the dose and schedule used, despite partial inhibition of FT activity and evidence of protein prenylation.¹²⁷ Other FTIs that have reached clinical trials, lonafarnib (SCH66336; Sarasar; Schering-Plough), BMS-214662 (Bristol-Myers Squibb), and L-778,123 (Merck) have not been evaluated in patients with prostate cancer.

PDK-1/Akt

The roles of PDK-1 and Akt in PI3K signaling and the importance of the pathway to prostate carcinogenesis and disease progression have been discussed. Despite the potential value of inhibiting PDK-1/Akt signaling, there exists a relative lack of clinically available agents directed at these molecules, in contrast to drugs targeting RTKs and the more downstream component of the pathway, mTOR (mammalian target of rapamycin; discussed below). Thus, the development of specific and effective small molecule inhibitors of PDK-1 and Akt (Fig. 1, sites 5 and 6, respectively) would fulfill a need in the development of therapies targeting this pathway. Industry efforts to develop drugs against the proximal components of the PI3K pathway include agents in preclinical studies targeting PI3K (Lilly, Iconix, Echelon-ComGenex), Akt (Kinetek, Celgene, Abbott, Kinacia), and integrin-linked kinase (ILK), a PDK-2 candidate (Kinetek).¹²⁸

Despite industrial and academic efforts, no direct, small molecule inhibitor of Akt is available. Worth noting, however, is recent preclinical work demonstrating the efficacy of a novel alkylphospholipid, perifosine, against PTEN-null human PC-3 prostate cancer cells in which Akt is identified as an important target of perifosine action.¹²⁹ Perifosine inhibited PC-3 cell growth at low micromolar concentrations and caused rapid loss of Akt phosphorylation and activity in association with induction of p21. This phospholipid analog had no direct effects on PI3K, PDK-1, or Akt activities, but decreased membrane localization of Akt. Myristoylated-Akt abrogated these drug-induced effects, leading the authors to hypothesize that perifosine targets Akt by interacting with its PH-domain, thereby inhibiting its recruitment to the cell membrane by 3'-phosphorylated products of PI3K. Currently, two Phase II clinical trials are evaluating perifosine in prostate cancer patients with recurrent hormone-sensitive, and metastatic androgen-independent disease.

The only inhibitor of PDK-1, the upstream, activating kinase of Akt, currently in clinical trials is UCN-01 (7-hydroxystaurosporine; Kyowa Hakko Kogyo). However, this agent is not specific, having been shown to inhibit multiple kinases, including PDK-1, PKC, and cyclin-dependent kinases.^{130,131} The apparent basis for this lack of specificity is its interaction

with the ATP binding site, a mechanism associated with the broad-spectrum kinase inhibition that characterizes other competitive inhibitors of ATP binding, such as flavopiridol.¹³² Nonetheless, Phase I evaluation of UCN-01 as monotherapy in patients with resistant solid tumors has been completed with some promising results in non-prostate cancer patients, and active Phase I trials are evaluating alternative dosing schedules and combinations with other chemotherapeutics.¹³²

Another class of novel small molecule inhibitors of PDK-1/Akt signaling is being developed by the authors of this chapter. Although not yet in clinical trials, the lead compound, OSU-03012, is in preclinical development as part of the NCI's Rapid Access to Intervention Development (RAID) program and will be described briefly. Development of OSU-03012 evolved from mechanistic studies on the antitumor effects of the cyclooxygenase-2 (COX-2) inhibitor, celecoxib, in prostate cancer cells, which demonstrated that the two pharmacological effects of celecoxib (COX-2 inhibition and apoptosis induction) were separable, and which identified the structural requirements for each of these activities.^{133,134} Moreover, blocking Akt activation through the inhibition of PDK-1 was shown to be a major COX-2-independent, antitumor target for celecoxib in prostate cancer cells.¹³⁵ These findings permitted the structure-based optimization of celecoxib to generate novel potent PDK-1 inhibitors that are devoid of COX-2-inhibitory activity. PDK-1 inhibition by these agents is achieved through interaction with the ATP binding site on the enzyme. OSU-03012 exhibits low micromolar IC₅₀ values for PDK-1 inhibition, Akt dephosphorylation, and apoptosis induction in PC-3 cells. Moreover, the drug suppresses the growth of established PC-3 xenograft tumors after oral administration (C.S. Chen, unpublished findings).

mTOR

mTOR (also called FRAP and RAFT-6) is a serine-threonine kinase that is a downstream component in the PI3K/Akt signaling pathway.^{136,137} Activation of mTOR by phosphorylation stimulates the translation of proteins important for cell cycle progression, such as cyclin D1, thereby coupling growth stimuli mediated by the PI3K/Akt pathway to cell

proliferation. Specifically, mTOR phosphorylates two regulators of translation, the 40S ribosomal protein S6 kinase (p70^{S6K}) and eukaryotic initiation factor 4E-binding protein-1 (4E-BP1). Activated p70^{S6K} is important for ribosome biogenesis, while 4E-BP1, a repressor of mRNA translation, is inactivated by phosphorylation causing the release of active eukaryotic initiation factor 4E (eIF-4E). As a component of the translation initiation complex, eIF-4E promotes ribosome recruitment to mRNA and stimulates the translation of proteins essential for cell cycle progression from G1 to S phase.^{63,138,139} The importance of dysregulated PI3K/Akt signaling in prostate cancer, particularly in advanced disease, as described above, suggests that mTOR is a relevant target for therapeutic intervention (Fig. 1, site 7). Indeed, both *in vitro* and *in vivo* studies, including those using prostate cancer cells, demonstrated that PTEN-inactive cells were preferentially sensitive to the growth inhibitory effects of mTOR inhibition.^{140–143} These findings suggest that patients with tumors characterized by elevated PI3K/Akt signaling, such as HRPC, may represent a population responsive to mTOR-targeted therapies.

Currently, two investigational drugs that inhibit mTOR are in clinical trials as anticancer agents. Both of these agents, CCI-779 (Wyeth) and RAD001 (everolimus; Novartis), are soluble ester derivatives of the macrolide rapamycin, which is currently approved as an immunosuppressant for organ transplant patients (Rapamune; sirolimus; Wyeth). Rapamycin and its derivatives inhibit mTOR by binding to the immunophilin, FK506-binding protein 12 (FKBP12). This complex subsequently binds mTOR and inhibits its activation, thereby suppressing phosphorylation of downstream targets and cell proliferation.¹⁴⁴ Preclinically, pharmacological inhibition of mTOR has been shown to suppress cancer cell growth and to overcome chemotherapeutic resistance conferred by PTEN inactivation in both *in vitro* and *in vivo* models of prostate cancer.^{140,145–149} CCI-779 has completed phase I studies without dose-limiting toxicity and with partial responses in some solid tumors (non-prostate).^{63,139} It is currently being evaluated in two Phase II trials in prostate cancer patients; an open trial in the neoadjuvant setting and a closed trial in patients with androgen-independent metastatic tumors. RAD001 is currently in Phase I trials.¹⁵⁰

Conclusions

Enthusiasm for the development of molecularly targeted agents has been spurred by the clinical successes of trastuzumab and imatinib mesylate, and the more recent approvals of gefitinib, cetuximab, and bevacizumab. One of the lessons learned from the development of these agents is the importance of target selection. Appropriate targets are the aberrant pathways or molecules upon which the cancer cell is critically dependent. The challenge here is whether targeting a single pathway or molecule will impact tumor burden given the redundancies in signaling pathways and the molecular heterogeneity inherent in the tumor cell population. In light of this problem, an approach that might prove effective is the targeting of convergence points of multiple signaling pathways, such as Akt. Modulation of such targets may provide more complete inhibition than those farther upstream, such as cell surface receptors. Of course, the potential benefits of such an approach will have to be balanced by careful evaluation of potential toxicities, as such points of signaling confluence are utilized in multiple cellular functions, including those in normal cell populations. Nevertheless, given the importance of signaling through PI3K/Akt in prostate cancer, targeting components of this pathway, either alone or in combination with cytotoxic agents or other targeted therapeutics, holds substantial promise.

Molecularly targeted agents that alter pathways critical to cancer cell survival and progression will undoubtedly compose, either alone or in combination, the foundation for future chemotherapeutic strategies for HRPC. The rate at which these strategies can mature and be translated to the clinical setting will rely in part on the identification and validation of appropriate targets, the consideration of more global signaling profiles or signatures in tumor cells that will facilitate the identification of critical pathways that can be targeted in combination, utilization of modern drug discovery/medicinal chemistry resources, access of clinician-scientists to new investigational agents developed by these resources, development of innovative means to identify patients who harbor a relevant target, and to assess drug responses in these patients, and on substantive and informative interactions among basic scientists and clinicians.

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CHEMOPREVENTION FOR PROSTATE CANCER

Noahiro Fujimoto

Department of Urology

*University of Occupational and Environmental Health
Iseigaoka, Yahatanishiku, Kitakyushu, 807-8555, Japan*

Introduction

Chemoprevention is defined as the use of specific agents to suppress or reverse carcinogenesis and to prevent the development of cancer.¹ Chemoprevention trials are ongoing for various cancers including prostate cancer. There are significant epidemiological differences related to the higher incidence of clinical prostate cancer observed in Western compared to Asian countries. However, the incidence of occult prostate cancer is very similar between Western and Asian countries. The development of prostate cancer is a long-term process involving multiple steps. Normal epithelia may require 10–30 years to develop into local clinical cancer and another 10–20 years to metastasize (Fig. 1).² Sex steroids, especially androgens, influence the development of prostate cancer. These findings provide multiple opportunities for preventing prostate cancer and investigation of chemoprevention is very attractive. The goal of prostate cancer chemoprevention is to find agents that modulate the progression from normal epithelium to clinically significant and localized cancer, and also prevent the progression from localized cancer to locally advanced, then to metastatic, and finally to hormone refractory cancer (Fig. 2).

The development of chemoprevention strategies against prostate cancer would have great overall impact both medically and economically. Various phase I–III chemoprevention trials were conducted by National Cancer Institute (NCI)³ and several other trials are ongoing (Table 1).

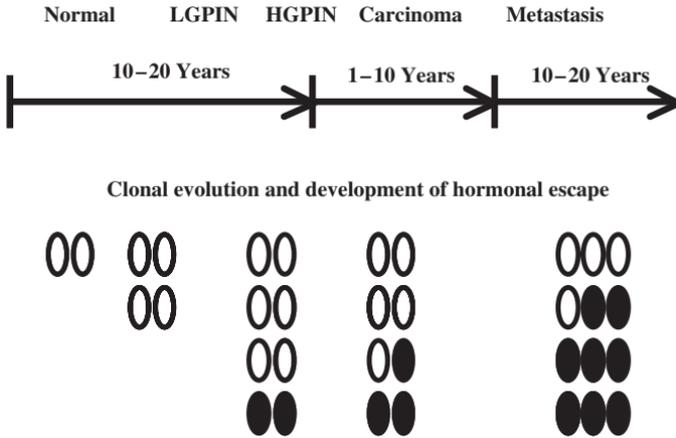


Fig. 1. Natural history of prostate carcinogenesis.² Early stages are androgen dependent. However, as disease progresses over time, androgen-dependent clones emerge, either *de novo* or in response to androgen deprivation therapy and clonal selection. Clear circles represent androgen-dependent or sensitive cells, filled circles represent androgen-independent or resistant cells. LGPIN: Low-grade prostatic intraepithelial neoplasia, HGPIN: high-grade prostatic intraepithelial neoplasia.

Chemopreventive Agents, Rationale and Clinical Trials

There are many candidate treatment strategies for prostate cancer prevention (Table 2).⁴ Here we will focus on several chemopreventive agents and clinical trials.

Sex Steroid Signaling: Antiandrogens and Antiestrogens

It is well known that sex hormones influence the development of prostate cancer. Thus, the strategies altering sex steroid signaling may be useful tools for preventing prostate cancer.

5- α Reductase Inhibitor (Finasteride)

Finasteride is an inhibitor of 5- α reductase, the enzyme that converts testosterone to the more potent androgen, dihydrotestosterone (DHT) and

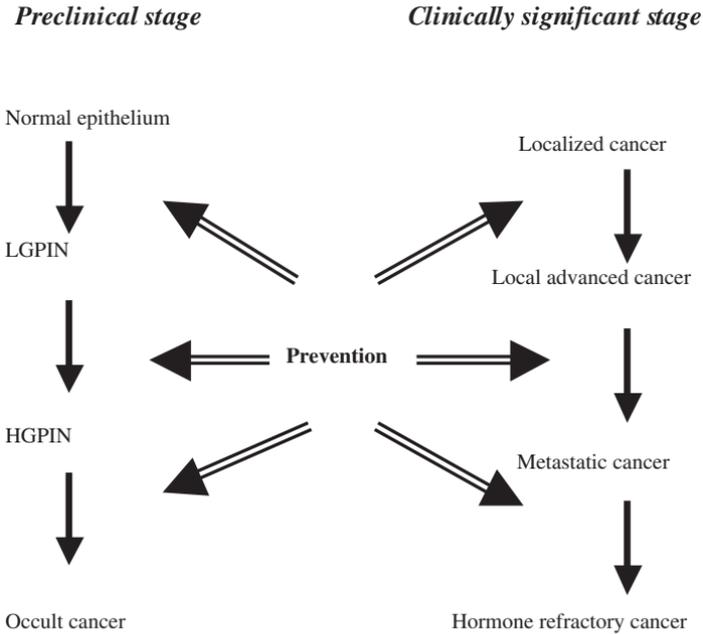


Fig. 2. Chemoprevention in the multiple-step process of prostate cancer progression. LGPIN: Low-grade prostatic intraepithelial neoplasia, HGPIN: high-grade prostatic intraepithelial neoplasia.

can lower the androgen level in the prostate. Thus, it was anticipated that finasteride would reduce the risk of prostate cancer. In 1993, The Prostate Cancer Prevention Trial (PCPT) was initiated and funded by the NCI to investigate chemoprevention of prostate cancer with finasteride. The PCPT was the first large-scale prospective phase III trial and demonstrated that finasteride significantly reduced the period-prevalence of prostate cancer. In this trial, 18,882 men, aged 55 years and older, with PSA level of 3.0 ng/ml or lower, and normal digital rectal examination, were randomized to treatment with finasteride (5 mg/day) or placebo for 7 years. In 2003, the PCPT was stopped and the results were published.⁵ Prostate cancer was detected in 803 of the 4,368 men in the finasteride group (18.4%), and in 1,147 of the 4,692 men in the placebo group (24.4%), for a 24.8% reduction in prevalence over the seven-year-period ($p \leq 0.001$). The difference in the incidence of prostate cancer between the two groups was observed early, and continued through late in the follow-up period. The early

Table 1. Ongoing Prostate Cancer Prevention Trials (National Cancer Institute)

Title of Trial	Protocol IDs
Phase I Randomized Study of Neoadjuvant Celecoxib Followed by Prostatectomy in Patients with Localized Prostate Cancer	JHOC-J0007, JHOC-00030801, NCI-N01-95129, NCI-P01-0186
Phase I Study of Lycopene for the Chemoprevention of Prostate Cancer	UIC-H-99-058, NCI-P00-0143, UIC-N01-CN-85081
Phase II Randomized Prevention Study of Fat- and/or Flaxseed-Modified Diets in Patients with Newly Diagnosed Prostate Cancer	CCUM-0202, DUMC-1385-02-7R3ER, NCI-P02-0235, UMCC-0202
Phase II Randomized Study of Dietary Soy in Patients with Elevated PSA Levels	CALGB-79806, NCI-P02-0207
Phase II Randomized Study of Doxercalciferol in Patients with Localized Prostate Cancer	WCCC-CO-99802, NCI-N01-CN-95130, NCI-P01-0188, WCCC-CO-2000169
Phase II Randomized Study of the Effects of a Low Fat, High Fiber Diet on Serum Factors in Patients with Prostate Cancer	UCLA-0001030, NCI-G01-1973
Phase II Randomized Study of Toremifene Followed by Radical Prostatectomy in Patients with Stage I or II Adenocarcinoma of the Prostate	PCI-00-105, NCI-P01-0181, PCI-N01-CN-75018
Phase II Randomized Study of Vitamin E, Selenium and Soy Protein Isolate in Patients with High-Grade Prostatic Intraepithelial Neoplasia	CAN-NCIC-PRP1
Phase IIB Randomized Chemoprevention Study of Eflornithine (DFMO) in Patients at High Genetic Risk for Prostate Cancer	UCIRVINE-97-18, NCI-P00-0164, UCIRVINE-U01-CA-81886-01
Phase III Randomized Study of Selenium and Vitamin E for the Prevention of Prostate Cancer (SELECT Trial)	SWOG-S0000, CALGB-S0000, CAN-NCIC-S0000, ECOG-S0000, NCCAM, NCI-P00-0172
Phase III Randomized Study of Selenium as Chemoprevention of prostate Cancer in Patients with High Grade Prostatic Intraepithelial Neoplasia	SWOG-S9917, CALGB-SWOG-S9917, ECOG-SWOG-S9917, NCI-P02-0203

Table 1 (Continued)

Title of Trial	Protocol IDs
Randomized Pilot Study of Isoflavones versus Lycopene Prior to Radical Prostatectomy in Patients with Localized Prostate Cancer	MCC-0105, NCI-3811, NCI-P02-0216
Randomized Study of Isoflavones in Reducing Risk Factors in Patients with Stage I or II Prostate Cancer	MCC-0002, NCI-4031, NCI-P01-0195

difference suggests that finasteride may have treated subclinical, microscopical cancer early in the study, and the fact that the difference continued to increase suggests that it prevented or delayed the onset of cancer.

However, the high-grade tumors (Gleason scores 7–10) were significantly common in the finasteride group. The high-grade tumors were noted in 6.4% of the men in the finasteride group, as compared with 5.1% of those in the placebo group. Possible explanations for this difference were (1) grading bias; histologic changes that mimic those of high grade disease are caused by androgen-deprivation therapy, and (2) finasteride might promote an environment that favors high-grade tumors, because there is clinical evidence that hypogonadal patients tend to have higher grade and more aggressive prostate cancers than patients with normal testicular function.^{6–8} Finasteride may select for high-grade tumors by selectively inhibiting low-grade tumors. Long-term follow-up is needed to elucidate the association between finasteride and high-grade prostate cancer. Sexual side effects were more common, but urinary symptoms were less common in finasteride-treated men.

In summary, finasteride prevents or delays the appearance of prostate cancer, but this possible benefit and a reduced risk of urinary problems must be weighed against sexual side effects and the increased risk of high-grade prostate cancer.

Antiestrogen and Selective Estrogen Receptor Modulators (SERMs)

Shibata *et al.* reported that an age-dependent decrease in DHT, with an increase in the estradiol/DHT ratio in the aging prostate, leads to a

Table 2. Candidates of Chemopreventive Agents for Prostate Cancer⁴

Sex steroid signaling	5 α Reductase inhibitors (finasteride) Antiandrogens (receptor antagonist) Selective estrogen receptor modulators (SERMs)
Differentiation/antiproliferation	Retinoids (RAR, RXR, selective agonists) Vitamin D analogs Ornithine decarboxylase inhibitors (DMFO)
Growth signaling pathways (angiogenesis)	PDGF receptor antagonists VEGF receptor antagonists Famestyl-protein transferase inhibitors Protein tyrosine kinase inhibitors (soy isoflavones)
Arachinoid acid-associated signaling (proapoptosis)	Nonselective cyclooxygenase inhibitors (NSAIDs) Selective cyclooxygenase-2 inhibitors (celecoxib, rofecoxib) 5-Lipoxygenase inhibitors Other anti-inflammatory agents (R-flurbiprofen) PPAR modulators (sulindac sulfone)
Gene therapy	Genetically modified vaccines <i>In situ</i> delivery of immunostimulatory genes <i>In situ</i> delivery of cytotoxic genes Replication-restricted cytolytic viruses
Growth factors	Endothelin-1 antagonists Matrix metalloproteinase inhibitors IGF-1 pathway inhibitors PSA protease inhibitors PPAR γ modulators (glitazones)
Antioxidants	Vitamin E Selenium Carotenoids Others (green tea polyphenols)

relatively estrogen dominant environment.⁹ African-Americans have the highest levels of serum estrone and estradiol, whereas Japanese man have the lowest, which parallels their risk for prostate cancer.¹⁰ Rising estrogens appear to increase sensitivity of the prostate tissue to androgens by up-regulation of the androgen receptor (AR).¹¹⁻¹³ Estradiol, in the

presence of androgens, has been shown to stimulate carcinoma in situ and adenocarcinoma of the prostate in Noble rats.¹⁴⁻¹⁷ Estradiol can induce high-grade prostatic intraepithelial neoplasia (PIN) and prostate cancer in the aging dog.^{18,19} Those observations suggest that estrogenic stimulation with decreasing androgen levels contributes to the genesis of prostatic dysplasia and subsequent prostate cancer.

Phytoestrogens

Phytoestrogens, such as isoflavonoids and lignans, have weak estrogenic activity. Soybean is a major source of isoflavonoids mainly in the form called genistein. Large amounts of soy are consumed in China and Japan, where the incidence of prostate cancer is low.²⁰ A direct inverse correlation was observed between serum isoflavonoids levels and prostate cancer incidence.²¹ Possible mechanisms of phytoestrogens' chemoprevention of prostate cancer are lowering the 5 α -reductase activity, increasing sex hormone binding globulin, lowering free testosterone, decreasing tyrosine specific protein kinase activity, and reducing p450 aromatase activity.²¹ Phytoestrogens are thought to prevent prostate cancer and are being investigated in NCI clinical trials.

SERMs

SERMs are nonsteroidal compounds that are generally considered to be weak estrogens and also possess cancer-suppressing activity. Toremifen is a chlorinated derivative of tamoxifen, widely used to treat breast cancer. In the transgenic adenocarcinoma of mouse prostate model, toremifene suppressed the development of high-grade PIN, decreased prostate cancer incidence, and increased survival. The molecular mechanism of toremifene's chemopreventive effects appears to be through estrogen receptor (ER)- α and to be androgen-independent.²² Estrogen stimulates cellular proliferation by inducing local production of stimulatory growth factors such as transforming growth factor (TGF)- α , insulin-like growth factor, and epidermal growth factor, and by suppressing inhibitory effects of factors such as TGF- β .^{23,24} Thus, SERMs would be expected to decrease the levels of stimulatory growth factors and augment the production of

TGF β . The antiproliferative effects of SERMs may also be mediated by binding and sequestration of calmodulin,²⁵ inhibition of protein kinase C,²⁶ and induction of p21.²⁷ In addition, SERMs can bind to ER and the formation of SERM-ER complexes results in the inactivation of the estrogen-regulated genes, thereby decreasing cellular proliferation. Toremifene has been evaluated in a phase II exploratory trial in men with high-grade PIN. A four-month toremifene treatment regimen significantly reduced high-grade PIN²⁸ and the chemopreventive effect of toremifene is being investigated by placebo controlled, randomized, dose-finding phase II–III clinical trials. Raloxifen, one of the SERMs, has been shown to induce apoptosis in androgen-independent prostate cancer cell lines²⁹ and could be a candidate agent for prostate cancer chemoprevention.

Antiproliferation/Differentiation

Difluoromethylornithine (DFMO)

As polyamines are ubiquitous and essential for cell survival, limiting or inhibiting polyamine synthesis results in reduced cycling of highly proliferative cancer cells. DFMO is an irreversible inhibitor of ornithine decarboxylase (ODC), and is involved in the synthesis of polyamines and, consequently, related to cell proliferation. DFMO has been demonstrated to inhibit carcinogenesis in animal models of epithelial cancers including urinary bladder, colon, skin and breast. The concentrations of some polyamines such as spermine and spermidine are higher in the prostate than in most other tissues. Heston *et al.* demonstrated that DFMO inhibited tumor growth in the prostate.³⁰ Because a significant limiting side effect of DFMO is ototoxicity, optimal doses must be determined. Thus, DFMO and other inhibitors of polyamine synthesis are thought to be chemopreventive agents of prostate cancer.

Vitamin A and Related Compounds

Vitamin A and metabolites (retinoids) possess a close relationship with important cellular functions such as morphogenesis, proliferation and differentiation. Peehl *et al.*³¹ demonstrated that clonal growth of prostatic

epithelial cells was inhibited by retinoic acid (RA). The cellular changes provided evidence that the retinoids play a role in prostate cell apoptosis and differentiation. Many vitamin A metabolites bind nonspecifically to both retinoic acid receptor (RAR) and retinoid X receptor (RXR). The receptor dimers, such as RXR-RAR, RXR-peroxisome proliferator activated receptor (PPAR), and RXR-vitamin D receptor (VDR), are known to induce differentiation when activated. Several retinoids, such as 9-cis retinoic acid (a pan-agonist for retinoic acid receptor and RXR) and an RXR selective agonist, are under investigation for treatment of prostate cancer.

Short-Chain Fatty Acids

This class of differentiating agents includes the bioactively stable analogs of sodium butyrate, phenylbutyrate (PB) and phenylacetate (PA). Samid *et al.*³² found that PA-treated human prostate cancer cells (PC-3, DU145, LNCaP) lost their ability to invade a basement membrane and showed diminished ability to form tumors when transplanted into athymic mice. Because a Phase I study did not show a benefit of PA, the focus of investigation turned to PB. PB is 1.5 to 2.5 times more active at inhibiting growth and inducing apoptosis than PA.^{33,34} The mechanisms of action for short-chain fatty acids are most likely not mutually exclusive. Two acceptable mechanisms of action are inhibition of histone deacetylase and activation of the PPAR nuclear receptor.³⁴ A Phase I study suggested that PB delayed the progression of heavily pretreated androgen-independent prostate cancer.³⁵ The activities and low toxicity profile make PB attractive for further investigation as a chemopreventive agent.

Arachidonic Acid Signaling/Proapoptotics

Anti-inflammatory Drugs, COX Inhibitors

Epidemiologic studies, preclinical models, and randomized clinical trials support an association between nonsteroidal anti-inflammatory drugs (NSAIDs) and decreased incidence of cancers, such as colon and prostate. NSAIDs are potent inhibitors of cyclooxygenases, enzymes that catalyze

the synthesis of prostaglandins from arachidonic acid. Cyclooxygenase 2 (COX2) is a key enzyme in the synthesis of proinflammatory prostaglandins (prostaglandin E₂, PGE₂). In humans, COX2 is upregulated in precancer and in prostatic carcinomas. NSAIDs were initially developed to suppress inflammation and pain by inhibiting the production of PGE₂ and its metabolites. NSAIDs are active against prostate cancer in laboratory and clinical studies. Inhibition of COX2 by celecoxib, the first COX2-selective drug, inhibits carcinogenesis and suppresses tumor growth.^{36,37} In addition, celecoxib is effective at inhibiting angiogenesis induced by basic fibroblast growth factor. Exisulind (sulindac sulfone) is a potent inhibitor of both COX1 and COX2. Anti-tumor activity was proven in clinical trials to treat familial adenomatous polyposis.^{38,39} Exisulind has proven to be active in rodent models of chemical carcinogenesis against breast, colon, lung and bladder.^{40–44} In mouse xenograft models of prostate cancer, exisulind induced apoptosis of cancer cells and showed anticancer activity.^{45,46}

Antioxidants

Epidemiological studies have provided evidence for an inverse association between exposure to antioxidant nutrients (e.g. lycopene, soy isoflavones, selenium, vitamin E, green tea polyphenols) and prostate cancer incidence and mortality.

Selenium

Selenium is a nonmetallic trace element recognized as a nutrient essential to human health. Selenium inhibits tumorigenesis in a variety of experimental models. Many animal models, including prostate cancer models, have shown reductions in tumor incidence in response to selenium supplementation.^{47,48} Possible mechanisms of selenium antitumorogenic effects are antioxidant effects, enhancement of immune function, induction of apoptosis, inhibition of cell proliferation, alteration of carcinogen metabolism, cytotoxicity of metabolites formed under high-selenium conditions, and an influence on testosterone production.^{49–55} Two large,

randomized trials have demonstrated that selenium supplementation could reduce overall cancer mortality, as well as mortality from stomach and esophageal cancers.^{56,57} In addition, a case-control study demonstrated that the highest selenium status tested reduced the risk of advanced prostate cancer.⁵⁸ A large scale, randomized study demonstrated the chemopreventive effect of selenium.⁵⁹ In this study, 1,312 subjects with a prior history of skin cancer were randomized to receive selenium or placebo, and followed for an average of 4.5 years. While no difference was found in rate of recurrence of skin cancer, prostate cancer incidence was reduced by two thirds among those in the selenium-supplemented group.

Vitamin E (α -Tocopherol)

Vitamin E functions as the major lipid-soluble antioxidant in cell membranes: it is a chain-breaking, free-radical scavenger and specifically inhibits lipid peroxidation, the biologic activity relevant to carcinogen-induced DNA damage.⁶⁰ α -Tocopherol is the most active form of vitamin E and may influence the development of cancer through several mechanisms such as antioxidation,⁶¹ inhibition of protein kinase C,^{62,63} induction of the detoxification enzyme NADHP, and inhibition of arachadonic acid and prostaglandin metabolism.^{64,65} A study of 2,974 subjects, over 17-year follow-up period, found low α -tocopherol to be associated with higher prostate cancer risk.⁶⁶ A large-scale, randomized, placebo-controlled trial also suggested that vitamin E is effective for chemoprevention of prostate cancer. This trial, the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Trial (ATBC), was conducted in Finland.⁶⁷ In this trial, 29,133 men, aged 50–69 years, were randomized and the median follow-up period was 6.1 years. A statistically significant 32% reduction in prostate cancer was observed in the α -tocopherol group.

As selenium and vitamin E are thought to be promising candidates for prostate cancer prevention, a new randomized, prospective, double-blind study was initiated in 2001.⁶⁸ This trial is called SELECT, the Selenium and Vitamin E Cancer Prevention Trial. SELECT was designed to determine whether selenium and vitamin E can reduce the risk of prostate cancer among healthy men, and the final results are anticipated in 2013.

Lycopene

Lycopene is a carotenoid naturally present in tomatoes and other fruits, is a potent antioxidant, and is the most significant free radical scavenger in the carotenoid family.⁶⁹ Mills *et al.* reported that tomato consumption was most strongly associated with reduced prostate cancer risk.⁷⁰ A nested case-control study within the context of the Health Professional Follow-up study showed that individuals consuming more than 10 servings per week of tomato-based products had an adjusted odds ratio of 0.65 for developing advanced/aggressive prostate cancers.⁷¹ These observations suggest that lycopene supplementation may be beneficial in preventing the progression of prostate cancer.

Others

Protein tyrosine kinase inhibitors, pan-inhibitors of platelet-derived growth factor, vascular endothelial growth factor, and fibroblast growth factor, endothelin-1 antagonist and matrix metalloproteinase inhibitors are candidate prostate cancer chemopreventive agents and are under investigation.

Conclusions

For prostate cancer prevention, Lieberman⁷² proposed a dual strategy; a public health approach and a cost effective pharmacologic, medically oriented, translational science strategy. A public health approach includes (1) changes in dietary practices with increased fruits and vegetables, decreased carbohydrates and charbroiled meat, (2) caloric restriction/obesity control, (3) increased physical activity and stress reduction, and (4) early detection of precancerous lesions. A pharmacologic, medically oriented, translational science strategy includes (1) identification of individuals at risk using clinical, histologic, genetic and proteomic profiles, (2) risk reduction by using chemopreventive agents to modulate surrogate endpoints, (3) suppression/reversal of promoter methylation in target genes and (4) suppression/reversal of signature protein patterns of early prostate cancer to delay the onset of clinically active prostate cancer.

As mentioned above, there are many candidate chemopreventive agents, and some clinical trials demonstrated promising results in the prevention of carcinogenesis and progression of prostate cancer. Large-scale, prospective, randomized trials are absolutely necessary to obtain useful strategies to prevent prostate cancer. However, we still need to overcome or better understand several issues. For example, which populations are optimal targets? When the target population is a low-risk group (general population), targets are easily definable, readily available, and results are widely applicable, but large study population and long follow-up periods are required. In contrast, when high-risk groups such as populations with HGPIN, are targeted, sample size and duration can be reduced, but the diagnosis is subjective, and sampling errors will occur, and results are not widely applicable. We also must consider when chemoprevention should be introduced and how long it should be continued. Are combinations of agents or strategies more useful than single ones? Furthermore, the principles of combination of various agents must be considered, including, (1) using two or more agents with different mechanisms of action, (2) using agents with non-overlapping toxicity and (3) reducing the dose of each agent.

The economic aspect of clinical trials should also be considered. To run a Phase III clinical trial in EORTC, the average cost per patient involved is between \$1,000 and \$2,000 USD. When a sample size is 30,000 men, \$20–\$40 million are needed.

In addition, it is necessary to evaluate multi-organ systems for effectiveness of any agent, because each agent may prevent cancer in one organ but may promote cancer in others. For example, tamoxifen reduced the risk of invasive breast cancer by 49%, but increased invasive endometrial cancer (2.53 times greater risk compared to placebo group).⁷³

Active basic and clinical research regarding the chemoprevention of prostate cancer is ongoing and will provide effective and safe strategies that result in clinical benefit.

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NEUROENDOCRINE DIFFERENTIATION AND ANDROGEN-INDEPENDENCE IN PROSTATE CANCER

Sonal J. Desai, Clifford G. Tepper and Hsing-Jien Kung

*Department of Biological Chemistry and Cancer Center
University of California at Davis
Sacramento, CA 95817, USA*

Introduction

One of the most troubling aspects of prostate cancer (PCa) is the emergence of hormone-refractory cells, which currently defy treatment.^{1,2} Increasing evidence suggests that a majority of hormone-refractory PCa cells, while androgen-independent, retain the expression and activity of the androgen receptor (AR).³ In fact, the level of AR or its coactivators in these tumors may actually be higher,^{4,5} suggesting that the receptor is activated by amplification or other cellular factors, independent of the action of androgen (or in the presence of castrate levels of androgen).⁶ Understanding these factors is the key to the identification of targets and the development of intervention therapy for androgen-independent tumors. Among the factors accompanying the progression of PCa to an androgen-independent state, are the appearance of an increased number of neuroendocrine (NE) cells and the increased serum level of chromogranin A (CgA), IL-6, and IL-8, soluble factors relevant to neuroendocrine differentiation (NED). In this chapter, we will describe the molecular pathways leading to NED and to androgen-independence, and their potential causal relationship. There have been excellent reviews on the subject of either NED or androgen-independence. The readers are referred to those publications^{2,7-14} for more details.

As discussed above, an association of increased NED as well as high serum concentrations of growth factors, cytokines, and neuropeptides has been frequently observed in androgen-independent tumors. Interestingly, growing evidence suggests androgen-withdrawal itself (and by implication androgen-ablation therapy)¹⁵ or in combination with circulating factors such as IL-6,^{16–19} epinephrine,^{20,21} or stromal factors such as HB-EGF,^{22,23} induces NED in the prostate. These differentiated cells become growth arrested, but are strongly resistant to apoptosis and at some point begin secreting factors (e.g., neuropeptides) that elicit signals that contribute to the survival and growth of surrounding, undifferentiated PCa cells, which would have otherwise died under androgen-deprived conditions. These surviving cells, upon further genetic aberrations evolve to become androgen-independent, untreatable clones. This is the central hypothesis of this chapter (Fig. 1) and we will elaborate on the current understanding of the molecular and cellular details of these processes.

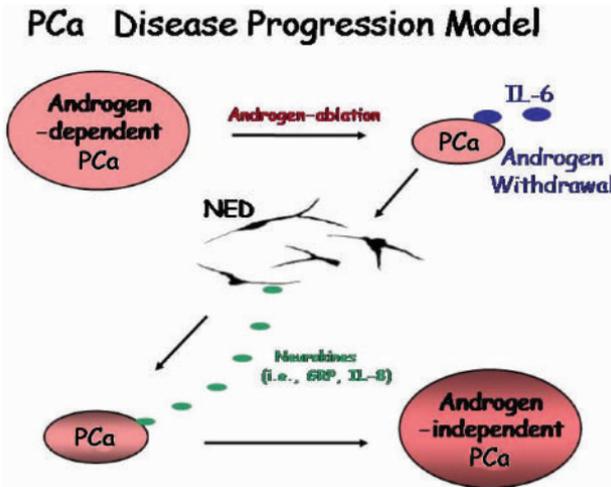


Fig. 1. PCa disease progression model. Initial occurrence of PCa is as an androgen-dependent tumor. Androgen-ablation therapy causes the tumor to regress, but, by itself or in combination with other soluble factors also induces NED of some cells. These NED cells then secrete neurokinines that act on surrounding non-NED cells to promote androgen-independent growth of the tumor.

Neuroendocrine Differentiation

NE cells are terminally differentiated, postmitotic cells. They are characterized by the presence of neuritic outgrowths and neurosecretory granules as well as by the expression and secretion of neuron-specific proteins.¹³ NE cells are present in all regions of the prostate at birth, but soon disappear from the peripheral regions. At the onset of puberty, NE cells reappear and increase to a low but apparently optimum level.^{1,24-26} The origin of NE cells is unclear. Based on cell line studies described in the next section, interconversion (or trans-differentiation) between prostate epithelial cells and prostate NE cells can occur. A current model is that the three cell types comprising the prostate epithelium (epithelial, basal, and NE) originate from a common endodermal pluripotent stem cell. The epithelial cells are androgen-dependent while the basal and NE cells are androgen-independent.^{24,25} Little is known about the function of NE cells in the prostate, but they appear to be essential for growth and differentiation as well as homeostatic regulation of the secretory processes in the mature prostate. Tumor cell populations have been reported to become enriched for NE cells and are correlated with elevated levels of NE markers.^{1,19,27} While the occurrence of purely NE, or small cell carcinoma and carcinoid or carcinoid-like tumors, is rare (1% of all prostate cancers),^{28,29} an increase in the number of cells in the prostate with NE characteristics has been reported as a marker for the development of androgen-independence in PCa. Furthermore, overexpression of SV40 large T antigen in prostatic tissue of mice models intended for prostate carcinoma gives rise to NE tumors, again suggesting a kinship of these two cell types in prostate carcinogenesis.³⁰⁻³² There has been a great deal of research in characterizing NE cells and identifying markers that could be used in determining the prognosis and possible treatment of PCa.³³⁻⁴² However, the factors that induce these changes are relatively unknown. NE cells found in PCa possess phenotypic traits of both normal NE cells, such as the expression of NE markers (i.e., CgA and NSE) as well as epithelial characteristics, such as PSA, and secrete neuropeptides, such as neurotensin, serotonin, and the bombesin homologue gastrin-releasing peptide (GRP). One theory is that, due to its origin from a pluripotent stem cell, a malignant cancer cell can mobilize a mix of genes that are normally differentially

expressed in basal, epithelial, or NE cells, which would explain the cell's ability to transform as well as the duality of characteristics within one cell and the ability of a usually postmitotic, terminally differentiated cell to proliferate. This would generate a cancer cell more adaptable to environmental changes, such as androgen depletion, and enhance its ability to respond to circulating aberrant growth signals.^{1,24,25} A summary of the signaling pathways involved in NED, which we will discuss below, is presented in Fig. 2A.

Androgen-Withdrawal Induced Neuroendocrine Differentiation

An increase in the number of NED cells in the prostate as evidenced by chromogranin A (CgA) staining has been associated with androgen-ablation therapy.^{43,44} The aggressive nature of these cells is underscored by the fact that while TUNEL staining demonstrated the occurrence of apoptosis in the exocrine cells of prostate tumors, CgA-positive NE cells were resistant to apoptosis.⁴⁵ This phenomenon was also seen in the canine prostate⁴⁶ and in human PCa xenograft PC-310^{47,48} grown in nude mice subjected to castration or anti-androgen treatment. In addition, *in vitro* withdrawal of androgen from the androgen-dependent PCa cell line, LNCaP, results in cell cycle arrest⁴⁹ and NE trans-differentiation¹⁵ characterized by distinctive morphological changes including rounding up of the cell bodies and the extension of neuritic processes (Fig. 3B). This is accompanied biochemically by an increase in the expression of NSE,⁵⁰ CgA,⁴¹ GRP³⁴ and neurotensin.⁵¹ Similar to *in vivo* observations, LNCaP cells are resistant to apoptosis when androgen is removed.⁵² While apoptosis appears to be initiated, ascertained by the appearance of DNA fragmentation, it is not completed. Instead, the cells are growth arrested and mRNA expression of anti-apoptotic proteins is seen. Substantial evidence supports the fact that suppression of AR signaling is obligatory for NED.⁵³ AR serves as the pivotal effector of androgen deprivation, as elimination of its ligand essentially shuts down the AR pathway by simultaneously terminating signals emanating from the receptor and causing a marked down-regulation in AR protein levels.⁵⁴ Accordingly, NED of LNCaP can be induced by antagonism of AR function with the anti-androgen bicalutamide (Casodex)^{49,55} or by AR silencing with siRNAs as evidenced by the appearance of the typical NE phenotype and expression of NE markers,

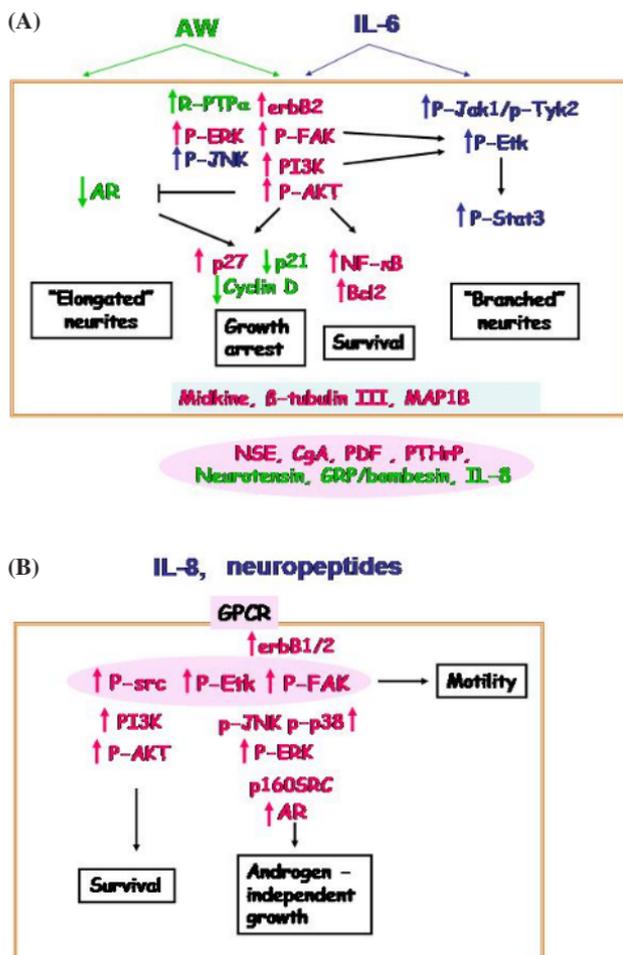


Fig. 2. (A) Signals associated with NED induced by androgen withdrawal (AW) or by IL-6. Molecules in red are activated (up arrow) or down-modulated (down arrow) by both AW and IL-6. Molecules in green are known to be modulated by AW only. Molecules in blue are modulated by IL-6 only. The phenotypes associated with individual signal pathways are indicated in boxes. Intracellular neuronal-related and secreted neurokinines modulated by AW and IL-6 are indicated. (B) Signals associated with neuropeptides and IL-8 induced androgen-independent growth. Both IL-8 and neuropeptides engage G-protein-coupled receptors (GPCR). ErbB family receptor tyrosine kinases, erbB1 and erbB2, are activated. The trimeric tyrosine kinase complex of Src, Etk, and FAK is also activated. The signals are then transmitted from tyrosine kinases to PI-3 kinase (PI3K) and serine/threonine kinases (JNK, p38MAPK, ERK, and Akt). Activated or phosphorylated ERK (P-ERK) is known to phosphorylate steroid receptor coactivator (p160SRC), which is co-recruited with androgen receptor (AR) to androgen response elements.

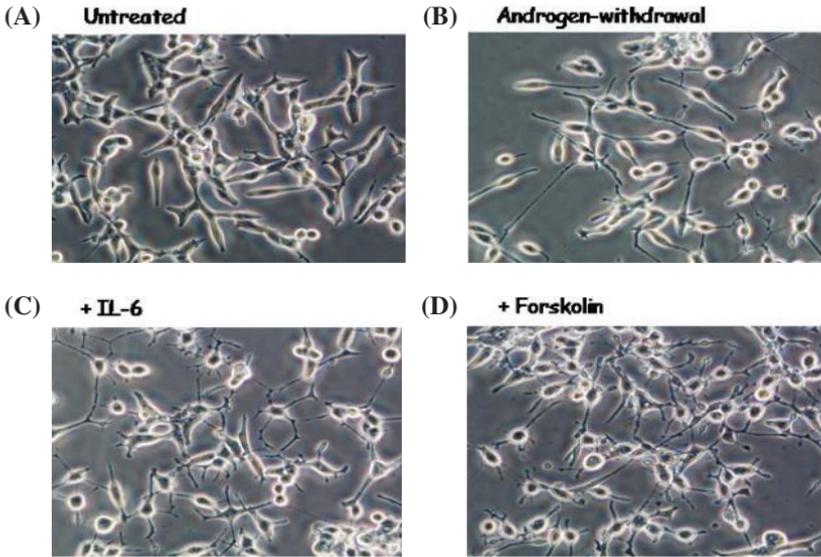


Fig. 3. Neuroendocrine differentiation in LNCaP cells. Cells were plated in RPMI 1640 supplemented with 2% FBS. The following day, cells were either **(A)** left untreated or treated with **(B)** androgen withdrawal (RPMI 1640 supplemented with 2% CDS-FBS), **(C)** IL-6 (50 ng/ml IL-6 in RPMI 1640 supplemented with 2% FBS), or **(D)** Forskolin (10 μ M Forskolin in RPMI supplemented with 2% FBS). Photographs were taken 96 hours after treatment.

such as NSE and beta-tubulin III.⁵³ Conversely, addition of androgen to hormone-depleted media inhibited NED of LNCaP and could restore basal expression levels of NSE and neurotensin.⁴⁹ In contrast, while androgen deprivation for 4–7 days induces a terminal differentiation by morphological criteria, the cells retain the capacity to respond to androgen and overcome growth arrest (Desai, unpublished data).

Investigation into signaling events that occur upon androgen withdrawal in LNCaP cells have implicated kinases and cell cycle regulators in the process of growth arrest and resistance to apoptosis. Notably, PI3K signaling is deregulated and augmented in the LNCaP model as a consequence of mutational inactivation of the PTEN lipid phosphatase.^{56,57} As a result, this aberrant signal poses as a dominant mediator of survival primarily through the resultant elevation in the basal level of active Akt/PKB,⁵⁸ a major PI3K target known to negatively regulate apoptosis via the phosphorylation and inactivation of pro-apoptotic molecules.^{59,60} Akt emerged as

a critical mediator of survival during androgen-deprivation by studies demonstrating its rapid hyper-activation observable within 8 hours of androgen withdrawal⁴⁹ and persisting for up to 60 days (Tepper, unpublished data). Although AR is a *bona fide* Akt substrate,^{61,62} Akt-mediated phosphorylation paradoxically promotes AR instability by targeting it for Mdm2-mediated ubiquitination and subsequent proteasome-mediated degradation.⁶³ Similar to other PCa models, chronic androgen ablation of LNCaP results in the transition from an androgen-dependent to -independent state in which growth arrest is overcome.^{49,64,65} In accord with elevated serum PSA serving as the clinical benchmark for the occurrence of hormone-refractory disease, reinstatement of the AR pathway (i.e., AR expression, PSA secretion) in experimental models is one hallmark of the transition to androgen-independence.^{49,64-67} The reappearance of AR is due, at least in part, to enhanced stability of the receptor.⁶⁸ In late passage and androgen-independent LNCaP cell lines (e.g., LNCaP-AI, LNCaP-rf, LNCaP-cds)^{49,64,67,69} this potentially involves a conversion of persistent PI3K/Akt activation from a degradation signal to an AR activator.⁶⁷

Consistent with androgen withdrawal-induced Akt activation, treatment of LNCaP cells with R1881, a synthetic androgen, can decrease PI3K and Akt activation in both parental LNCaP and LNCaP-rf cell lines.⁴⁹ The mechanism by which this occurs is yet to be determined. The receptor tyrosine kinase erbB2, a member of the EGFR family, is an upstream activator of the PI3K pathway. ErbB2 is found expressed in NE cells of patients who have undergone radical prostatectomy⁷⁰ and is both upregulated and activated in androgen-deprived LNCaP cells (Tepper, unpublished data). However, it is unlikely that erbB2 is solely required for Akt activation by androgen withdrawal since functional knockout of erbB2, by its sequestration in the ER via expression of an erbB2-specific single chain antibody,⁷¹ did not diminish Akt phosphorylation (Tepper, unpublished data). Therefore, multiple pathways for PI3K activation probably exist. ErbB2, a known activator of MAPK, could be responsible for constitutive activation of MAPK after androgen withdrawal (Desai, unpublished data).⁷² RPTP- α also increases in androgen deprived NE differentiated LNCaP cells and contributes to MAPK activation.^{50,55} One interesting possibility is that erbB2 and RPTP- α may cross-talk during the differentiation process leading to MAPK activation. The induction of

PI3K and Akt appears to be responsible for LNCaP resistance to apoptosis in the absence of androgen. Inhibiting PI3K either by treatment with pharmacological agents such as wortmannin or LY294002 or by overexpressing PTEN can selectively induce apoptosis in LNCaP cells during androgen deprivation.⁴⁹ This is accompanied by decreased Akt activation and triggering of the apoptotic proteolytic cascade, evidenced by caspase-3 activation. Short-term (3-day) androgen withdrawal prior to the addition of PI3K inhibitors further sensitizes LNCaP to apoptosis induced by PI3K inhibitors. PI3K inhibition might translate into enhanced apoptosis susceptibility via down-regulation of the anti-apoptotic protein Bcl-2, which increases upon androgen withdrawal.⁷³ In contrast, LNCaP-rf cells are remarkably resistant to treatment with PI3K inhibitors which might be explained by their markedly elevated levels of activated Akt and by Bcl-2 only being decreased by 50% upon treatment with the inhibitors (Tepper, unpublished data). Together, this suggests that immediate signaling stimuli are replaced by other factors as LNCaP cells progress to androgen-independence. These factors may be secreted by NE cells themselves, in a paracrine or autocrine fashion, allowing differentiated cells to eventually obtain signaling and growth autonomy.

PI3K signaling may contribute to overcoming growth arrest, in addition to suppressing apoptosis. Downstream effects of PI3K pathway dysregulation can certainly manifest as inappropriate progression through the Rb-mediated restriction point. Specifically, while cyclin-dependent kinase inhibitor p27^{kip1} and p16^{Ink4a} protein levels increased upon acute androgen withdrawal in LNCaP cells, they are significantly decreased in androgen-independent LNCaP and CWR22 cells and xenografts.^{49,54,69} Although PI3K/Akt activation can suppress forkhead-mediated p27^{kip1} transcription,⁷⁴ the primary mechanism for p27^{kip1} protein down-regulation in this model was via increasing its rate of ubiquitin-mediated degradation,⁷⁵ as LY294002 treatment of androgen-independent LNCaP cells in the presence of the protein synthesis inhibitor cycloheximide markedly enhanced p27^{kip1} stability.⁴⁹ At present, it is rather unclear as to how androgen withdrawal augments p27^{kip1} expression level in the presence of PI3K/Akt activation. However, the expression of p27^{kip1} seems to be a determining factor for the growth arrest of NE cells. p21^{Waf1/Cip1} exhibits the opposite expression pattern to p27^{kip1}, decreasing during acute androgen

withdrawal followed by reinstatement in the refractory stage.⁴⁹ This seems to contradict the conventional role of p21^{Waf1/Cip1} as an inducer of cell cycle arrest. However, it may be explained by recent findings that p21^{Waf1/Cip1} can be either cell-cycle inhibitory or mitogenic, depending on its cellular localization.^{76–78} In the nucleus, p21^{Waf1/Cip1} binds to and inhibits cyclin/CDK activity, thereby blocking cell cycle progression.⁷⁹ Upon phosphorylation of p21^{Waf1/Cip1} by Akt, p21^{Waf1/Cip1} translocates to the cytoplasm, where it promotes cell growth.⁸⁰ In addition, p21^{Waf1/Cip1} has been demonstrated to be critical for the assembly of cyclin D-cdk4 complexes.⁸¹ Thus the restoration of p21^{Waf1/Cip1} expression might be a pivotal event for the resumption of G1-S progression in the refractory stage. The AR and PI3K pathways play complimentary roles in positively regulating p21^{Waf1/Cip1} expression. While androgen stimulates transcription of the p21^{Waf1/Cip1} gene through AR binding to a consensus binding site in the p21^{Waf1/Cip1} promoter,⁸² Akt stabilizes the translated product by phosphorylation on residues Thr145 and Ser146.⁸³ Accordingly, inhibition of PI3K causes a decrease in p21^{Waf1/Cip1}. Usurping p53-mediated growth arrest through mutational inactivation⁸⁴ or reduction in p53 expression^{54,85} also figures as a prominent mechanism underlying androgen-independent growth. Recent data implicates PI3K as a potent suppressor of the p53 pathway as Akt-mediated phosphorylation of Mdm2 promotes its nuclear localization, high affinity binding to p53-p300 complexes, ubiquitination, and enhanced targeting of p53 for proteasome-mediated degradation.⁸⁰ However, the role of these cell cycle proteins and their requirements during NED has yet to be uncovered. Importantly, the theme of PI3K activation promoting hormone-refractory PCa appears to be conserved even in models that do not possess genetic defects in PI3K pathway regulation, such as CWR22. For instance, microarray analyses of CWR22 recurrent tumors demonstrated elevated expression of genes encoding growth factors (e.g., HGF, VEGFC, FGF2, PDGFA) known to stimulate PI3K/Akt.⁸⁶

IL-6-Induced Neuroendocrine Differentiation

Another potential inducer of NED in PCa is Interleukin-6 (IL-6), a 212 amino acid glycoprotein that was first identified as a regulator of immune and inflammatory responses, but later shown to be involved in cell growth,

differentiation, and metastasis.⁸⁷ IL-6 has been implicated as a driving factor in the progression of PCa and elevated serum IL-6 levels are often found in PCa patients.⁸⁸ The receptors for IL-6, an 80-kd IL-6 specific receptor subunit (alpha chain) and a 130-kd signal transducer called gp130,^{87,89,90} are expressed in prostate carcinomas and the PCa cell lines LNCaP, PC-3, and DU145.⁹¹ The androgen-independent PCa cell lines PC-3 and DU145 express IL-6, but LNCaP cells do not.⁹¹ An antibody to either IL-6 or the 80-kd subunit IL-6 receptor can inhibit growth of PC-3 cells suggesting that IL-6 is part of an autocrine loop promoting the growth and androgen-independence of PC-3 cells.⁹² In contrast, IL-6 treatment of LNCaP cells induces p27^{kip1} and consequent growth arrest^{18,19} and NED, as evidenced by morphological criteria (Fig. 3C) and the expression of NE markers NSE, CgA, and beta-tubulin III.^{18,19,50,93} The receptors for IL-6 are found in cholesterol rich lipid rafts of the plasma membrane and the formation of these rafts appear to be required for IL-6 induced signaling and NED.⁹⁴ IL-6 stimulation of gp130 has been shown to interact with and activate erbB2 and erbB3 receptors in LNCaP cells. Under these conditions, erbB2 appears to be required for IL-6 induced mitogen activated protein kinase (MAPK) phosphorylation.⁹⁵ IL-6 also induces expression of erbB2 (Desai, unpublished data). The above properties are thus shared with androgen-deprived LNCaP cells. Signaling in LNCaP cells induced by IL-6 utilizes the customary JAK-STAT family as mediators of signal transduction.⁹⁶ The binding of IL-6 to its receptor complex results in the autophosphorylation and activation of JAK1, JAK2, and Tyk2, which in turn leads to the phosphorylation of and transcriptional activation by STAT3. STAT1, STAT5a, and STAT5b are expressed in LNCaP, but do not become activated by IL-6 signaling.¹⁶ STAT3 appears to be the key player in NED induced by IL-6, as ectopic expression of a dominant-negative form of STAT3 in LNCaP cells abrogate their differentiation into NE cells and the expression of NSE and CgA.⁹³ IL-6 does not induce differentiation or NSE expression in PC-3 cells, which do not express STAT3. However, STAT3 overexpression in PC-3 cells results in an NED like morphology. Treatment with IL-6 does not further increase the NED phenotype, but does slightly induce NSE expression. Consistent with IL-6 induced NED, STAT3 overexpression in PC-3 cells leads to constitutive activation of STAT3 and growth inhibition of PC-3.⁹³

IL-6 can also mediate PCa aggressiveness through antagonism of apoptosis. Similar to androgen withdrawal, IL-6 triggers the PI3K pathway. IL-6 has previously been reported to protect Hep3B hepatocarcinoma cells from TGF- β induced apoptosis through PI3K-Akt activation and by inhibiting caspase-3 activation.¹⁷ Accordingly, inhibition of IL-6 signaling sensitizes PC-3 cells to etoposide-mediated cytotoxicity.⁹² In LNCaP cells, IL-6 activates Etk, a member of the Btk tyrosine kinase family,⁹⁷ in a PI3K-dependent manner.¹⁹ Etk overexpression protects LNCaP cells from apoptosis induced by oxidative stress or thapsigargin.⁹⁸ This protection was abolished by treatment with LY294002 prior to challenge with either stress. Indeed, Etk poses as a critical anti-apoptotic component of the IL6R-PI3K axis parallel to Akt signaling. IL-6 induced STAT3 activation has been implicated as an anti-apoptotic signal by virtue of affording protection to Hep3B cells,¹⁷ and by gp130-STAT3-mediated induction of Bcl-2 in BAF-B03 mouse pro-B cells.⁹⁹ However, the role played by STAT3 in IL-6-treated LNCaP cells with respect to apoptosis has yet to be determined.

Etk appears to play a critical role in modulating the cytoskeletal changes that manifest as the characteristic NE morphology induced by IL-6. Treatment of LNCaP cells with IL-6 promotes the extension of short neuritic processes having a high level of branching complexity. While this can be recapitulated by overexpression of Etk in the absence of ligand, abrogation of Etk function using a dominant-negative approach alters the morphological changes to long, unbranched outgrowths¹⁹ more typical of the response to androgen withdrawal. Consistent with these observations, we have been unable to detect Etk activation in the latter scenario (Desai, unpublished data) thereby implicating Etk as a strong effector of branching seen in IL-6 induced NED. Currently, the mechanism via which Etk activation translates into extension of neurites is speculative. The small GTPase RhoA, but not Rac1 or Cdc24, has recently been shown to be activated by Etk.¹⁰⁰ Since RhoA controls the organization of cytoskeletal actin and mediates the formation of stress fibers, RhoA activation by Etk would be consistent with the requirement of Etk in the IL-6 induced NED phenotype. Etk can also associate with focal adhesion kinase (FAK) in LNCaP cells.^{101,102} FAK has been shown to promote neurite outgrowth in PC12 cells¹⁰³ and appears to be necessary for this process in LNCaP cells since expression of a dominant negative form of FAK prevents differentiation by

IL-6 stimulation or androgen withdrawal (Desai, unpublished data). As with nonmalignant cells, cellular morphology and the processes regulating cell shape have functional implications. This is exemplified by the Etk-FAK association also serving as a migration signal in response to GRP or integrin stimulation of LNCaP or HUVEC cells, respectively.^{101,102} Interestingly, Etk is highly expressed in PC-3 cells, which have a greater migratory potential compared to LNCaP cells.¹⁰² Etk has been shown to interact with Cas, a docking protein, and enhance the interaction of Cas with Crk.¹⁰⁴ The Cas/Crk complex is involved in regulating actin and cell motility and loss of Etk inhibits migration in COS-7 cells. Both Cas and Crk are also recruited by FAK, and it will be interesting to see whether an Etk/FAK/Cas/Crk complex is formed at the cellular membrane during IL-6 induced NED of LNCaP cells. This suggests that the final NE morphology and aggressiveness of the cell might be determined by levels of Etk, FAK, and the molecules with which they interact.

Other Factors Inducing Neuroendocrine Differentiation

While other factors have been shown to induce NED, less is known about the pathways involved. Inducers of cyclic AMP, such as epinephrine, forskolin (Fig. 3D), and PAPCA, can induce NED of LNCaP cells.^{16,20,21,105,106} Cyclic AMP elevation leads to growth arrest and expression of NSE, CgA, PTHrP, neurotensin, and serotonin. PKA and MAPK activation are thought to be involved^{16,20} and may lead to activation of the transcription factor CREB.¹⁰⁶ Contrary to IL-6 or androgen-withdrawal-induced differentiation, cAMP-induced differentiation does not appear to be terminal. Removal of cAMP inducers results in the loss of the NED phenotype and an increase in protein kinase activities.²¹ Co-treatment of IL-6 with cAMP enhances the extent of NED suggesting that these agents may cooperate to induce differentiation in PCa.¹⁶ HB-EGF, a prostate stromal-derived factor that promotes survival, can induce differentiation and increase NSE expression through MAPK activation.²³ In contrast to other NED inducers, HB-EGF does not induce cell cycle arrest,²³ but instead promotes androgen-independent growth of LNCaP cells and down-modulates AR.²² IL-1 β and IL-2,^{107,108} and anticancer

agents that induce growth arrest such as jolkinolide B,¹⁰⁹ the COX-2 inhibitor NS-398,¹¹⁰ and silibinin¹¹¹ also induce NED and an increase in NSE and CgA expression in LNCaP cells suggesting that NED may be a default pathway in response to cellular stress in androgen-dependent cells.

As discussed above, a wealth of clinical and experimental data implicates NED as a common feature of hormone-refractory disease. Although NED can be induced by circulating cytokines or passively as a result of androgen ablative therapy, NE cells possess the potential to actively and potently drive the metastatic growth of PCa cells in the absence of androgens and/or in the presence of AR antagonists. This is primarily accomplished through the paracrine, and possibly autocrine, secretion of polypeptide factors such as neurotrophins and cytokines. Below, we will discuss the signaling pathways triggered by several of these polypeptide ligands and how they engender one or more of the vital components of androgen-independent growth such as reinstatement of AR signaling and stimulation of metastasis through enhanced motility, invasiveness, and angiogenesis. Since there is significant diversity in these ligands, the receptor-mediated signals they evoke, and their ultimate biological effects, the combinatorial outcome of “Neurokine” signaling might be sufficient to support androgen-independent growth. A summary of the signal pathways involved in neurokine-induced androgen-independence is presented in Fig. 2B.

Androgen-Independent Growth

The absence of androgen and subsequent down-regulation of AR during androgen-ablation therapy would suggest that AR is no longer required in androgen-independent tumors. However, AR is still expressed in almost all androgen-independent tumors³ and this suggests that AR is involved in androgen-independent growth of PCa. Amplification of AR and its coactivators such as p160 SRCs⁴⁻⁶ are also detected in relapsed and metastatic tumors. Androgen-independent LNCaP cell lines, which can grow in the absence of androgen, show an increased level of AR expression.^{49,64,112} Mutations in AR can lead to the activation of AR by other steroids such as estrogens,¹¹³ glucocorticoids,¹¹⁴ and even nonsteroidal anti-androgens

used to treat PCa.¹¹⁵ However, the low frequency of mutations in androgen-independent tumors is leading investigators to investigate alternative mechanisms of AR activation, either being ligand-independent or in synergy with castrate levels of androgen.

Three classes of activators have been identified (1) circulating growth factors that bind to transmembrane receptor tyrosine kinases (EGF, IGF-1, and KGF); (2) neuropeptides (GRP and neurotensin) and chemokines (IL-8) secreted by neuroendocrine cells that activate G-protein-coupled receptors (GPCR); and (3) interleukins (e.g., IL-6) that bind to cytokine receptors. These factors, while differing in the detailed signal pathways they transmit, share certain common traits. For instance, they all induce phosphorylation cascades and activate tyrosine and serine/threonine kinases. It has been suggested that phosphorylation of AR, or its activators, facilitates the assembly of a transcriptional complex that mimics the recruitment by androgen-bound AR, and may be an alternate route to AR activation.¹¹⁶ Since AR and its coactivators are known to be phosphorylated only at serines and threonines, serine/threonine kinases are thought to be immediate activators of the AR complex, whereas tyrosine kinases are the upstream activators of serine/threonine kinases. Phosphorylation and activation of AR by the serine/threonine kinases ERK,^{117,118} Akt,⁶² PKA,^{119,120} PKC,^{121,122} and CAK¹²³ have been reported. Examples of serine/threonine kinase activation of AR coactivators include ERK phosphorylation of SRC-1⁶⁴ and phosphorylation of SRC-2⁴ by IL-6 and EGF stimulation, respectively. Most of these studies were based on *in vitro* studies or transient expression systems. The *in vivo* phosphorylation sites of AR have recently been identified,^{124–126} and ironically none of the sites are consensus phosphorylation sites of ERK or Akt,¹²⁴ raising the possibility that other novel kinases are involved. Studies to identify protein kinases associated with or induced by AR have resulted in the discovery of several novel kinases, ANPK,¹²⁷ SPAK¹²⁸ MAK,¹²⁹ and PAK6.^{130,131} These kinases modulate (ANPK, SPAK, and MAK activate, while PAK6 represses) AR activity by directly binding to AR, but do not seem to directly phosphorylate AR or affect cellular growth induced by androgen. Despite these new twists, it seems clear that AR activity can be enhanced by kinases. In the ensuing sections, we will review how soluble factors induce the activation of these kinases.

Growth Factors

Growth factors such as KGF, IGF-1, and EGF have been shown to activate AR in the absence of androgen,¹³² via the activation of their respective receptor tyrosine kinases (FGF-R, IGF-R, and EGFR). ErbB2, when over-expressed, can induce PSA expression in an androgen-independent, but not AR-dependent manner.^{117,118} All of the receptor tyrosine kinases are known to activate serine/threonine kinases, such as MAPK^{117,118} and Akt,⁶² which in turn activate AR activity as discussed above.

Neuropeptides

Of growing interest is elucidating the role of neuropeptides in the androgen-independent growth of PCa. Since neuropeptides are secreted by NE cells, the potential contribution of the prostate micro-environment to cancer growth becomes significant. As mentioned in the previous section, NE cells in the prostate and NE-differentiated LNCaP cells secrete neuropeptides such as GRP and neurotensin. Androgen-independent PCas and PCa cell lines also express GRP and neurotensin, as well as chemokines such as IL-8, that appear to be involved in an autocrine loop that stimulates growth. GRP receptors^{133,134} and neurotensin receptors¹³⁵ are found in PCas and PCa cell lines. GRP and neurotensin have been shown to stimulate androgen-independent growth in PC-3, DU145, and LNCaP cells.^{51,101,135–138} Conversely, GRP antagonists^{139–142} and neurotensin antagonists¹³⁵ have been shown to inhibit PC-3 and DU145 growth. The majority of information about the mechanism mediating neuropeptide-induced growth of PCa pertains to GRP signaling. GRP binds to and activates GPCR proteins (Fig. 2B), which engage the heterotrimeric G proteins α , β , and γ , which in turn leads to sequential Ca^+ mobilization, PLC and PKC activation, and ultimately cellular proliferation.¹⁴³ The specific response in the cell is dependent on which G protein is activated and whether it is stimulatory or inhibitory. In the case of GRP, GRP-R has been shown to activate $G_{\alpha q}$, which activates the $\text{PLC}\beta$ pathway and $G_{\alpha 12}$, which activates the Rho pathway. However, activation of $G_{\alpha q}$ and $G_{\alpha 12}$ by GRP in PCa cells has not been verified. In PC-3 and DU145, cross-talk between GPCR proteins and receptor tyrosine kinases¹⁴⁴ has been

demonstrated by the ability of GRP to transactivate EGFR and that it requires Src activation and Ca^+ mobilization. Once EGFR is activated it leads to Ras and MAPK activation and DNA synthesis. The transcription factor Elk-1, and immediate early genes *c-fos* and *c-myc* are also up-regulated by GRP presenting a possible pathway for growth induction.¹⁴⁵ Evidence for GRP as a progression factor is provided by its ability to up-regulate proangiogenic factors such as MMP-9,^{146,147} VEGF, and IL-8,¹⁴⁸ the latter two being mediated through NF- κ B. Furthermore, cross-talk between GRP signaling and β 1 integrin results in enhanced processing of pro-MMP-9 into its active form as a result of Src and PI3K activation culminating in the up-regulation of membrane-bound uPA.¹⁴⁶ GRP activation of the PKC pathway in PC-3 cells leads to the activation of FAK and increased motility suggesting that GRP may also play an important role in tissue invasion, increasing the potential for metastasis.¹⁴⁹

GRP and neurotensin have also been shown to activate AR and induce androgen-independent growth of LNCaP cells.¹⁰¹ GRP can act synergistically with low levels of androgen to activate AR, leading to the possibility that residual circulating androgen present after androgen-ablation therapy could cooperate with GRP in achieving an androgen-independent state.¹⁵⁰ GRP-induced activation and complex formation of Etk, FAK, and Src are required for AR activation. Interestingly, Etk activation is dependent upon Src and FAK, but not on PI3K, as is the case with IL-6 stimulated Etk activation. Activation of AR supports the notion that neuropeptides might mediate the transition from an androgen-dependent to -independent state in PCa. Further indication that GRP may be a transition-promoting factor is the evidence that neutral endopeptidase (NEP), which cleaves neuropeptides, is down-regulated in androgen-independent tumors,¹⁵¹ and is down-regulated by GRP in LNCaP.¹⁵² Reintroduction of NEP in androgen-independent cells interferes with GRP signaling¹⁵³ and cellular migration.¹⁵⁴

Chemokines

As mentioned above, the chemokine IL-8 is an angiogenic factor involved in the migration and invasion of cancer cells. Serum IL-8 levels are elevated in PCa patients¹⁵⁵ and IL-8 is overexpressed in PC-3 cells.¹⁵⁶ The

IL-8 receptors, CXCR1 and CXCR2, are expressed in DU145 and PC-3 cells¹⁵⁷ and antisense-mediated suppression of IL-8 expression reduces invasion and metastatic potential of PC-3 xenografts in nude mice.¹⁵⁸ Many studies have focused on the role of IL-8 in tissue invasion and metastasis.^{158–160} However, a recent finding showed that IL-8 could stimulate the androgen-independent growth of LNCaP cells via recruitment of the AR transcription complex to the proximal region of the PSA promoter.¹⁶¹ Analogous to GRP signaling, IL-8 induces Src, FAK, and MAPK activation (Fig. 2B) and promotes migration of LNCaP cells. Src and MAPK appear to be required for AR activity and LNCaP proliferation, while Src and FAK are required for LNCaP migration. Together, these data indicate that GRP and IL-8 may be progression factors of PCa contributing to the androgen-independence and metastatic potential of advanced cancers.

Cytokines

As described in the previous section, our work, as well as that of others, demonstrated that IL-6 treatment of LNCaP induced NED and growth arrest.^{19,162} Paradoxically, IL-6 has also been found to induce growth of PCa cells, including LNCaP. When subjected to prolonged IL-6 treatment, eventual selection of proliferating LNCaP cells occurred. While the molecular details of the genetic adaptation caused by IL-6 is not clear, once achieved, the cells become androgen-independent and can grow in the presence of IL-6. This may be related to the observation that in low-passage LNCaP cells, activated Akt down-modulates AR, whereas in high-passage LNCaP cells, it enhances AR activity.⁶⁷ In fact, a very interesting aspect of the pleiotropic influence of IL-6 upon the transition to androgen-independence is its ability to directly regulate AR.^{163–167} Although IL-6 activation of AR can occur without androgen, IL-6 can also act synergistically with low levels of androgen.^{163–165} The pathway of IL-6-mediated AR activation is complex and involves multiple signal transducers. Two reports demonstrated that JAK-STAT3 activation is the principal pathway responsible for AR activation via direct STAT3-AR interaction.^{163,164} This was confirmed by inhibition of AR activation in the presence of over-expressed dominant negative STAT3. However, a third report implicated

MEK/MAPK, PKA, and PKC, since AR activation could be inhibited with pharmacological inhibition of each of these kinases.¹⁶⁵ Yet another paper showed that IL-6 activation of AR depends on ERK and p300.¹⁶⁷ One reason for this diversity may lie in differences in cellular milieu and the outcome of competition for cellular resources that can activate AR.¹⁶⁶ For instance, STAT3 is the dominant mediator of IL-6 signaling and AR activation in LNCaP cells, but PKA or PKC might predominate to activate ectopically-expressed AR in the STAT3-deficient cell line PC-3. An intriguing question is how IL-6 and AR cooperate under diverse conditions of NED. In contrast to down-regulation of AR activity and protein levels during androgen withdrawal, IL-6 might contribute to cell survival during androgen ablation therapy through its ability to activate the AR. This could result in maintenance of AR expression so that other growth factors are able to utilize AR to circumvent androgen dependence.

Summary

In summary, there is considerable evidence that NED represents an integral and potentially important part of prostate carcinogenesis. The propensity of prostate carcinoma cells to undergo NED under a variety of conditions including androgen withdrawal suggest that it is an innate property of prostate epithelial cells to survive and to overcome adverse conditions. Although the cellular signals involved in this trans-differentiation process vary with different inducing conditions, the differentiated cells have three common characteristics: resistance to apoptosis, growth arrest, and expression of neuronal phenotypes. The release by these cells of neurokines, which are survival and growth factors, is likely to have an impact on surrounding undifferentiated prostate cancer cells undergoing androgen-deprivation crisis. Accumulating evidence suggests that these neurokines are capable of activating AR, presumably via kinase signaling. This may be one way a fraction of PCas survive androgen ablation and eventually develop into androgen-independent clones. If true, targeting neurokines and the downstream protein kinases in conjunction with hormone therapy offers a new intervention strategy for treatment of prostate cancer and in particular, androgen-independent tumors. (This work from the authors' labs was supported by grants from the NIH and DOD.)

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BIOLOGY OF PROSTATIC ACID PHOSPHATASE AND PROSTATE-SPECIFIC ANTIGEN AND THEIR APPLICATIONS IN PROSTATE CANCER

Suresh Veeramani*, Ta-Chun Yuan*, Siu-Ju Chen*
Fen-Fen Lin* and Ming-Fong Lin*,†,‡

**Department of Biochemistry and Molecular Biology*

†Department of Surgery/Urology, College of Medicine and

‡Eppley Institute for Cancer Research

University of Nebraska Medical Center

Omaha, NE 68198, USA

Introduction

Prostatic acid phosphatase (PACp) and prostate-specific antigen (PSA) are two well-known biomarkers of prostate epithelial cells and are of immense value historically in the detection of prostate cancer (PCa). Because both are elevated significantly in the circulation of patients with PCa, correlating with the stages of clinical PCa, PACp is an old marker for PCa diagnosis and PSA is the new gold standard. Early detection of PCa has been accomplished in recent years utilizing PSA-based screening methods that have led to the early treatment of PCa and decreased incidence of PCa death. In this chapter, we focus our discussion on the recent advances in the biology of PACp and PSA, and include a brief overview of the history of PACp and PSA in PCa diagnosis. Various clinical tests based on PSA that have revolutionized the field of PCa diagnosis are beyond the scope of this chapter but are described elsewhere.¹⁻³ In the first section, we focus on the role of PACp in androgen-regulation of cell proliferation and the tumorigenicity of PCa cells. In the second section, we discuss the biological function of PSA. The major impediment in the treatment of PCa is

the development of hormone-refractory cancer, which is indicated by the rebound of the circulating PSA level. We focus our discussion, in the latter part of the chapter, on the possible mechanisms of androgen-independent PSA secretion by hormone-refractory PCa.

Historical Review — Discovery and Clinical Applications of PACP and PSA

PCa has become the most predominant cancer in American men, with approximately 30,000 deaths each year.⁴ With increased testing and screening programs, most PCa cases are diagnosed while they are still confined to the prostate.^{1,2} Since the early 1930s, the quest for a suitable marker for the diagnosis of PCa has been the center of attraction. In 1936, Gutman and his colleagues made the seminal observations that the activity of PACP is increased in the circulation of patients with PCa, especially those with bone metastasis, to a level higher than that in normal adult males.⁵ Subsequent studies confirmed that secretory PACP (sPACP) activity correlates with prostate tumor progression and can serve as an indicator of treatment.⁶ Since then, sPACP had been studied extensively as a marker for PCa diagnosis and therapeutic efficacy until the usefulness of PSA was appreciated.^{1,6}

PSA was discovered by Chu and associates in a major research effort in search of promising markers for PCa diagnosis.⁷ Quantitative assessments of PSA in the early 1980s revealed that the level of PSA is elevated in the circulation of patients with PCa^{1,8} and correlates with cancer stage. PSA is of immense value in predicting the survival rate of patients with advanced PCa.⁹ Within a few years of its discovery, the clinical potential of PSA was fully appreciated and PSA test kits shortly became available. Those basic efforts have led to early diagnosis and decreased PCa death rates.¹

Although there is a general concern that PSA may detect too many clinically insignificant tumors, it should be emphasized that with the available modifications to increase the specificity of PSA-based tests, the detection of organ-confined clinical cancer has increased 2-fold, with reduction in disseminated cancer between 1986 and 1992, and reduction in death rates from 34,902 to 31,078 in the years 1994 and 2000.^{1,4} Hence, the introduction of PSA-based tests has not only led to the increased

detection of early PCa but also led to timely therapeutic intervention resulting in reduced mortality rates.

PACP

Biochemical Properties of PACP

The active form of PACP protein contains two subunits with a similar molecular size of approximately 50 kDa. The PACP subunit was initially translated as a proprotein with 386 amino acids containing a 32-amino acid signal peptide.¹⁰ Post-translational modifications in the endoplasmic reticulum are apparently required for the stability of PACP protein because the expression of a signal peptide-null PACP from a cDNA expression vector occurs at very low levels (X. Q. Zhang and M. F. Lin, unpublished observations). Post-translational processings generate several intermediate species of PACP proteins.¹¹

PACP is the major acid phosphatase in normal, well-differentiated prostate epithelial cells.¹²⁻¹⁴ There are two forms of PACP: the cellular (cPACP) and the sPACP forms. Both forms of PACP can hydrolyze a broad variety of small organic phosphomonoesters *in vitro* in acidic conditions.¹²⁻¹⁵ Physiologically, cPACP level is negligible before adolescence in males. In normal adults, cPACP is found at a high level of approximately 0.5 mg/gm wet tissue.^{12,16} sPACP is secreted into seminal fluid at concentrations of approximately 1 mg/ml.¹⁷ Thus, sPACP may have a functional role in fertility. Interestingly, sPACP might also exhibit amidolytic activity on seminal proteins.¹⁸ Further studies are needed to clarify the roles of sPACP.

PACP — An Authentic Protein Tyrosine Phosphatase

PACP efficiently hydrolyzes small organic compounds, including phospho-amino acids, with *K_m* values at mM ranges.¹⁹ Interestingly, PACP preferably hydrolyzes aromatic compounds over aliphatic compounds.²⁰ PACP was initially shown to be co-purified with the major protein tyrosine phosphatase (PTP) activity in non-cancerous prostatic cells.²¹ Further studies revealed that biochemically, PACP exhibits dual-specific protein

phosphatase activity.^{19,22,23} It has a very high affinity for the p-Tyr linkage in protein substrates with K_m values in the nM range, which is over 50-fold higher than for p-Ser/p-Thr linkages and several orders of magnitude higher than for free phospho-amino acids.¹⁹ Unexpectedly, a low V_{max} is observed, which could be due to high affinity interactions between PACP and p-Tyr protein substrates resulting in a delay in the release of the dephosphorylated substrate.

The PTP activity of PACP has been demonstrated by various approaches.^{24–29} The site-directed mutagenesis approach confirmed that PACP indeed exhibits authentic PTP activity.³⁰ It is interesting to note that PACP contains neither the characteristic signature motif of the PTP superfamily nor the signature motif of the dual-specific protein phosphatase.^{10,31} Based on the three-dimensional structure of rat PACP, it was proposed that the Cys¹⁸³ residue of PACP functions as the phosphate acceptor and participates in the dephosphorylation reaction.³² The reactivity of Cys¹⁸³ was confirmed by titration experiments.³³ Additionally, Asp²⁵⁸ is conserved in the PTP family.³² Nevertheless, His¹² and Asp²⁵⁸, but not Cys¹⁸³ or Cys²⁸¹, are required for the PTP activity of PACP.³⁰ The results thus indicated that PACP is a novel PTP and uses the same active site and catalytic mechanism of acid phosphatase (AcP) to execute its PTP activity.

ErbB-2/HER-2/Neu (ErbB-2) — An In Vivo Substrate of PacP

cPACP is implicated to be the major PTP in non-cancerous prostate epithelial cells.^{19,21} Using the autophosphorylated epidermal growth factor receptor as a substrate, p-Tyr dephosphorylation by PACP was optimal at a neutral pH.³⁴ The putative substrate of cPACP was established in the mid-1990s. Incorporation of PACP protein into PACP-null DU145 PCa cells showed decreased tyrosine phosphorylation of a 185 kDa phosphoprotein³⁵ that was later identified to be the ErbB-2 protein. Thus, PACP interacts with ErbB-2 and dephosphorylates the p-Tyr linkage of ErbB-2.³⁶ Initial analyses showed that cPACP dephosphorylates ErbB-2 protein primarily at the Tyr¹²⁴⁸ residue³⁰ that interacts with p52^{Shc} protein, which is involved in regulating androgen action.³⁷ ErbB-2 is a putative growth factor receptor with an intrinsic tyrosine kinase activity.³⁸ Thus, the low cPACP activity in prostate carcinomas^{29,39–41} may infer unregulated

tyrosine phosphorylation of ErbB-2, leading to aberrant tyrosine phosphorylation signaling.²⁹ The function of cPAcP in PCa cells is, at least in part, to cause p-Tyr dephosphorylation of ErbB-2 protein.^{29,30,36}

Biological Activity of cPAcP

Effect of cPAcP on the Growth and the Tumorigenicity of PCa Cells

In PCa cells, the level of cPAcP inversely correlates with the proliferation rate and the tumorigenicity (Table 1).^{42,43} For example, growth stimulation of LNCaP cells by various stimuli results in decreased cPAcP activity. Furthermore, the introduction of exogenous PAcP into PC-3 cells, by a cDNA expression vector, diminishes growth rates and tumorigenicity (Table 1).^{26,29}

Table 1. Correlation of cPAcP with the Proliferation and the Tumorigenicity of PCa Cells

	C-33 20 → 33	C-51 33 → 80	C-81 80 → 125	LN-28 and -40	PC-3	PC-411 and -416
Androgen Sensitivity	+++	++	+	++/+++	-	+
AR	+++	+++	+++	+++	+	+
cPAcP Expression	+++	++	+	++	-	+
pErbB-2 Level	+	++	+++	+	+++	+
ErbB-2 Protein	+++	+++	+++	+++	++	++
Cell Growth*	112	57	49	86	33	52
PSA Secretion (Steroid-Reduced Condition)	+	++	+++	+	N/A [†]	N/A [†]
Tumorigenicity [‡]	+	++	+++	+	++++	++/++++

*The doubling time in hours was estimated in the steroid-reduced condition.³⁶

[†]Not applicable because PC-3 cells do not express PSA.

[‡]Tumorigenicity was analyzed utilizing xenograft animal models.²⁹

Note: LNCaP C-33, C-51 and C-81 cells are LNCaP-FGC cells that have passage numbers between 20–33, 35–80 and 81–125, respectively. LN-28 and LN-40 cells are stable subclones of C-81 cells transfected with a PAcP cDNA expression vector, while PC-411 and PC-416 cells are stable subclones of PC-3 cells transfected with PAcP cDNA expression vector.^{27,36} PAcP expression gradually decreases upon passage in LNCaP cells. This results in aberrant tyrosine phosphorylation of ErbB-2 protein leading to increased cell growth, PSA secretion, and tumorigenicity even under androgen-depleted conditions. The LNCaP cell model closely resembles the observations in the clinical PCa specimens.

We have established an interesting cell model system using the LNCaP cell line, which recapitulates PCa progression in the clinic.^{27,43} Prolonged passage of androgen-sensitive parental LNCaP C-33 cells (starting at passages 20–33) in regular culture conditions resulted in loss of androgen-sensitivity and development of androgen independence by passage 81 (LNCaP C-81) (Table 1). Although C-81 cells express a similar level of functional androgen receptor (AR) as their parental C-33 cells, they behave as androgen-independent cells, resembling the clinical progression to advanced hormone-refractory PCa.⁴³ Importantly, even in the absence of androgen, PSA secretion in C-81 cells is much higher than in C-33 cells.⁴³ Interestingly, C-81 cells showed decreased cPacP expression, and increased proliferation rate and malignancy as compared to the C-33 cells.

Several lines of evidence show that cPacP exhibits the characteristics of a tumor suppressor. First, the expression of cPacP is decreased or absent in prostate carcinomas, correlating with cancer progression.^{39–41} Second, the loss of cPacP expression in PCa cells correlates with increased tumorigenicity.²⁹ Finally, ectopic expression of cPacP directly suppresses tumor growth in xenograft animal models.^{28,29} Collectively, decreased expression of cPacP correlates with PCa progression, rapid tumor formation, and androgen-independent cell proliferation. Thus, the level of cPacP may serve as a useful marker for predicting the metastatic potential of PCa cells.

Effect of cPacP on Androgen Sensitivity of PCa Cells

In AR-positive PCa cells, the expression of cPacP correlates with androgen sensitivity (Table 1). In AR-positive, androgen-independent PCa cells, ectopic expression of cPacP restores androgen-stimulated proliferation.^{27,44} One possible explanation of this phenomenon is that in AR-positive, cPacP-expressing cells the cell growth is decreased, in part, due to ErbB-2 dephosphorylation (Fig. 1). Upon androgen stimulation, cPacP activity decreases, resulting in increased tyrophosphorylation of ErbB-2 and subsequent promotion of cell proliferation.^{25,26,44} In PacP-null PCa cells, ErbB-2 is hyper-tyrophosphorylated, which promotes cell proliferation and decreases androgen dependence, despite the expression of functional AR.^{27,44} These results provide an explanation of the clinical phenomenon of advanced

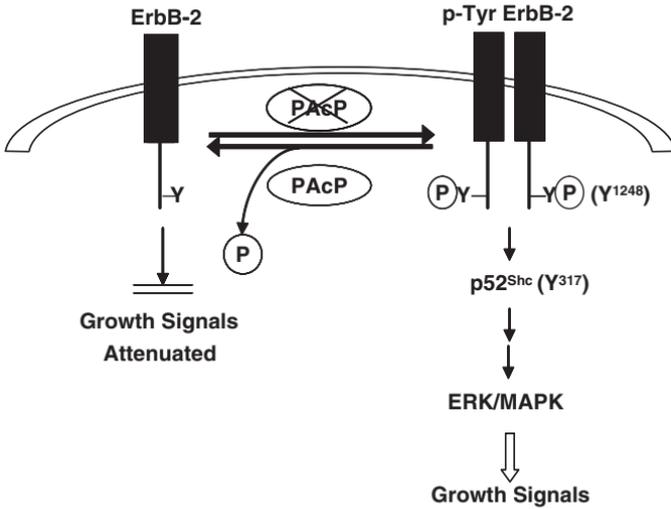


Fig. 1. Proposed roles of cPACP and ErbB-2 in the regulation of prostate cell growth. cPACP is involved in dephosphorylating the ErbB-2 protein, at least at Tyr¹²⁴⁸, thereby blocking the growth signals from ErbB-2. In the absence of or low levels of cPACP, e.g., upon dihydroxytestosterone stimulation and in advanced PCa, ErbB-2 can be aberrantly tyrophosphorylated, which can transduce signals to the ERK/MAPK through p52^{Shc}, leading to growth promotion.

hormone-refractory PCa, in which AR is functional and the expression of cPACP is decreased,^{29,40} while the ErbB-2 gene is not amplified.⁴⁵ Nevertheless, the detailed molecular mechanism by which cPACP and ErbB-2 regulate androgen-stimulated cell proliferation remains to be further elucidated.

PSA

Biological Activity of PSA

PSA, a member of kallikrein family, is a serine protease and exhibits activity and specificity similar to that of chymotrypsin.⁴⁶ PSA is predominantly expressed and secreted by prostate secretory epithelia at approximately 1 mg/ml in the seminal plasma of healthy men.⁴⁷ PSA is also expressed in a wide range of tissues.^{48–51} In these tissues, PSA may be involved in growth regulation by steroid hormones.⁵²

The synthesis and control of PSA expression in prostatic cells are well documented. Naïve translated PSA, or proPSA, is a 244-amino acid protein with a 17-amino acid leader sequence.⁵³ Several inactive truncated forms of PSA are found to be increased in benign prostatic hyperplasia (BPH).^{54,55} The ratio of truncated PSA to total PSA (PSA index) aids in the discrimination between non-cancerous and cancerous prostate gland.¹ Serum PSA may form complexes with α -antichymotrypsin and α_2 -macroglobulin³ and circulate as complexed PSA.^{1,2}

PSA may be involved in the proteolytic cleavage of seminal proteins. Semenogelin I/seminal plasma motility inhibitor (SPMI) precursor and its active product SPMI may be the substrates for PSA.^{56,57} Other putative substrates of PSA include IGFBP-3,⁵⁸ PTHrP,⁵⁹ and extracellular matrix (ECM) proteins, such as fibronectin and laminin.⁶⁰ Since PSA can modulate *in vitro* interactions between IGF-I and IGFBP-3, PSA may play a role in regulating the growth of prostatic fibromuscular cells.⁶¹

Effect of PSA on Prostate Tumorigenicity

The highly invasive property of androgen-independent PCa cells could be due in part to the degradation of the ECM proteins by increased secretion of PSA, since a monoclonal antibody (mAb) against PSA blocked PCa invasion in a dose-dependent manner.⁶⁰ This is in parallel with the clinical observation that patients with metastatic PCa have elevated levels of serum PSA. On the contrary, PSA exhibits anti-angiogenic activity⁶² and has a direct suppressive effect on new blood vessel formation in PCa.⁶³ Furthermore, recombinant PSA inhibits endothelial cell proliferation and tube formation, which are essential features of angiogenesis.⁶² It is also suggested that anti-angiogenic drugs such as TNP-470 might act through increasing PSA secretion.⁶⁴ Future studies are needed to clarify the role of PSA in PCa metastasis.

Androgen-Independent PSA Secretion

Androgens are well documented as key factors regulating PSA gene expression.⁶⁵ Since PCa cells initially depend on androgens for

proliferation, androgen ablation therapy is the mainstay of treatment for advanced PCa, and leads to a drop in circulating PSA. Unfortunately, cancer cells eventually escape from the steroid requirement and progress to androgen independence, which is indicated by the rebound of PSA levels in circulation.⁶⁶ The ability to treat these patients is limited. Interestingly, most of the hormone-refractory PCa cells express functional AR and PSA. The molecular mechanism of androgen-independent PSA secretion deserves careful analysis.

Biochemically, upon androgen binding, AR exhibits conformational changes, induced by modifications including phosphorylation and dimerization, for its activation.⁶⁷ It is proposed that in hormone-refractory cancers, in the absence of androgen, AR is also activated by phosphorylation.⁶⁸ Hence, a direct cross-talk between AR and cellular kinases may be an important factor involved in the elevation of androgen-independent PSA secretion. Our understanding of the cross-talk between AR and protein kinases is discussed briefly as follows.

ErbB-2/HER-2/Neu (ErbB-2) → ERK/MAPK Pathway

Data from our lab strongly indicate that ErbB-2 plays a critical role in hormone-refractory proliferation of PCa cells as well as in their enhanced androgen-independent PSA secretion, and are of clinical relevance (Fig. 2). This notion of ErbB-2 regulation is shown in ErbB-2 cDNA-transfected C-33 cells in which the growth rate and PSA secretion are elevated, even in the absence of androgen.^{38,44,69} Conversely, in C-81 cells in which ErbB-2 is activated and PSA secretion is increased, the expression of a dominant-negative mutant of ErbB-2, or addition of ErbB-2 inhibitors, reduces the elevated androgen-independent PSA secretion.³⁸

In androgen-deprived environments, ErbB-2, via the ERK/MAPK or Akt pathway, might activate AR by serine phosphorylation (Ser⁵¹⁴ for ERK/MAPK and Ser⁷⁹¹ for Akt).^{69–71} Our data indicate that ERK/MAPK plays a key role in the early stage of androgen-independent PSA secretion, as well as in tumor progression. For example, in androgen-depleted conditions, elevated PSA secretion is decreased by MEK1 inhibitor, PD98059, in C-81 cells, while PSA secretion is increased by MEK1 cDNA transfection in C-33 cells.³⁸ Furthermore, in ErbB-2 cDNA-transfected C-33 cells,

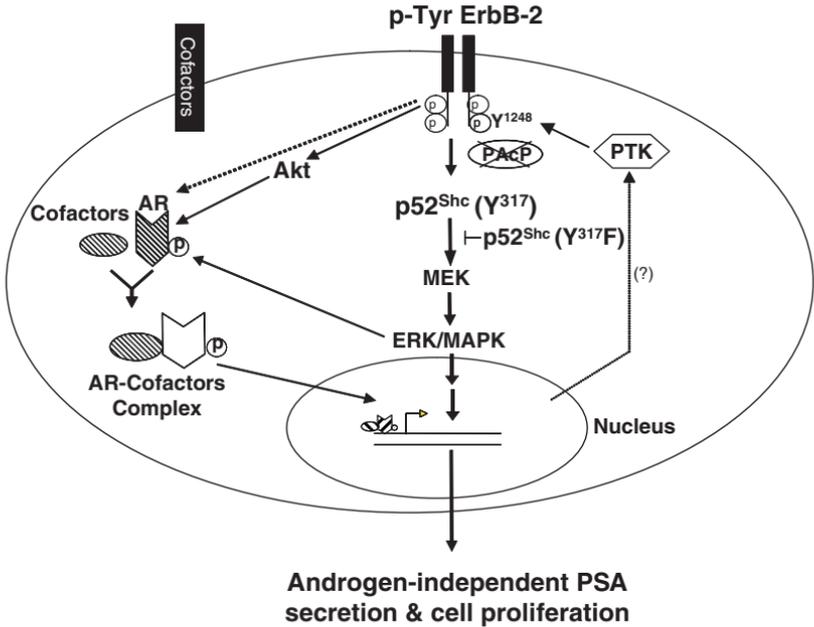


Fig. 2. Proposed model of regulation of PSA expression and secretion by ErbB-2 in hormone-refractory PCa. In androgen-independent human PCa cells, ErbB-2 is activated by hyper-tyrophosphorylation, in part, due to the loss of cPacP expression. The activated ErbB-2 can transduce its signals *via* p52^{Shc} to activate the downstream ERK/MAPK, which may lead to AR phosphorylation, resulting in an increase in androgen-independent expression and secretion of PSA, as well as cell proliferation. Activated ErbB-2 may also phosphorylate AR *via* Akt.

PD98059 effectively abolishes the increased PSA secretion in steroid-reduced conditions. This mechanism (Fig. 2) thus provides an explanation of the clinical observations of hormone-refractory prostate carcinomas in which cPacP is decreased,^{29,39–41} the ErbB-2 gene is not amplified, while the ErbB-2 protein could be either activated by tyrophosphorylation and/or elevated.^{45,72} Subsequently, ERK/MAPKs are activated by phosphorylation,^{73,74} leading to AR activation by phosphorylation,⁷⁵ as well as the rebound of PSA levels in the circulation of most patients.¹ Collectively, our data strongly indicate that the ErbB-2(→)ERK/MAPK pathway is crucial in androgen-independent PCa cell proliferation, as well as PSA secretion.

ErbB-2 can up-regulate PSA secretion at two levels: the transcriptional level and the post-translational level in the secretory pathway. In C-81 cells in which ErbB-2 is activated by hyper-tyrophosphorylation, the level of PSA mRNA is elevated in the steroid-reduced condition, compared to C-33 cells,^{27,43} which may be due in part to the activation of the PSA promoter by ErbB-2 *via* ERK/MAPK and AR.⁶⁹ Alternatively, ErbB-2 may activate MEK1, leading to the up-regulation of a transcription factor(s) that subsequently activates the PSA promoter.⁷⁶ ErbB-2 may also activate the secretory pathway of PSA in those cells (M. S. Lee and M. F. Lin, unpublished observations). Nevertheless, further experiments are needed.

Akt Pathway

AR phosphorylation might also occur through the PI3K → Akt pathway in hormone-refractory PCa cells.⁷¹ Androgen-responsive, low passage LNCaP cells have a lower Akt activity than the androgen-independent high passage cells,⁷⁷ which implies that this pathway could be important in later stages of advanced metastatic cancer, where PTEN mutation leads to constant Akt activation. Nevertheless, it should be noted that pathways other than PTEN could be involved in activating Akt in higher passage LNCaP cells because PTEN is also mutated in the lower passage LNCaP cells.^{78,79} Interestingly, the anti-androgen flutamide blocks Akt-induced PSA secretion in IL-4-treated LNCaP cells,⁸⁰ while it stimulates the growth of LNCaP cells,⁸¹ indicating the differential roles of Akt. Nevertheless, IGFBP-5 cDNA-transfected LNCaP cells express hyperactivated Akt and exhibit higher growth rates and PSA secretion levels in castrated mice than do control cells.⁸² Further experiments are required to clarify the role of Akt in androgen-independent PSA secretion *vs.* hormone-refractory PCa cell proliferation and its clinical relevance.

Cytokine Pathway

Interleukin-6 (IL-6) has been proposed to play a role in the development of androgen-independence in PCa cells because IL-6 enhances the growth of LNCaP cells in androgen-depleted environment and serum IL-6 levels are found to be elevated in some patients with advanced PCa. Since

androgen-independent LNCaP-IL-6+ cells, which were selected by continuous passage in media containing 5 ng/ml IL-6, are resistant to the cell cycle arrest mediated by IL-6,⁸³ it is inferred that IL-6 may contribute to PCa progression, and *via* the ErbB-2(→)ERK/MAPK pathway, stimulate androgen-independent PSA secretion.^{84,85} IL-4 might also have a role in the androgen-independent, AR-mediated PSA secretion,⁸⁰ because IL-4 level is elevated in some PCa patients with hormone-refractory cancer.⁸⁶

PKA Pathway

Forskolin, a PKA activator, can increase the activity of the PSA promoter in the absence of androgen.⁸⁷ The activation of PKA by forskolin also results in increased PSA expression in androgen-depleted LNCaP cells.⁸⁸ Butyrate, a differentiation agent, increased circulating PSA levels in castrated animals. Electromobility shift assay showed an increased AR-ARE complex formation in butyrate-treated LNCaP cells that is partially inhibited by a PKA blocker, indicating that butyrate action on LNCaP cells might, in part, involve the PKA pathway.⁸⁸

Other Downstream Mechanisms

Protein phosphorylation may activate other regulatory factors, e.g., AR cofactors, leading to increased PSA secretion. Alternatively, aberrantly regulated transcriptional factors may play a role in this mode of regulation. For example, nuclear factor-Kappa B (NF-κB), which is increased in androgen-independent PCa cells, can activate PSA expression,⁸⁹ while the growth of those cells is inhibited by NF-κB inhibitors.⁹⁰ A 45-kDa cell-specific transcription factor (p45) has also been implicated in androgen-independent expression of the PSA gene.⁹¹

Future Perspectives

The decreased expression of cPacP is implicated as a crucial step in carcinogenesis and/or progression of human PCa. More studies of the basic biology of PacP may provide new insights into its potential roles in tumor

suppression and potential uses for therapy of PCa. In xenograft animal models, a single intratumoral injection of an expression vector encoding the wild type PAcP resulted in the growth suppression of prostate tumors.²⁸ The restoration of cPAcP expression in PCa may provide a new avenue for treating patients with advanced PCa in which the expression of PAcP is lost.

Functional molecules involved in androgen-independent pathways may serve as potential targets for clinical therapy. Inhibitors of androgen-independent pathways such as ErbB-2 and ERK/MAPK blockers might prove valuable in the treatment of hormone-refractory PCa. Studies indicated that co-administration of the ERK/MAPK inhibitor PD98059 with docetaxel increases the apoptotic death of androgen-independent PCa cells.⁹² Chemointerventions for hormone-refractory PCa might be made more useful with co-administration of these pathway blockers with the regular therapeutic regime.

Immunotherapy for PCa also has potential value. Phase I studies using PSA expressing vaccinia viral vectors indicated that T cells could be induced in the patients.⁹³ Vaccination with dendritic cells preloaded with PSA mRNA resulted in the reduction of circulating PSA levels in 80% of the patients.⁹⁴ Similar experiments with PAcP also showed some efficacy.⁹⁵ Future work is needed in the clinical utilization of PAcP and PSA as targets of immunotherapy against PCa.

The proteolytic property of PSA has been exploited in the development of therapeutic interventions for PCa. For example, peptide-conjugated -doxorubicin⁹⁶ and vinblastine,^{97,98} which can be activated in prostate tissue by PSA, has shown some promising results. Similar strategies have been applied for PAcP (M. F. Lin, unpublished observations). Nevertheless, promotion of such strategies to clinical application requires further studies. In conclusion, due to the recent advances in understanding the biology of PAcP and PSA, as well as their functional roles in PCa progression and metastasis, new avenues through which to develop novel strategies for improving the efficacy of treating hormone-refractory PCa are expected.

Acknowledgments

This project is in part supported by NIH, NCI, CA88184, and UNMC Graduate Student Fellowship (T. C. Y.; S. J. C). We thank all group

members for their efforts toward conquering this cancer. We are also indebted to Dr. T. M. Chu and his colleagues for the discovery of PSA.

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EPIGENETICS IN PROSTATE CANCER

Jose A. Karam, Elie A. Benaim, Hong Chen,
Rey-Chen Pong and Jer-Tsong Hsieh

*Department of Urology, UT Southwestern Medical Center
5323 Harry Hines Blvd., Dallas, TX, USA*

Introduction

Prostate cancer (PCa) is the most common non-cutaneous cancer affecting males in the USA. One in six American men will be diagnosed with PCa in their lifetime. In 2004, a total of 230,110 new cases and 29,900 deaths are estimated to occur in the USA.¹ It has been well documented that alterations in genomic DNA, such as point mutations, homozygous deletions and loss of heterozygosity are linked to the pathogenesis of cancer,² including PCa.³ However, the majority of studies have focused on the DNA sequence and to a lesser extent on DNA structure and its surrounding environment. Recently, investigators started looking at epigenetics as an alternative and complementary mechanism in the pathogenesis of cancer. The term “epigenetic” refers to the heritable changes in gene expression that are caused by mechanisms other than the alteration in the nucleotide sequence.⁴ This concept generated tremendous new knowledge in understanding gene expression in mammalian cells.

The Role of CpG Dinucleotides in DNA Methylation

CpG dinucleotides can be either clustered as CpG islands or dispersed. In the era before completion of the human genome project, a CpG island was defined as a stretch of 200-bp of DNA with a C + G content of 50% and

an observed CpG/expected CpG in excess of 0.6.⁵ However, this definition included inactive and viral sequences within the human genome. When corrected for these “intrusive” sequences, a more biologically appropriate and more stringent definition of a CpG island would be a stretch of 500-bp of DNA with a C + G content of 55% and an observed CpG/expected CpG of more than 0.65.⁶ Therefore, the definition of a CpG island is still arbitrary. More than half of the human genes are associated with unique CpG islands.⁷ Reports from the human genome project have estimated that the human genome contains almost 30,000 CpG islands.⁸

CpG dinucleotides are present throughout the genome and are usually methylated when not associated with CpG islands. However, when the CpG dinucleotides are found in CpG islands, they are usually unmethylated, in particular if they are found in the promoter region of an active gene,⁹ or methylated, when found in the 3' end of the gene. However, CpG methylation at the 3' end of the gene does not affect gene transcription.¹⁰ In general, it is a rare event to find *de novo* CpG methylation in normal somatic cells. However, as cells age, especially when cultured *in vitro*, an increase in methylation has been documented.^{11,12}

Cytosine methylation prevents gene expression by interfering with transcription initiation. DNA methylation has no effect on base pairing but can alter protein-DNA interaction by protruding into the major DNA groove.¹³ Another explanation is that methylation results in decreased binding affinity of the transcription factors to gene promoter regions.¹⁴

Methyl Binding Proteins (MBP)

The first true MBP to be identified was MeCP2 (Methyl CpG-binding protein 2). It was found to specifically bind to the methyl group at position 5 of cytosine and to be active in somatic mammalian cells but not necessarily in embryonic stem cells.¹⁵ This protein has been found most abundantly in the pericentromeric heterochromatin region¹⁶ and its localization to this region has been shown to be dependent on the presence of methylated DNA.¹⁷ MeCP2 specifically represses methylated promoters through a transcription repressor domain (TRD). MeCP2 can bind to DNA that contains one symmetrically methylated CpG pair and does not need chromatin disassembly in order to bind to DNA.¹⁸ In fact, MeCP2 can displace histone H1 from

DNA and then exert its repression function, at least *in vitro*.¹⁹ MeCP1 can also discriminate between methylated and unmethylated DNA, although needing a high amount of methylated CpG for its binding (more than 10 methylated CpGs).²⁰

Four other methyl-binding domain (MBD)-containing proteins were identified by comparing the MBD domain of MeCP2 to Expressed Sequence Tags (EST) databases.²⁰ MBD1-mediated activity is Histone Deacetylase (HDAC)-dependent, as its actions are partially inhibited by HDAC inhibitors (HDIs). However, the identity of this HDAC is still unknown.²¹ MBD1 interacts with a histone H3 methyltransferase as well as heterochromatin protein 1 (a methyl-lysine binding protein) resulting in chromatin compaction and transcription repression.²² In the prostate, MBD1 is highly expressed in benign prostate hyperplasia and low grade PCa. However, MBD1 expression decreases in high grade PCa.²³ MBD2, which is a part of the MeCP1 complex, has transcription repression activity²⁴ as well as DNA demethylase activity.^{25,26} MBD3 is similar in structure to MBD2, however no demethylase activity has been demonstrated.²⁷ MBD4 has DNA glycosylase activity, therefore effectively removing uracil or thymidine from a mismatched CpG location, at least *in vitro*. Binding experiments showed that mutations at methyl CpG sites can be reduced by the activity of MBD4.²⁸

DNA Methyltransferases (DNMTs)

The conversion of cytosine (C) into 5-methylcytosine (5mC) is catalyzed by enzymes termed DNA methyl transferases (DNMTs), using S-adenosyl-methionine (SAM) as a methyl donor.²⁹ Three active DNMTs have been characterized. DNMT1 is responsible for methylating daughter DNA strands during the S-phase, through its interaction with Proliferating Cell Nuclear Antigen (PCNA).³⁰ DNMT1 is involved in maintaining the methylation status of genes and this task is possible due to its high affinity to hemi-methylated DNA.³¹ When DNMT1 is absent in mice, DNA methylation is severely affected, resulting in an embryonic lethal phenotype.³² DNMT3a and DNMT3b have some structural similarities, having the N-terminal as the regulatory subunit and the C-terminal as the catalytic subunit. In contrast to DNMT1, both DNMT3a and DNMT3b can methylate unmethylated and hemi-methylated DNA.³³

An elegant study in *Drosophila* showed that DNMT3a, but not DNMT1, acts as a *de novo* methyltransferase.³⁴ Therefore, DNMT3a establishes the gene methylation pattern and then DNMT1 maintains this pattern in the future generations. When DNMT3a or DNMT3b were knocked out, the resulting mice died at 4 weeks of life or during embryonic development, respectively.³⁵ Increased DNMT mRNA has been documented in colon and lung cancer.^{36–39} However, DNA hypermethylation is not necessarily due to overexpression of DNMT.⁴⁰

DNMT Inhibitors

DNMT inhibitors act mainly by inhibiting methylation; however, other mechanisms are involved. After phosphorylation, these compounds incorporate into DNA or RNA.⁴¹ After incorporating into DNA, they covalently bind DNMT resulting in its inhibition^{42–44} without causing DNA demethylation *per se*.⁴⁴ Additionally, their incorporation into DNA causes structural instability resulting in DNA damage.⁴⁵

Currently, five DNMT inhibitors have been used in preclinical/clinical trials, namely 5-azacytidine (azacytidine), 5-aza-2'-deoxycytidine (decitabine), dihydro-5-azacytidine (DHAC), arabinofuranosyl-5-azacytosine (fazarabine),⁴⁶ and most recently zebularine. Zebularine is the only DNMT inhibitor that can be given orally, while the other DNMT inhibitors are given parenterally.⁴⁷

DNMT inhibitors have been used in preclinical studies with very good tumor control rate. However, in clinical trials, the results are not so dramatic, especially with solid tumors, where limited efficacy has been encountered.⁴⁶ Decitabine has been used in phase II trial in patients with hormone-independent metastatic prostate cancer, however, the results were not encouraging.⁴⁸ Most of the DNMT inhibitors have a relatively high cytotoxicity profile and can only demethylate genes as long as they are present in the cell surroundings, therefore limiting their potential clinical usefulness, as they cannot be administered for prolonged periods of time. However, a recent *in vitro* study showed that zebularine can suppress DNMT1 expression, demethylate p16, and restore p16 expression for up to 40 days in continuously treated T24 bladder cancer cells, without causing pronounced cytotoxicity. In addition, when zebularine is given at a lower

dose following an initial dose of decitabine, a profound demethylation of p16 is observed. These results suggest the usefulness of combining an initial bolus of a parenteral DNMT inhibitor followed by a maintenance dose of the oral agent zebularine.⁴⁹ However, proof of their usefulness in a preclinical and ultimately clinical setting is yet to be established.

Recently, RNA interference (RNAi) has been used to knock out DNMT1,⁵⁰ which composes the majority of DNMT activity in cancer cells,⁵¹ resulting in reactivation of repressed genes. Another study, however, showed that both DNMT1 and DNMT3b need to be shut down to re-express silenced genes.⁵² These results imply the need to treat cancer cells with compounds that can affect more than one type of DNMT, as cancer cells might be able to overcome the deficiency in one DNMT.⁵³ Two phase I clinical trials using MG98, an antisense oligodeoxynucleotide against DNMT1, have been recently conducted to study the MG98 safety profile and dosage regimens in patients with cancer.^{54,55}

Histones

In mammalian cells, genomic DNA is always associated with histone proteins to form chromatin structure. The basic unit of chromatin is the nucleosome, which is composed of 146 base pairs of DNA that are wrapped twice around a disk-like complex made of 8 histone proteins (2 of each of histones H2A, H2B, H3, and H4). A stable nucleosome core is assembled by H3 and H4 heterodimerization, followed by H3 dimerization resulting in a (H3-H4)₂ tetramer.⁵⁶ Subsequently, H2A and H2B heterodimerize and each dimer attaches to one side of the (H3-H4)₂ tetramer.⁵⁶ This yields an octamer of histones on which DNA can be wrapped.⁵⁷ Adjacent nucleosomes are brought together by histone H1. The N- and C-termini of histone H1 are able to bind to DNA within the nucleosome in addition to the “linker” DNA between the nucleosomes. This binding and subsequent neutralization of the acidic DNA results in the formation of higher order chromatin.^{58–60} The amino acids at the N-terminal of the histone protrude out of the nucleosome, thereby allowing for specific reversible modifications and interaction with surrounding factors. This N-terminal is rich in the basic (positively charged) amino acids like lysine and serine, which allows for reversible histone modification by methylation, acetylation,

phosphorylation, ubiquitination, and most recently sumoylation. When this positively charged N-terminal of the amino acid is free, it results in tight binding of histones to DNA phosphates, which are negatively charged, therefore inhibiting access of transcription factors to the gene promoter region and subsequently silencing gene expression.

Acetylation of histones is mediated by histone acetyltransferases (HATs) using acetyl CoA as the acetyl moiety donor, resulting in activation of gene transcription. HATs do not directly bind to DNA, instead, they are subunits in coactivator complexes that mediate transcriptional activity recovery. Examples of such coactivator HATs in humans are p300/CBP⁶¹ and TAFII250,⁶² which is a component of human RNA polymerase II, an enzyme involved in transcription. Steroid Receptor Coactivator-1 (SRC-1)⁶³ and ACTR⁶⁴ are also coactivators with HAT activity that interact with nuclear receptors in a hormone-dependent fashion. pCAF⁶⁵ has the ability to associate with p300/CBP,⁶⁶ ACTR⁶⁴ and SRC-1,⁶³ resulting in a multitude of HATs in the same complex. Acetylation of histones has been linked with transcriptional activation.

The second histone modification is phosphorylation of the Serine-10 of histone H3. This modification has been implicated in both activation of transcription⁶⁷ and chromosomal condensation in mitosis. This phosphorylation corresponds to the activation of early-immediate genes, such as c-Fos⁶⁸ and has been shown to occur through the Rsk-2,⁶⁹ Msk-1⁷⁰, and Snf-1 kinases.⁷¹ Interestingly, the latter histone kinase works in concert with HAT to activate gene transcription.^{71,72} Also, I kappa B kinase α (IKK α) has been reported to phosphorylate histone H3, subsequently allowing for specific H3 acetylation through CREB-binding protein (CBP), resulting in activation of nuclear factor kappa B (NF- κ B) responsive genes.⁷³ It is not clear how histone phosphorylation activates gene transcription; however, it could be related to the fact that adding the negatively charged phosphates to histone N-termini disrupts the histone-DNA interaction, resulting in increased access of transcription factors to promoter regions.⁷⁴

The third histone modification is due to methylation of either arginine or lysine residues. Arginine methylation at histone H3 and H4 is mediated by the histone methyltransferase (HMT) CARM1^{75,76} and PRMT1,⁷⁷ respectively, through transfer of a methyl group from SAM. The end product

results in gene activation. Histone lysine methylation, on the other hand, can result in either gene transcription activation or repression. Suvar3-9 has HMT activity, specifically methylating Lysine-9 of histone H3 (K9-H3),⁷⁸ subsequently attracting the heterochromatin protein HP1^{79,80} resulting in heterochromatin assembly⁸¹ and gene silencing. Human HMTs at K9-H3 include SUV39H1⁸² (causing retinoblastoma (Rb)-mediated transcription repression) and EZH2 (a member of the polycomb group of transcriptional repressors).^{83,84} In addition to methylation at K9-H3, EZH2 has the ability to methylate K27-H3, resulting also in transcription repression.⁸⁵ EZH2 expression was reported to be increased in metastatic prostate cancer and in localized prostate cancer. In the latter case, it indicates a poor patient prognosis and outcome.⁸⁵ Interestingly, both monomethylation⁸⁶ and trimethylation⁸⁷ at K9-H3 can control and trigger DNA methylation *per se*. Recently, it has been shown that methylation levels of K9-H3 differ depending on their location in the chromosome and that different HMTs are responsible for their methylation at each of these sites: monomethylated and dimethylated K9-H3 are localized in silenced euchromatin regions and their methylation is mediated by G9a HMT, while trimethylated K9-H3 was abundant in pericentromeric regions, with SUV39H1 and SUV39H2 responsible for their methylation.⁸⁸ In contrast, methylation at Lysine-4 of histone H3 (K4-H3) by SET7/Set9^{89,90} results in transcription activation. In LNCaP prostate cancer cells, the activated androgen receptor (AR) binds to the androgen responsive element (ARE) of the prostate specific antigen (PSA) gene, resulting in decreased methylation of K4-H3 of the PSA enhancer and promoter and increased PSA transcription.⁹¹

The fourth covalent histone modification is ubiquitination.⁹² Ubiquitin is a 76-amino acid peptide that can attach to the C-terminus of histone H2A and likely to that of H2B as well. Ubiquitination of histone H2A coincides with transcriptional activity.⁹³ However, ubiquitination of histone H2B results in histone H3 methylation, thereby causing transcription repression.⁹⁴

Recently, histone sumoylation has been implicated in transcriptional repression of gene activity. SUMO (small ubiquitin-related modifier) is involved in post-translational modification of several proteins and it is mediated by the same enzymatic cascade that catalyzes ubiquitination. Sumoylation of histone H4 results in recruitment of HDAC and the

heterochromatin protein HP1, therefore causing transcriptional repression.⁹⁵ Collectively, these histone modifications may constitute a “Histone code” that is able to specify patterns of gene expression.⁹⁶

Histone Deacetylases (HDACs)

In mammals, HDACs have been grouped into 3 classes. Class I HDACs (HDAC 1, 2, 3, 8) have catalytic site homology at their C-termini and class II HDACs (HDAC 4, 5, 6, 7, 9,10) share homology at their catalytic C-termini and regulatory N-termini.⁹⁷ Class III is the conserved NAD-dependent Sir2 group of deacetylases.⁹⁷ Interestingly, class I and II, but not class III HDACs, are inhibited by Trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA). Similar to HATs, there is evidence that HDACs do not directly bind to DNA. Instead, they are part of the transcription regulator complexes.⁹⁸ HDACs do not show complete redundancy in their functions. Class I HDACs are almost exclusively located in the nucleus. On the other hand, class II HDACs translocate between the cytoplasm and nucleus.^{97,99}

Histone Deacetylase Inhibitors (HDIs)

HDIs can be classified on the basis of their different structures into hydroxamates (TSA and SAHA), cyclic tetrapeptides (apidicin depsipeptide and depudecin), carboxylates (sodium phenylbutyrate and valproic acid) and benzamides (CI-994 and MS-27-275).^{100–108}

The inhibition of HDACs, as mediated by HDIs via interacting with a zinc active site in the HDAC moiety,¹⁰⁹ could result in induction of differentiation, growth arrest, and/or apoptosis.¹¹⁰ These effects are attributed to a subset of genes whose expression has been altered, or returned back to normal upon exposure to HDIs and histone hyperacetylation,¹¹¹ as seen in single-gene,¹¹² as well as high-throughput studies.¹¹³

Several HDIs, including SAHA, phenylbutyrate, and depsipeptide have been used in Phase I and Phase II clinical trials.¹¹⁴ SAHA can be administered orally or intravenously and has been shown to increase the levels of acetylated histones in peripheral blood mononuclear cells and reduce tumor activity in pretreated patients with hematologic and solid

cancers.¹¹⁵ SAHA was also effective in increasing the intratumor acetylated histone levels and inhibiting the growth of prostate cancer xenografts in nude mice.¹¹⁶

The Interaction Between DNA Methylation and Histone Modifications in Gene Regulation

Recently, an interaction between histone modifications and DNA methylation has been discovered and has been intensively studied. Jones and colleagues described a system where methylated cytosine attracts the methyl-binding protein MeCP2, which in turn binds a HDAC-corepressor complex, therefore resulting in transcription inhibition.¹¹⁷ In addition, DNA methylation and histone methylation also interact, as MeCP2 can recruit a HMT, resulting in methylation of K9-H3.¹¹⁸ More recently, it has been shown that DNMT1 and DNMT3a interact with SUV39H1 (a K9-H3 HMT) as well as HP1, providing more evidence for the close association between DNA and histone methylation.¹¹⁹

A question that has been recently addressed is whether methylation of DNA precedes histone modifications or *vice versa*.^{120,121} Most of the currently available data supports the dominant role of methylation in the epigenetic control of promoter activation, as DNMT inhibitors are more important than HDAC inhibitors in initiating gene reactivation.¹²² In these cases, even if HDAC inhibitors cause histone acetylation, methyl binding proteins such as MeCP2 are still bound to methylated cytosine, thereby needing a DNMT inhibitor to release the methylation and subsequently disengaging from MeCP2.¹²³ However, recent data showed that methylation of K9-H3 and subsequent gene silencing can occur prior to methylation of CpG in the gene promoter.⁵³ Both phenomena are present to some extent and the relative importance of one or the other could be dependent on specific tumor tissue, cell type, experimental conditions, and the specific gene under study.

The fact that DNA methylation and histone deacetylation cooperate to silence genes has been exploited to develop new therapeutic regimens. DNA microarray analysis¹¹³ experiments show that the use of DNMT inhibitors and HDAC inhibitors results in a synergistic activation of specific tumor suppressor genes,^{122,124,125} as well as affecting global gene

expression. In addition, low doses of DNMT inhibitors and HDIs are combined in order to decrease the occurrence of side effects and cause a synergistic inhibition of tumor growth.¹²⁶ Decitabine and phenylbutyrate was found to have a synergistic effect in preventing the formation of lung tumors in mice.¹²⁷

Epigenetics in the Pathogenesis and Diagnosis of PCa

Patra and colleagues have demonstrated that elevated DNMT and HDAC expression is associated with PCa, indicating that epigenetic regulation plays an important role in PCa pathogenesis.¹²⁸ Several genes have been described as being methylated in PCa and only to a limited extent or not at all in normal prostates. Some genes are methylated in early stages of PCa, potentially making them useful as diagnostic markers. Other genes are methylated in advanced stages of PCa and can be used as prognostic markers.

GSTP1

The most commonly studied epigenetically-controlled gene in PCa is the Glutathione-S-transferase- π 1 (GSTP1) gene. GSTP1 is responsible for intracellular detoxification reactions by conjugating free radicals to glutathione.^{129,130} The first evidence of GSTP1 methylation in PCa was demonstrated by Lee and colleagues,¹³¹ when they described that GSTP1 was not expressed in several PCa cell lines due to promoter hypermethylation.

The revolution in detection of methylated DNA came in 1996 when Herman and colleagues described methylation-specific PCR (MSP), which can offer higher sensitivity and specificity in detecting minute amounts of methylated DNA.¹³² Recently, Harden and colleagues employed a quantitative MSP (qMSP) assay for GSTP1 in conjunction with routine prostate biopsy histology in order to improve the PCa detection. They found that histology alone detected 39/61 tumors (64% sensitivity), while GSTP1 qMSP with histology detected 48/61 tumors (79% sensitivity) with a 100% specificity. However, when the threshold of qMSP is decreased, 4/11 samples that were labeled as normal on histology were positive on qMSP. This raises the question that such patients should be monitored more intensively, as

preneoplastic lesions can harbor GSTP1 methylation. However, more controlled trials are needed to justify such a follow up.¹³³

By combining laser capture microdissection and MSP, recent data demonstrated the absence of GSTP-1 methylation in patients with normal or benign hyperplastic prostates, while GSTP1 was methylated in 6.3% with prostatic proliferative inflammatory atrophy (PIA), 68.8% with high-grade prostatic intraepithelial neoplasia (HGPIN), and 90.9% with PCa. These results suggest a progressive increase in GSTP1 methylation during prostate carcinogenesis and PIA as a potential precursor of HGPIN/PCa based on the presence of GSTP1 methylation.¹³⁴

MSP has also been used to detect GSTP1 methylation from several sources with good success, including blood, bone marrow, urine, ejaculate, lymph nodes (LNs) after pelvic LN dissection, prostate biopsies, TURP, and prostatectomy specimens (Table 1). MSP was able to detect GSTP1 methylation in all of these tissues, with a low rate of false positive results.

Table 1. GSTP1 Detection in Human Tissue/Body Fluids using MSP

Sample Origin	Patient Diagnosis	% Methylation	Reference No(s).
Prostate			
Biopsy	Cancer	89	133
Biopsy washings	Cancer	100	180
	PIN	67	
	BPH	0	
Prostate resection	Cancer	88	134
	HGPIN	69	
	PIA	6	
	BPH	0	
	Normal	0	
Pelvic lymph nodes	Cancer	90	181
	Normal	11	
Bone marrow	Cancer	40	181
Urine	Cancer	27–39	182, 183
	Normal	0–3	
Plasma	Cancer	36–72	184, 185
	Normal	0	
Ejaculate	Cancer	50	184
	Normal	0	

Detection of cancer in LN allows for a more accurate assessment of metastatic disease and better adjuvant therapy. Detection of methylated GSTP1 in blood, urine, and ejaculate potentially allows for earlier detection of PCa. In addition, its presence in blood, urine, or bone marrow could prove to be a useful prognostic marker, surrogate marker for predicting cancer volume during therapy and a means of follow up after definitive therapy.

Membrane Receptors

CD44

CD44 is an integral membrane glycoprotein involved in cell to cell adhesion and cell-extracellular matrix interaction.¹³⁵ It has been shown that CD44 promoter hypermethylation inversely correlates with the expression level of CD44 in PCa cell lines.¹³⁶ The methylation status of CD44 was examined in prostatectomy specimens, which revealed that 77.5% of PCa specimens harbored CD44 methylation, in contrast to only 10% of the matched normal controls, suggesting the possible involvement of CD44 methylation and transcriptional repression in the prostate carcinogenesis.¹³⁷ Additionally, the hypermethylation of CD44 has been found to be more common in advanced stages of PCa.¹³⁸ Recently, Woodson and colleagues reported that hypermethylation of CD44 was 1.7 times more common in black patients than in white patients with PCa.¹³⁹ Ekici and colleagues studied CD44 expression in a cohort of patients with PCa and reported that the presence of CD44 is significantly higher in non-metastatic versus metastatic groups, correlates inversely with pathologic stage and disease progression and positively with PSA-free survival.¹⁴⁰

CAR

Coxsackie and adenovirus receptor (CAR), first identified as the high affinity receptor for both coxsackie and adenovirus (type 2 and 5)^{141,142} is a typical immunoglobulin (Ig)-like membrane protein with two Ig domains that may have adhesion activity.¹⁴³ Like many other cell adhesion molecules, the loss of CAR is often detected in several cancer types.¹⁴⁴⁻¹⁴⁹ In PCa, decreased expression of CAR is found in primary tumors¹⁴⁶ and increased expression of CAR can reduce tumor growth of

PCa *in vitro* and *in vivo*.¹⁵⁰ Data from our laboratory indicate that the CpG islands in the CAR promoter are unmethylated; however, the decreased expression of CAR is due to histone deacetylation at the CAR promoter.¹⁵¹ In PCa, the use of HDIs increases the expression of CAR, which further enhances the adenovirus susceptibility of PCa cells.¹⁵¹ Thus, combining HDIs with recombinant adenovirus could lead to a more effective treatment regimen for PCa patients.

Nuclear Receptors

AR

The AR has been shown to be down-regulated as a result of its promoter methylation.^{152,153} AR is methylated in only 8% of PCas and in none of the normal prostates. The PCas with methylated AR were exclusively high stage, indicating that AR methylation is a relatively late event in prostate carcinogenesis.¹⁵⁴

Estrogen Receptor (ER)

When studied in clinical specimens, ER methylation was detected in 60% of BPH, 80% of low-stage PCa and 100% of high-stage PCa specimens. The difference of ER methylation was highly significant in PCa compared to BPH.¹⁵⁵ Another study investigated the different subtypes of ERs in paired PCa samples and discovered that ER α -A and ER α -B gene promoters were methylated in 98% and 92% of PCa samples, respectively, while they were unmethylated in normal prostate samples. ER α -C was not methylated in any PCa samples. In addition, ER β was methylated in 78% of PCas, with no methylation in normal prostates.¹⁵⁴ Such differences in ER gene methylation could imply a different role of each subtype of ER in the pathogenesis of PCa.

Retinoid Acid Receptor- β 2 (RAR β 2)

RAR β 2 is a nuclear receptor that binds to the retinoic acid responsive element (RARE) found in retinoic acid pathway genes and other transcription factor genes.¹⁵⁶ The hypermethylation of RAR β 2 has been

reported in 53%–83% of PCAs, compared to 0%–3% in normal tissues/BPH.^{157–159} It has been shown that decitabine and TSA can induce RAR β 2 gene expression, indicating that histone deacetylation and DNA methylation are responsible for silencing of the RAR β 2 gene.¹⁵⁷

Ras Effector Proteins

In PCa, enhanced expression of Ras protein correlates with increased tumor grade.¹⁶⁰ Several studies indicated that most metastatic tumors expressed Ras protein, while only a fifth of primary tumors did.^{160–162} Surprisingly, PCa cells demonstrate an extremely low rate of mutation in the *Ras* gene.^{162–164} This implies that other effectors regulating Ras activity may be involved in increasing Ras protein levels in PCa.

RASSF1A

RASSF1A, a Ras effector homologue, is a tumor suppressor gene that is down-regulated due to its promoter hypermethylation in several cancers^{165,166} including PCa.^{158,167} *RASSF1A* hypermethylation was found in 53%–71% of PCa specimens and it was more prevalent in patients with Gleason scores 7–10.^{158,167} These results suggest that *RASSF1A* gene methylation may reflect an aggressive PCa phenotype and could potentially be used as a marker in this context.

Human Disabled-2 Interacting Protein (hDAB2IP)

hDAB2IP is a new member of Ras-GTPase activating protein family that maintains Ras inactivation status.¹⁶⁸ The down-regulation of hDAB2IP is often associated with many androgen-independent PCa cell lines and increased expression of hDAB2IP can suppress the growth of PCa.¹⁶⁸ Analysis of hDAB2IP gene promoter reveals that it is hypermethylated in PCa cell lines but not in normal epithelial cells. Both DNMT inhibitors and HDIs can induce the expression level of hDAB2IP,^{169,170} indicating that the epigenetic machinery plays an important role in modulating hDAB2IP expression during PCa progression.

Nuclear Proteins

Cyclin D2

Cyclins are proteins involved in cell cycle regulation by interacting with cyclin-dependent kinases.¹⁷¹ Cyclin D2 is involved in the transition from G1 to S phase during mitosis.¹⁷² Padar and colleagues found that PCas are more commonly methylated (32%) than normal prostate tissue (6%) independent of age. In addition, methylation was significantly higher in patients with Gleason scores >7 .¹⁷³ This finding suggests that loss of cyclin D2 could be involved in PCa progression.

ZNF185

ZNF185, or Zinc Finger 185 is a protein that belongs to the LIM domain protein family.¹⁷⁴ LIM proteins play a role in cellular growth and differentiation.¹⁷⁵ In a recent study, ZNF185 was found to be down-regulated in a genomic screening of PCas. After treatment of PCa cell lines with a DNMT inhibitor, ZNF185 expression was re-established. Using MSP, ZNF185 hypermethylation was detected in PCa, but not in any normal prostate samples. When stratified into different stages, ZNF185 is methylated in 36.3%, 50% and 100% of PCa samples with Gleason 6, Gleason 9 and from metastatic sites, respectively. These data indicate a role for ZNF185 promoter hypermethylation in the progression of PCa and its potential application as a tumor marker for PCa.¹⁷⁶

Bone Morphogenic Protein-6 (BMP-6)

BMP-6 belongs to the transforming growth factor- β superfamily, which is involved in the formation of bone and cartilage.¹⁷⁷ In contrast to the above genes, where their expression has been silenced by promoter hypermethylation, BMP-6 gene methylation is decreased in PCa. This loss of BMP-6 promoter methylation and subsequent gene activation leads to overexpression of BMP-6 protein in both primary and secondary sites of PCa with advanced metastasis.¹⁷⁸ BMP-6 expression in PCa is

associated with a decreased bone-metastasis-free survival as compared to those with no BMP-6 expression.¹⁷⁹ The re-expression of BMP-6 by aggressive PCa could be one way through which these cancers metastasize to bone.

Conclusion

In addition to the genetic mutations of tumor suppressor genes associated with PCa, recent data clearly indicate that epigenetic alterations are also involved in this silencing. Unlike genetic changes, epigenetic modifications are potentially reversible, which can open a new avenue of cancer therapy; several inhibitors of DNMT and HDAC are currently being studied in clinical trials for several cancers. Even so, more controlled studies on epigenetics in PCa are needed to develop new methods for early cancer detection, to predict patient prognosis and to ultimately treat patients with PCa.

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10

SIGNIFICANCE OF 5 α -REDUCTASE IN PROSTATE CANCER

Jun Shimazaki

*Department of Urology, Graduate School of Medicine
Chiba University, Chiba, Japan 262-8670*

Introduction

Since Huggins and Hodges¹ alleviated the pain of patients with prostate cancer by androgen removal, endocrine therapy has become an important strategy for treating this disease. Of the androgens in circulation, more than 90% is testosterone secreted from the testis, and the remainder is dehydroepiandrosterone and its sulfate, and androst-4-ene-3,17-dione, from the adrenal gland. These androgens are converted to dihydrotestosterone (DHT, 5 α -androstane-17 β -ol-3-one) by 5 α -reductase (EC 1.3.99.5) in the prostate, and then bound to androgen receptor, which mediates the effects of androgens. Since DHT regulates prostate growth, including the growth of malignant prostate tissues, the role of 5 α -reductase in cancer development has become a focus of research.

5 α -Reductase

Two types of 5 α -reductase have been cloned.² In the human enzymes, type 1 (SRD5A1) has a deduced molecular weight of 29,000 and 259 amino acids, and type 2 (SRD5A2) has a deduced molecular weight of 28,000 and 254 amino acids. Amino acid sequence identity is approximately 50% between types 1 and 2. Sequence identity shared by human and rat isozymes is 60% for type 1 and 77% for type 2. The kinetic parameters of

human enzymes are different between the two types: optimal pH and K_m 's for testosterone are pH 6–8.5 and 1–5 μM for type 1, and pH 5.0–5.5 and 0.075–1.0 μM for type 2.³ Enzyme reactions of both types require NADPH as cofactor.

In the rat prostate, SRD5A1 is found in the basal cells⁴ or tall secretory cells⁵ of the glandular epithelium, and SRD5A2 is detected in the stromal cells. In the human prostate, early reports did not detect SRD5A1 but found SRD5A2 in the basal and stromal cells of non-malignant and cancerous prostates.^{6,7} Subsequently, both types of isozymes have been shown in normal and abnormal prostates by means of immunohistochemistry,⁸ RT-PCR,⁹ and microarray.¹⁰ To date, SRD5A1 and SRD5A2 have been found in many tissues such as prostate, genital and non-genital skin, and male genital organs.

Expression of SRD5A2 mRNA correlated well with the level of dihydrotestosterone estimated by 5α -reductase activity in the prostate, but no association was found between the expression of SRD5A1 mRNA and enzyme activity.¹¹ Using biochemical measurement, values of K_m and V_{max} in stroma were higher than those in epithelium from both non-malignant and cancerous tissues, indicating a higher activity of stromal 5α -reductase, although the tissue concentrations of DHT were in similar ranges between the two fractions.¹² Cancer cells reveal some 5α -reductase activity: SRD5A1 in LNCaP, PC 3 and DU145,^{13–16} and SRD5A2 in LNCaP.^{14,16} When cancer cells obtained from surgery were grown in culture, expression of both types of 5α -reductase could be detected, however, SRD5A1 was preferentially expressed.¹⁷ Of the cancer specimens studied, 28% showed high intensity staining for SRD5A1 in the tumor epithelium, but the hyperplastic tissues showed less intense staining in the epithelium.¹⁸ Although some cancer cells exhibit SRD5A1, SRD5A2 seems to act as the main enzyme in the prostate, as suggested by clinical evidences. For example, the small prostate of a patient with a specific type of male pseudohermaphroditism who had normal SRD5A1 but mutated SRD5A2,¹⁹ shrunk as the result of treatment with the SRD5A2 inhibitor, finasteride. Therefore, SRD5A2 in the stromal component may mainly act in the formation of DHT in both non-malignant and cancerous prostates, thus the SRD5A2 gene, in relation to DHT metabolism, has been widely studied in order to understand the development of prostate cancer.

Formation and Metabolism of DHT in Cancerous Prostate

In 1965, Shimazaki *et al.* first found that incubation of radiolabeled testosterone with tissue extracts of rat prostate or minced tissues of normal and pathological human prostates formed radiolabeled DHT and androst-4-ene-3,17-dione.^{20,21} Three years later, Bruchovsky and Wilson found that DHT could be detected in isolated prostatic nuclei 2 hours after administration of ³H-testosterone to rats.²² Several months later, Anderson and Liao confirmed that nuclei of both rat ventral prostate and seminal vesicles selectively retained ³H-DHT.²³ The metabolic rate of DHT formation is high in hyperplastic tissues, moderate in normal tissues, and low in cancerous tissues.^{20,21} Reduced activity of 5 α -reductase in the cancerous tissues was further confirmed by various procedures: incubation with minced tissues²⁴ and with homogenate,^{25,26} RT-PCR,^{11,27} and microarray.¹⁰ Poorly differentiated cancerous tissues showed less activity than well differentiated ones. Metabolic foci in lymph nodes revealed scant activity²⁸ and no expression of SRD5A1 or SRD5A2 mRNA.²⁹ Although a few opposing findings are present,^{8,9} decreased 5 α -reductase activity in cancer tissues has been indicated in many other reports. In this context, hormone concentrations (pM/dry weight) in normal, benign hyperplastic, and cancerous prostate tissues were: 12.6 \pm 2.3, 14.1 \pm 2.4, and 39.6 \pm 6.2 of testosterone; 12.9 \pm 1.9, 45.5 \pm 5.8, and 22.4 \pm 2.4 of DHT; and no detectable level, 30.0 \pm 7.6, and 42.0 \pm 7.9 of androst-4-ene-3,17-dione, respectively.³⁰

DHT is further metabolized to 5 α -androstane-3 α ,17 β -diol and, to a lesser extent, 5 α -androstane-3 β ,17 β -diol in the prostate. The former is converted to androsterone.³¹ To assess the activity of 5 α -reductase, measurements of 5 α -androstane-3 α ,17 β -diol glucuronide and androsterone glucuronide in serum are commonly used. The oxidative reaction of 5 α -androstane-3 α ,17 β -diol has been discussed, since dog prostate hyperplasia can effectively be induced with this steroid, perhaps by its conversion to DHT. However, in cases of prostate cancer, this reaction does not seem to be substantial.³²

Percutaneous administration of dehydroepiandrosterone to healthy aged male volunteers caused an increase of androst-4-ene-3,17-dione and conjugated metabolites of DHT (glucuronides of androsterone, 5 α -androstane-3 α ,17 β -diol and 5 α -androstane-3 β ,17 β -diol) in serum,

despite unchanged levels of testosterone and DHT.³³ In patients who underwent castration, the serum ratio of DHT/testosterone increased,³⁴ and 58% of DHT remained in the serum, in contrast to the 92% reduction of serum testosterone,³⁵ suggesting that adrenal androgen is a precursor of DHT. The prostate contains metabolizing enzymes for the conversion of dehydroepiandrosterone and its sulfate, and androst-4-ene-3,17-dione to DHT, including sulfatase,³⁶ 3 β -hydroxysteroid dehydrogenase,³⁷ and 17 β -hydroxysteroid dehydrogenase.^{38,39} An estimated 30%–50% of total androgens in men are synthesized in peripheral tissues, including the prostate, from inactive adrenal precursors.⁴⁰ In a 14-year follow-up of 1008 men, the total testosterone, estrone, estradiol, and sex hormone-binding globulin levels did not correlate with the incidence of prostate cancer, but androst-4-ene-3,17-dione showed a positive dose-response gradient, suggesting an adrenal contribution.⁴¹ Although the proportion of adrenal androgen acting as precursors of DHT is uncertain, adrenal androgen must be considered in striving for complete block of DHT formation for prostate cancer treatment. It is a clinical issue whether endocrine therapy can be achieved by suppression of the testicular hormone alone or whether elimination of influence from adrenal androgens is necessary.

Risk of DHT Formation

Men castrated at a young age and patients with male hypogonadism seldom experience prostate cancer. Ethnic differences in the incidence of clinical prostate cancer are evident, although differences in the incidence of latent cancer among the races are small. Therefore, the activity of 5 α -reductase in the formation of DHT may be an etiological and/or progressive factor in clinical cancer.⁴²

The levels of total and free testosterone in African-Americans were 19% and 21% higher than those in whites (both $p = 0.02$), respectively.⁴³ Three reports, in which testosterone, DHT, estradiol, and sex hormone-binding globulin were measured in prospective cohort studies, were reviewed by meta-analysis.⁴⁴ Men whose total testosterone level was in the highest quartile were 2.34-fold more likely to develop prostate cancer than men in the lowest quartile. Levels of DHT and estradiol did not differ significantly, but low sex hormone-binding globulin was revealed as a

risk factor. Thus, levels of circulating bioavailable testosterone may be associated with prostate cancer. On the contrary, the serum testosterone level in Japanese youths was not different from that of Caucasian and African-American youths in the USA, but the latter two groups had increased serum levels of 3 α ,17 β -androstane diol glucuronide and androstane diol glucuronide, suggesting high DHT formation.⁴⁵ Racial differences in the activity of SRD5A2 continue as men age.⁴⁶ A high serum testosterone level correlates with high grade cancer⁴⁷ and increased risk of relapse.⁴⁸ Conversely, patients with high grade cancer had correlated low level of serum testosterone,^{49,50} however, the initial step in the development of prostate cancer may be proceeded more by increased levels of androgen. The activity of 5 α -reductase in the prostate increased by various androgens via feed-forward control.⁵¹⁻⁵³ Therefore, an increased level of testosterone, even a small increase, serves as a substrate and raises the activity of 5 α -reductase, which consequently elevates the DHT level.

The gene encoding SRD5A2 contains five exons and is located on chromosome 2p23. No specific mutations or polymorphisms of 5 α -reductase have been detected in the families of patients with hereditary prostate cancer.⁵⁴ Therefore, polymorphisms of SRD5A2 in a comparison between ethnic groups or in a case-control study have widely been examined. The number of dinucleotide repeats (TA)_n in the 3' untranslated region of the 5 α -reductase gene was discussed in relation to the risk of prostate cancer,⁵⁵ because longer alleles may cause a modest reduction in the activity of the enzyme. Initially, differences in the number of repeats were found among the races, but no evidence revealed any correlation between the differences and the risk.⁵⁶ The (TA)_n repeats were not correlated with the level of androstane-3 α ,17 β -diol glucuronide.⁵⁷ On the contrary, in the Chinese population, which has the lowest incidence of prostate cancer, men who are heterozygous for the (TA)₀/(TA)_n allele had a modest risk reduction compared with men who are homozygous for the (TA)₀ allele and have higher serum DHT levels.⁵⁸ Men who are homozygous for the (TA)₉ or (TA)₁₈ alleles and men who are of the (TA)₉/(TA)₁₈ genotype have a modestly reduced risk.⁵⁹ The number of (TA)_n repeats was related with age at the onset of prostate cancer.⁶⁰ Of 208 patients with localized prostate cancer, 30 were (TA)_n heterozygotes in peripheral lymphocytes, and this genotype was then compared with that of tumor DNA. Fifty-seven percent of the

tumors showed loss of heterozygosity (LOH) or microsatellite instability in this marker. Tumors showing these somatic mutations tend to be of a high grade and stage.⁶¹ Together with these reports, (TA)_n repeat length may partly be viewed as an etiologic/progressive factor.⁶²

Screening among healthy, racially/ethnically diverse male populations detected 10 missense substitutions and three double mutations that are all naturally found in human males.⁶³ Of the ten single amino acid substitutions, V89L (substitution of leucine for valine at codon 89) is on almost 33% of the chromosomes, followed by A49T (substitution of threonine for alanine at codon 49) on 2.0%. Other mutations are rare, at less than 2%. The V_{max} of A49T is 5-fold higher than that of the wild type, and the V_{max} of V89L shows 50% reduced activity.

In men with the LL genotype of V89L, the serum levels of testosterone and free testosterone were reduced by 12% and 16% respectively, and that of 5 α -androstane-3 α ,17 β -diol glucuronide was reduced by 10%.⁶⁴ The frequency of the VV genotype was the highest in African-Americans at 58.9%, lower in Caucasians at 57.1%, and the lowest in Asians at 29.4%.⁶⁵ The VV genotype is associated with a higher serum level of 5 α -androstane-3 α ,17 β -diol glucuronide than the VL and LL genotype, thus a correlation with the higher incidence of prostate cancer in African-Americans was suggested. Men with the V allele are at a 2-fold greater risk for prostate cancer development and an additional 2-fold increase in the risk of progression when compared to men with the LL genotype.⁶⁶ Similarly, men with the VV or VL genotypes had an increased risk for prostate cancer compared with those with the LL genotype.⁶⁷ On the contrary, no correlation between the V89L genotype and prostate cancer was reported,^{58–60,68} or conversely, the L allele may be of a risk but not significantly.^{69–72} Finally, meta-analysis from nine studies concluded no association between SRD5A2 genotype and prostate cancer risk.⁶²

An association between A49T polymorphism and prostate cancer was reported in African-Americans and Hispanic men.⁷³ The missense mutation in healthy men of both populations is rare, but men with AT/TT genotypes have increased risk of clinically significant cancer at 7.2-fold in African-Americans and 3.6-fold in Hispanic men. The polymorphism was correlated with extracapsular extension in Caucasians.⁷⁴ On the contrary, no association was found between this polymorphism and tumor stage,

grade, or family history.⁷⁵ Men with one or two copies of the variant T allele had a 24% lower androstenediol glucuronide level than men homozygous for the wild type allele.⁵⁷ Meta-analysis, however, revealed a modest effect of T allele on cancer.⁶² The T allele was not detected in the Japanese population, indicating racial differences in the polymorphism. R227Q (substitution of glutamine for arginine at codon 227) significantly reduces enzyme activity, but men with RQ phenotype are rare, and this polymorphism was not associated with prostate cancer.⁵⁸

From these reports, it seems to be difficult to determine the risk for prostate cancer attributable to polymorphisms of SRD5A2. In each case-control study, selection of the control group could not completely exclude men with tiny foci of cancer or benign hyperplastic prostate, which is an androgen-dependent disease. The Japanese population is considered to be ethnically homogeneous, and the incidence of prostate cancer was formerly low. However, the incidence is increasing at the fastest rate of all male malignancies, therefore, the different incidences among countries can not be explained by racial differences only. Finally, reports of genetic association are influenced by study size and time of publication, thus results have been variable.⁷⁶

The diet of Japanese men has changed to include foods with high fat and protein compositions since World War II. A diet of 25% reduced fat and the same total calories caused a 10% decrease in serum testosterone of healthy volunteers.⁷⁷ Isoflavonoids (genistein and daidzein) derived from soybean and green tea gallates (epigallocatechin-3-gallate and epicatechin-3-gallate) are favorite foods for Oriental men, and are also 5 α -reductase inhibitors.^{78,79} γ -Linolenic acid,⁸⁰ eicosapentaenoic acid,⁸¹ and free fatty acids⁸² are also inhibitors. Ingested foods may influence androgen metabolism, and consequently the incidence of prostate cancer.

Treatment with Inhibitors

The 5 α -reductase activity in rat prostate and human skin fibroblasts was inhibited by estrogen,^{83,84} progesterone, and deoxycorticosterone acetate.⁸⁵ 6-Methyleneprogesterone is also an inhibitor.⁸⁶ These inhibitors are natural steroids or their derivatives and have other hormonal effect. Subsequently, pure inhibitors have been synthesized; some specifically

inhibit one type of enzyme (SRD5A1 or SRD5A2), while others inhibit both types of enzyme.⁸⁷

4-aza-3-oxo-1-ene (finasteride, dutasteride, PNU157706), 4-aza-3-oxo-4-methyl (4MA, turosteride), 3-carboxylic steroid (SKF105657), and other androstane derivatives⁸⁸ are steroidal inhibitors, and benzoquinolone (bexlosteride, izonsteride), ONO 3805, and FK 143 are nonsteroidal inhibitors. Bexlosteride, a SRD5A1 inhibitor, inhibits testosterone-stimulated LNCaP (possessing SRD5A1) cell growth in culture at 10 nM and 100 nM, but the compound shows no effect without testosterone.⁸⁹ In transplantable androgen-dependent tumors in animals, growth retardation was shown with the following systems; 4MA for the Noble tumor,⁹⁰ and PNU 157706 and turosteride for the Dunning R3327 tumor.^{91,92} Treatment with inhibitors evokes retardation of tumor growth, but the effect is rather weak when compared with castration, suggesting that the remaining testosterone promotes tumor growth. Alternatively, weak retardation of the growth may be due to incomplete suppression of DHT formation, especially in cases where a specific SRD5A2 inhibitor is used.

Asymptomatic patients with stage D prostate cancer were treated with 10 mg of finasteride daily for up to 12 weeks, and their clinical data were compared with those from the placebo group.⁹³ Treatment with finasteride decreased the serum levels of PSA and DHT, but did not affect prostatic acid phosphatase, testosterone, prostate volume, or appearance of bone scan. Patients with untreated M1 cancers were treated with luteinizing hormone-releasing hormone (LHRH) analog and finasteride, flutamide, or both. After 12 weeks, PSA levels decreased in all three groups, but no differences were noticed on bone scan scores or in performance status.⁹⁴ Treatment with finasteride did not change the histological pattern of cancer.⁹⁵ Therefore, finasteride may be, if necessary, applied with other drugs to bolster the effects. Treatment with finasteride (5 mg/day) and flutamide (250 mg tid) for relapsed or metastatic cancer evoked a considerable result, accounting for 65% of the overall survival at 5 years.⁹⁶

Prevention

The ACI/Seg rat has a 63% incidence rate of spontaneous prostate cancer by 140 weeks of age. Treatment with 20 ppm of FK 143 in the diet reduced

the incidence of tumor formation to some extent, but treatment with 200 ppm of the same inhibitor showed no effect, suggesting that the lack of effect is due, at least partly, to increased testosterone in the prostate.⁹⁷ In F344 rats, treatment with dimethylaminobiphenyl causes prostate cancer. Finasteride reduced the number of visible tumors, but the total number of visible and microscopic tumors was the same as that for the control, thus finasteride may delay the development of tumors to macroscopic size.⁹⁸ Finasteride reduced prostate tumor incidence by 10% in Wistar rats treated with methylnitrosourea/testosterone propionate, and the chemopreventive activity is suspected to occur by suppression of prostatic polyamine synthesis.⁹⁹ These animal experiments suggest rather weak, if any, inhibiting effect of 5 α -reductase inhibitors on the development of prostate cancer.

During treatment of benign hyperplastic prostate with a daily dose of 5 mg finasteride, occurrence of prostate cancer was surveyed. Incidence of cancer was 1% and 2% in the finasteride and placebo groups, respectively, during the 2 years of a Canadian trial.¹⁰⁰ In the USA, both groups showed the same cancer incidence at 5% for 4 years.¹⁰¹ Fifty-two men with increased PSA but negative biopsy were divided into the finasteride and placebo groups.¹⁰² The finasteride group showed a decrease in serum PSA by 48% and in DHT by 67%, but an increase in testosterone by 21%. The incidence of cancer was 4% (one man) in the placebo group but 30% (eight men) in the finasteride group. These results indicate that the short-term administration of finasteride cannot restrain the clinical outbreak of prostate cancer.

The Prostate Cancer Prevention Trial was started in the USA in 1993; 18,882 men 55 years of age or older with normal digital rectal examination and 3 ng/ml or less of PSA were divided into the finasteride (5 mg daily) or placebo groups.¹⁰³ After 7 years, prostate cancer was detected in 18.4% men in the finasteride group and in 24.4% of men in the placebo group, thus a 24.8% reduction in the occurrence of cancer was calculated during this period. Tumors of Gleason score 7–10 accounted for 37.0% in the finasteride group and 22.2% in the placebo group, thus the rate of malignancy increased by 1.7% with finasteride. Additionally, more sexual side effects and fewer urinary symptoms were noticed in the finasteride group. This raises the question as to whether finasteride retarded the growth of low grade cancers but could not suppress high grade cancers.

From this report, an argument on balancing diminished incidence rate and increased tumor malignancy due to finasteride has arisen. A consensus of opinion has not yet been reached (see *New Engl J Med* 2003, 349: Scardino *et al.*, p. 297, and Rubin *et al.*, p. 1569). However, chemopreventative treatments involving 5 α -reductase inhibitor remain in use.

Conclusion

It is widely accepted that DHT, formed by 5 α -reductase in the prostate, plays a pivotal role in the development of prostate cancer. Therefore, metabolism of DHT in the prostate is an etiologic and progressive factor. At present, the association between structural variants of 5 α -reductase and prostate cancer risk might not clearly be demonstrated from the results of studies considering ethnicity as well as case-control differences. Other factors involved in androgen synthesis and metabolism may cooperate to influence the risk of prostate cancer. Application of 5 α -reductase inhibitors in therapy and prevention seem to be ineffectual, thus further studies are required.

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ROLES OF VITAMIN E IN PROSTATE AND PROSTATE CANCER

Shuyuan Yeh, Jing Ni, Eugene Chang, Yi Yin and Ming Chen

*Departments of Urology and Pathology
University of Rochester Medical Center, Rochester, New York*

Introduction

In the United States, prostate cancer is the second leading cause of new cancer cases, accounting for approximately 33% of all new cases in males and is the second leading cause of cancer-related death of men, behind lung cancer.¹ Factors such as diet, exercise, and environment are all aspects related in the subsequent development of cancers. For example, statistics from the American Cancer Society show that the death rates of prostate cancer are greater in the United States compared to China.¹

Epidemiological studies have indicated the protective roles of certain vitamins and minerals in prostate cancer.²⁻⁴ Among these vitamins and minerals, vitamin E has been identified by researchers as a potent anticancer agent in the delay or prevention of prostate cancer. There is a variety of factors that affect the performance and effectiveness of vitamin E in the body. The method by which vitamin E is taken into the body is an area of concern. Another consideration is the efficacy of different isoforms of vitamin E including four tocopherols and four tocotrienols.⁵ There are also numerous synthetic vitamin E analogs that have been proven to be effective *in vivo*, such as α -vitamin E succinate (VES).

In vivo animal and clinical studies support *in vitro* data on the effectiveness of vitamin E in curbing the growth of cancers through a variety of mechanisms including cell cycle inhibition, apoptosis, gene regulation, and

antioxidation. This review discusses anticancer mechanisms of vitamin E other than its antioxidant activity, and supporting animal and clinical data. Upcoming clinical trials and a look at future study directions are also included.

Vitamin E and Its Analogs

Vitamin E refers to a family of compounds called tocopherols and tocotrienols. Both groups are further divided into four isoforms: α , β , γ , and δ . α -Tocopherol is the most commonly found natural form of vitamin E as it accounts for about 90% of all tocopherols in most mammalian tissues.⁵ It has several esterified analogs including α -tocopheryl acetate (α -vitamin E acetate, VEA), α -tocopheryl nicotinate (α -vitamin E nicotinate, VEN), and α -tocopheryl succinate (α -vitamin E succinate, VES). Of all these forms, VES (vitamin E succinate) is the most effective in terms of its anticancer properties. Both *in vitro* and *in vivo* studies have shown that VES is capable of inducing apoptosis and inhibiting cell proliferation in cancer cells without affecting the proliferation of most normal cells.⁶ VES can be hydrolyzed by esterase in the gastrointestinal tract and may thus lose some of its potency. *In vivo* animal studies have shown that an intraperitoneal injection is an effective delivery strategy of the VES.⁷ A non-hydrolyzable ether forms, α -tocopheryl oxybutyric acid and ether acetic acid analogs, have been created as a solution for this loss of potency.⁸⁻¹⁰

In addition, Birringer *et al.* have shown that the modification of different functional moieties of the vitamin E molecule can enhance its proapoptotic properties. Analogs of VES with a lower number of methyl substitutions on the aromatic ring were less active than VES itself. Replacement of the succinyl group with a maleyl group greatly enhanced the activity. However, methylation of the free succinyl carboxyl group on VES completely abolished the apoptogenic activity of these compounds.¹¹

Vitamin E Absorption and Transport

Major dietary sources of vitamin E include vegetable oils, margarine, nuts, seeds, whole grains, soybeans, eggs, and avocados. The recommended dietary allowance of vitamin E is 15 mg/day (15 mg = 22.5 IU),¹² however,

common doses range from 100 to 800 IU/day with no significant adverse side effects, and have not been associated with mutagenic or teratogenic properties.¹³

Vitamin E is absorbed in the intestine and circulates through the lymphatic system. It is absorbed together with lipids, packed into chylomicrons, and transported to the liver. This process is similar for the various forms of vitamin E. After transport to the liver, α -tocopherol will be absorbed and released to the plasma. Most other ingested β -, γ -, and δ -tocopherols and tocotrienols are secreted into bile, or not absorbed and excreted in the feces.⁵

In the American diet the level of consumption of γ -tocopherol ranges from two to four times higher than the level of α -tocopherol. Both forms are equally well absorbed by the intestines, bound with chylomicron lipoprotein, and transported to liver, yet the plasma level of α -tocopherol is five to ten times higher than plasma levels of γ -tocopherol. This is attributed to α -tocopherol's higher affinity to a liver cytosolic tocopherol transfer protein (TTP) compared to γ -tocopherol. Therefore, TTP is a major determinant of plasma tocopherol levels. There have been studies that show that dietary supplementation with α -tocopherol will actually decrease levels of γ -tocopherol in the blood and adipose tissue^{14,15} due to the limited binding capacity of the hepatic TTP.

Furthermore, Bonina's study indicates that α -tocopherol and VES (α -tocopheryl succinate) are incorporated into erythrocyte membranes with the help of specific transport proteins. The study suggested that other vitamin E transfer or binding proteins could exist and differences in membrane incorporation of α -tocopherol and VES might contribute to their variant cytoprotective properties.¹⁶ Indeed, other vitamin E binding proteins, the tocopherol associated protein and tocopherol binding protein, were identified, but their functions were not fully characterized.¹⁷⁻¹⁹

Functional Mechanisms of Vitamin E in Prostate Cancer

Currently, a new clinical trial, SELECT,^{20,21} has been initiated in the US, and an earlier epidemiological study also indicated that daily supplements of Vitamin E could reduce the incidence and mortality of prostate cancer.² However, the functional mechanisms remain largely unclear. We summarize the functional mechanisms of vitamin E as follows.

Vitamin E and Its Analogs Induce Proapoptotic Properties in Prostate Cancer Cells

α -Vitamin E (α -tocopherol) has been shown by researchers to have proapoptotic properties in human prostate cancer LNCaP cells. Their data showed that vitamin E administration resulted in reduced DNA synthesis and enhanced DNA fragmentation, as well as a general inhibition of cell proliferation.²²

Another study by Gunawardena and colleagues, showed that α -tocopherol stimulated apoptosis in three different human prostate cancer cell lines: DU-145 (androgen-unresponsive), LNCaP (androgen-responsive), and ALVA-101 (moderately androgen-responsive). The group cited nucleosome fragmentation as evidence of apoptosis following α -tocopherol treatment in these different prostate cancer cell lines.²³

Furthermore, results from other researchers indicated that the apoptosis-triggering properties of VES may be due to its modulation of Fas signaling. Fas belongs to the tumor necrosis factor receptor and nerve growth factor receptor superfamily, and contains a cytoplasmic death domain that can initiate an apoptotic cascade. Using two prostate cancer cell lines (LNCaP and PC-3) and the normal prostate epithelial cells (PrEC) the investigators showed that VES induced apoptosis only in cancer cells. They also showed that VES administration enhanced Fas ligand expression and increased Fas levels in the membrane, both of which are important events in Fas-induced apoptosis.²⁴

Vitamin E and Its Analogs Inhibit Cell Cycle Progression

VES has been shown to inhibit the proliferation of prostate cancer cell lines through the inhibition of cell cycle. Our group has found that VES effectively inhibits prostate cancer LNCaP cell growth by causing cell cycle arrest in the G1 phase with a reduction of cells in S phase. VES decreases the expression of several cell cycle regulatory proteins such as cyclin D1, D3, E, cdk2, and cdk4, but not cdk6.²⁵ In addition, Ni *et al.* also found that VES can inhibit the phosphorylation of retinoblastoma (Rb), and consequently inhibit the E2F activity.²⁵ Another group, Venkateswaran *et al.*, has observed similar inhibitory effects of VES. Their data shows VES-induced

G1 arrest in LNCaP and G2/M arrest in PC-3 prostate cancer cells. Their data also showed a G1 phase arrest, and the mechanism credited for the increased amounts of p27 by VES.²⁶

In addition to α -tocopherol, γ -tocopherol is another isoform of vitamin E that has demonstrated anticancer properties. A study by Gysin and colleagues showed that γ -tocopherol inhibits cell proliferation more significantly than α -tocopherol in DU-145 and LNCaP cells. They also showed that the mechanisms of γ -tocopherol are through inhibition of DNA synthesis, defects of cell cycle with decreased S-phase cell population, and down-regulation of cyclin D1 and cyclin E levels. Based on their results, γ -tocopherol is more potent than α -tocopherol in those two cell lines.²⁷ However, the absorption efficiency of γ -tocopherol is lower than that of α -tocopherol in human.¹⁴

VES Inhibits the Expressions of PSA and the Androgen Receptor (AR)

A functional AR is essential for the development and progression of prostate cancer.²⁸⁻³⁰ In prostate, AR can bind to the promoter of and regulate the expression of prostate-specific antigen (PSA), the most popular detection marker for prostate cancer. Results from our earlier report suggested that VES, at a non-toxic concentration and *in vivo* achievable level, could selectively inhibit the expression of both AR and PSA, but not retinoid X receptor α (RXR α) and peroxisome proliferators-activated receptor α (PPAR α), in prostate cancer LNCaP cells. Results from further investigation indicated that VES can affect the translational efficiency of AR. Overall, the results suggested that VES-mediated inhibition of prostate cancer cell growth can be partly due to the inhibition of AR function.³¹

VES Inhibits Activity of MMP9 Secreted From Prostate Cancer Cells

Most of the tumors of prostate cancer patients become incurable once their cancers progress to metastatic stage. Metastasis of cancer cells employs complicated processes including degradation of extracellular matrix, migration, homing and angiogenesis. Our group has found that

VES can affect the invasiveness of prostate cancer PC-3 and DU-145 cells. Results from mechanism investigation suggested that VES could affect the matrix metalloproteinase-9 (MMP-9) activity, but not the tissue inhibitor of metalloproteinases (TIMPs). The inhibition of MMP-9 activity and cancer metastasis through matrigel could be observed with 24 h treatment of VES. This time frame is shorter than the event of VES mediated disturbance of the cell cycle distribution and cell growth, which takes action upon longer VES treatment. Thus, the inhibition of MMP-9 activity could be an event independent of VES-mediated cell growth inhibition and cell cycle progression of prostate cancer cells.³²

***In vivo* Animal Study of Vitamin E's Role in Prostate and Prostate Cancer**

One study has investigated the effects of vitamin E deficiency on pubertal growth and maturation of the rat prostate. The rats were placed on a vitamin E deficient diet at four weeks of age and were followed for 15–26 weeks. The study showed that vitamin E deficiency in the body led to a less significant increase in weight, DNA, and protein in the lateral lobe of prostate compared with control rats. The group also concluded that vitamin E deficiency may contribute to the delay of prostate differentiation.³³

In spite of the constructive role of vitamin E in the development of prostate, the role of vitamin E in the incidence and progression of prostate cancer are controversial in animal models. In 3,2'-dimethyl-4-aminobiphenyl (DMBA)-initiated rat prostate carcinogenesis, the modifying effects of six naturally occurring antioxidants, including α -tocopherol, were investigated. Atypical hyperplasias and carcinomas of the prostate were observed in the ventral lobe in all groups treated with DMAB. However, the incidence of these lesions was not significantly different between carcinogen control and antioxidant-treated groups. There were also no significant increases or decreases in the incidence of tumors in any other organs.³⁴

Fleshner *et al.* also investigated the effects between vitamin E and a high-fat diet on prostate cancer development. Tumors were induced by subcutaneous injection of LNCaP cells in nude mice and they were treated with dl- α -tocopherol at 11.4 mg/kg body weight/day while divided into four different groups (regular diet, high-fat diet, regular diet with supplemental

vitamin E, high-fat diet with supplemental vitamin E). They concluded that the mechanism for fat induced growth of prostate cancer cells is mediated by oxidative stress, thus making vitamin E an effective solution.³⁵

Vitamin E has been shown to enhance the growth-inhibitory effects of adriamycin on human prostatic carcinoma cells *in vitro*. Vitamin E used in combination with adriamycin was evaluated in the treatment of Nb rat prostate adenocarcinoma. The adriamycin-vitamin E treatment groups had the lowest average final tumor volume, but the mortality rate increased. These results suggest that vitamin E may play a role in enhancing the cytotoxic effects of adriamycin.³⁶ Recently, the antineoplastic effects of VES, selenium, and lycopene were tested on Lady mouse prostate cancer model. The results suggested that oral feeding of VES, selenium, and lycopene will elicit a better antineoplastic effects.³⁷ Overall the antineoplastic effects of vitamin E and its analogs need further evaluation in animals.

Clinical Study of Vitamin E in Prostate Cancer

There have been numerous studies focusing on the effect of vitamin E supplement and its correlation to cancer occurrence. Results from a finished Alpha-Tocopherol, Beta-Carotene (ATBC) Cancer Prevention Study (n = 29,133) of male smokers suggested that the daily supplement of α -tocopherol could reduce the incidence and mortality of prostate cancer.²

In addition, the association between prostate cancer and baseline vitamin E and selenium was evaluated in the trial-based cohort of the ATBC study. During nine years of follow-up, 317 men developed incident prostate cancer. The report found that there were no significant associations between baseline serum α -tocopherol, regular dietary vitamin E, or selenium and prostate cancer.³⁸ Overall, their results indicated a protective effect for total vitamin E among those who received the α -tocopherol intervention.³⁸ In another study, the investigators examined the associations between prediagnostic blood levels of micronutrients and prostate cancer risk in two nested case-control studies of 9,804 and 10,456 male residents of Washington County, Maryland, who donated blood in 1974 (CLUE I) and 1989 (CLUE II), respectively. They found serum α -tocopherol might be weakly associated with prostate cancer risk.³⁹ Furthermore, a study was done on 10,456 male residents from Washington County, Maryland using

donated blood samples. The results showed that the risk of prostate cancer declined with increasing concentrations of γ -tocopherol although not linearly. Men having γ -tocopherol levels in the top fifth had a five-fold reduced risk compared to the bottom fifth. In this study, statistically significant protective effects are associated with high levels of selenium and α -tocopherol are observed only when γ -tocopherol concentrations are high.^{3,40}

An additional population-based, case-control study in King County, Washington examined supplement dietary use in 697 incident prostate cancer cases (ages 40–64). The results suggested that individual supplements of zinc, vitamin C, and vitamin E may be protective.³ However, the suggested supplemental vitamin E may not have a beneficial effect on the risk of prostate cancer in non-smokers.⁴¹

Furthermore, the relation of baseline levels of serum α -tocopherol and serum sex hormones in older men was studied.³⁶ Their results suggested that serum α -tocopherol was significantly inversely associated with serum androstenedione, testosterone, sex hormone-binding globulin, and estrogen levels. These results indicated that α -tocopherol may affect concentrations of several sex hormones in older men and may have implications for the protective effect of vitamin E in relation to prostate cancer in the ATBC Study.⁴² Moreover, another group analyzed whether long-term supplementation with α -tocopherol affected VEGF levels, a cytokine integrally involved in endothelial cell proliferation, vascular permeability, and regulation of angiogenesis. Two hundred Finnish men were randomly assigned to placebo and α -tocopherol pills (50 mg) daily. There was an 11% reduction in VEGF levels in the α -tocopherol group compared to a 10% increase for the control group. The result suggests that α -tocopherol may suppress prostate cancer angiogenesis through reducing VEGF levels.^{3,43}

Due to controversial data from various studies, whether the supplement of α -tocopherol and its analogs could lower the risk of prostate cancer remains to be elucidated. Currently, there is a big clinical trial, Selenium and Vitamin E Clinical Trial (SELECT) in the US. The SELECT trial is a randomized, prospective, double-blind study that intends to determine whether selenium and vitamin E, singly or in combination, can reduce the risk of prostate cancer in healthy men. The study will provide 200 μ g

L-selenomethionine and 400 mg racemic α -tocopheryl acetate. The trial began in 2001 and final results are expected in 2013.²¹ The outcomes of this clinical trial will provide a comprehensive investigation of the association between the prostate cancer risk and the supplement of vitamin E and/or selenium.

Summary

Although some controversial data exists, the epidemiological and clinical studies suggested that the incidence and mortality of prostate cancer may be reduced with daily supplement of α -tocopherol analogs. We and other researchers have devoted efforts on exploring these underlying mechanisms. Currently, the identified mechanisms include inhibition of DNA synthesis,²² inducing apoptosis and FAS ligand activity,²⁴ affecting the expression and function of AR and PSA,³¹ targeting on cell cycle molecules,^{25,26} and inhibiting the invasiveness of prostate cancer cells.³² There are still several fields have not been addressed. First, it is of great interest to know whether α -tocopherol and its analogs can affect the growth factor and kinase signals, oncogene function, bone metastasis, and angiogenesis in prostate cancer cells. Second, as most of the published results rely on the *in vitro* cell line studies, it is important to use animal models to test the anticancer effects of α -tocopherol and its analogs. The application of animal models, including cancer cell xenografts on nude mice, transgenic mice such as TRAMP model,⁴⁴ LADY prostate cancer model,^{45,46} knockout mice models such as NKX3.1knockout mice,⁴⁷ pTENknockout mice⁴⁸ and others, could advance our insights of how α -tocopherol affects the development of prostate cancer *in vivo*. Third, there is a need to identify the α -tocopheryl derivatives with better efficacy and stability than the parental chemical version. Fourth, the exploration of the roles of tocopherol-associated protein (TAP) and tocopherol transfer protein (TTP) are also important. Till now, TTP is the only protein that has been linked to the absorption of tocopherol into liver and then subsequently into circulating systems.^{49,50} However, the roles of another tocopherol binding protein, TAP, have not been well-explored. Fifth, among these fat-soluble vitamins, A, D, E, and K, the receptors of vitamin A and vitamin D have been identified. It is interesting to investigate whether vitamin E has a specific receptor, which can bind

and propagate the function and signal of vitamin E. Overall, the anticancer effects of vitamin E and its analogs have been observed, yet the underlying mechanisms need more intensive investigations. Although there have been many advances in radiotherapy, chemotherapy, and surgery, the use of complementary therapies for cancer remains of key interest. Understanding the functional mechanisms of vitamin E are important, as they will provide a base for the combinational therapy with other compounds. In the long run, cancer patients could benefit from a cocktail therapy via combining different treatments with complementary or synergistic effects.

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12

VITAMIN D AND PROSTATE CANCER

Yi-Fen Lee, Huei-Ju Ting and Bo-Ying Bao

*Departments of Pathology & Laboratory Medicine and Urology
University of Rochester Medical Center
Rochester, New York, USA*

Introduction

Nutritional factors have been hypothesized to be critical in the development of numerous cancers, and this holds true for prostate cancer. On the basis of geographic patterns of ultraviolet radiation throughout the contiguous United States, and on epidemiological data on prostate cancer incidence, the hypothesis was raised by Schwartz and colleagues that vitamin D deficiency may be a prostate cancer risk factor, and that increased exposure to sunlight may protect against clinical prostate cancer.¹ *In vitro* studies have shown that treatment with $1\alpha, 25(\text{OH})_2\text{D}_3$ (1,25-VD), a active form of vitamin D, decreases proliferation and increases differentiation of prostate cancer cells. Prostate cancer cells contain a specific receptor for 1,25-VD (the vitamin D receptor, VDR) that is known to mediate inhibition of proliferation, tumor invasiveness, the induction of apoptosis, and differentiation of prostate cancer cells. In this chapter, we will summarize recent progress in the study of vitamin D in prostate cancer, including epidemiological analyses, the vitamin D anti-tumor mechanisms, and vitamin D-based treatment in clinical trials. Understanding how vitamin D acts in the prostate is of great importance for the continued advancement of prostate cancer therapy and prevention.

Epidemiology Study

On the basis of geographic patterns of ultraviolet radiation throughout the United States and epidemiological data on prostate cancer incidence, a hypothesis was raised by Schwartz and colleagues that vitamin D deficiency might be a risk factor and that increased sunlight exposure may protect against prostate cancer.¹ It was initially found that prostate cancer mortality rates were higher in northern latitudes. Because casual exposure to sunlight results in the production of vitamin D, it was postulated that vitamin D promotes differentiation of prostate cells and that inadequate levels of vitamin D may permit the growth of prostate cancer. Later, it was reported that prostate cancer mortality rates by county within the United States were inversely correlated with the availability of UV radiation, lending further support to the vitamin D hypothesis.² Men with prostate cancer have been found to have lower serum levels of 1,25-VD before diagnosis of the disease.³ This is further supported by the Ahonen group study comprised of a 13-year follow-up of about 19,000 middle-aged men who were free of clinically verified prostate cancer. They found that among those who developed prostate cancer, more than half had vitamin D deficiency (serum level of vitamin D3 [1,25-VD₃] below 50 nmol/l). Low 1,25-VD₃ concentrations in the younger men were associated with more aggressive prostate cancer, and high 1,25-VD₃ levels delayed the appearance of clinically verified prostate cancer by 1.8 years. Therefore, Vitamin D deficiency may increase the risk of initiation and progression of prostate cancer.⁴

One of the most recent studies of 200,000 samples from Nordic men, including men from Norway, Finland, and Sweden, found that the normal average serum concentration of 25(OH)-vitamin D (40–60 nmol/l) was associated with the lowest risk of prostate cancer, and that both low (≤ 19 nmol/l) and high (≥ 80 nmol/l) 25(OH)-vitamin D serum concentrations were associated with higher prostate cancer risk. High vitamin D levels might enhance expression of 24-hydroxylase, the enzyme that inactivates 1,25-VD. Therefore, the U-shaped risk of prostate cancer might be due to the availability of 1,25-VD within the prostate.⁵

Vitamin D Action

1,25-VD, the bioactive form of vitamin D, was identified as a nutrient a century ago, and is the major regulator of calcium and phosphate homeostasis, responsible for maintenance of bone integrity, and also plays an important role in hair follicle cycling and mammary gland development. The biological effects of 1,25-VD are mainly mediated through the VDR, a member of the nuclear receptor superfamily, which heterodimerizes with the retinoid X receptor (RXR) and binds to response elements located in the promoters of target genes in various tissues, such as bone (osteocalcin, osteopontin, and beta 3 integrin), kidney (24-hydroxylase), and intestine (calbindin).^{6,7} Like other nuclear receptors, VDR contains an amino-terminal activation function 1 (AF-1) domain, a DNA binding domain (DBD), composed of two hexameric half-sites organized as direct repeats that recognize specific DNA response elements, and a ligand binding domain (LBD) that contains a ligand-regulated transcriptional activation function 2 (AF-2) region.⁸ Ligand-induced conformational changes in the LBD of VDR create an interface for protein-protein interaction between receptors and a group of intermediate proteins, termed coregulators, that may connect liganded receptors to basal transcription machinery, or that remodel the chromatin structure to facilitate transcription initiation.⁹

Several coregulators that modulate VDR activity have been identified. Included are positive coregulators such as SRC-1, TIF2/GRIP-1, SRC-3/RAC3/AIB-1, HMG-1/2, TAF(II)55, Smad3, and TRAP220, as well as negative coregulators such as NCoRs, Smad7, and Stat1.¹⁰⁻¹⁸ The VDR-interacting protein (DRIP) complex, identified by affinity column assay, consists of several components, promotes transactivation of VDR *in vitro*, and demonstrates chromatin remodeling activity.¹⁹ Large numbers of coregulators have been identified, and it is believed that they may form several multiprotein complexes to serve particular functions in modulating receptor transactivation. For example, the SWI/SNF complex possesses ATP-dependent chromatin remodeling activity, the CBP and p/CAF complexes possess histone acetyltransferase activity, and the DRIP complex functions to recruit basal transcription factors.²⁰

The phosphorylation status of VDR is also important for modulation of receptor function. For example, phosphorylation of VDR by casein kinase-II promotes its transactivation activity, while phosphorylation of VDR by PKC is thought to negatively regulate its activity. In addition, the tyrosine-phosphorylated VDR exerts increasing affinity for DRIP205, a component of a coregulator complex that acts as a bridge between the basal transcription machinery and VDR.²¹ A recent report showed that the p38 and JNK-triggered c-Jun/AP-1 pathways transactivate VDR and sensitize breast cancer to vitamin D-induced growth inhibition.²² These lines of evidence indicate that cross-talk between growth factors and VDR-mediated vitamin D action may be involved in modulating vitamin D-mediated growth arrest in cancer cells.

Mechanism of Anti-tumor Action in Prostate Cancer by Vitamin D

A key feature of cancer cells is their increased rate of growth relative to normal cells, and this can be attributed to increased cell proliferation, decreased cell death, or a combination of the two. 1,25-VD has been demonstrated to regulate cellular differentiation and proliferation in a number of normal and malignant cells, including prostate cancer cells, however the response to 1,25-VD in cancer cells appears to be cell type-specific. The majority of the 1,25-VD-mediated signals function through the VDR. The sensitivity and responsiveness of cells to vitamin D are therefore partly dependent on the activation of VDR. Miller *et al.* first demonstrated the presence of VDR in the LNCaP human prostate cancer cell line.²³ VDRs have since been found in other prostate cancer cell lines, as well as in normal prostate epithelial and stromal cells grown in culture.²⁴ Several studies have demonstrated that 1,25-VD and its analogs inhibit the growth of prostate cancer cell lines as well as primary prostate cancer cells in culture.^{23–26} The 1,25-VD responsiveness is variable among cell lines tested, however the context of VDR and the 1,25-VD-mediated VDR transcriptional activity did not fully explain the different vitamin D anti-proliferative responses among cell lines. Our data and that of other investigators show that the cross-talk between vitamin D and androgen/AR signaling contributes to some degree of growth inhibition. The vitamin D anti-proliferation effect is greater in AR-expressing

prostate cancer cells, such as LNCaP and CWR22R, than in AR-negative prostate cancer cells, such as DU145 and PC-3. Treatment with vitamin D enhances the AR expression, and inhibition of AR expression by antiandrogen-treatment, AR RNAi, or AR targeted disruption resulted in diminished vitamin D growth inhibition effects.²⁷⁻²⁹ Therefore, androgen/AR signaling plays important roles in the anti-proliferative action of vitamin D in prostate cancer cell lines as summarized in Fig. 1.

Different mechanisms have been proposed for the inhibition of cell growth by vitamin D. The most common feature of response to 1,25-VD in cancer cells is the induction of G1/G0 cell cycle arrest, however, the 1,25-VD-induced G1/G0 arrest pathways are likely multi-factorial. In LNCaP cells, vitamin D treatment decreases retinoblastoma protein phosphorylation, represses E2F transcriptional activity, slightly increases levels of cyclin-dependent kinase (CDK) inhibitor p21, and decreases CDK activity, finally resulting in G0/G1 accumulation.^{29,30} In other cancer models, such as U937 myelomonocytes, p21 appears to be the most highly up-regulated

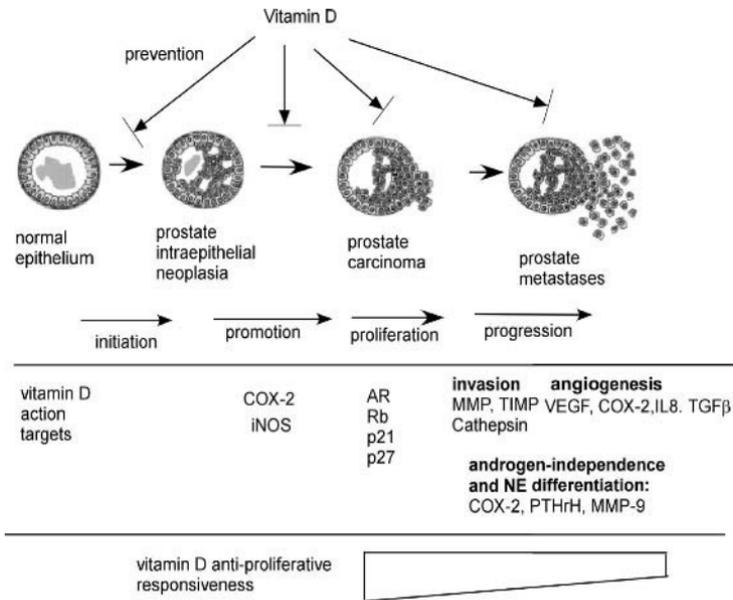


Fig. 1. Illustration of vitamin D anti-tumor effects and of putative vitamin D responsive genes.

gene, yet, in head and neck squamous cell carcinoma (SCC), p21 is not increased, but p27 is up-regulated. Identification of a vitamin D response element (VDRE) in the p21 promoter regions suggested direct regulation of p21 activity by vitamin D/VDR, and the cell context is likely to be another determining factor. In addition to G1/G0 arrest, induction of apoptosis and differentiation of the prostate cancer cells may also contribute to the antiproliferative activity of vitamin D in some prostate cancer cells.³¹ Studies in breast cancer cells suggest that the vitamin D/VDR pathway seems to be involved in stimulating the mitochondria to release cytochrome C rather than a pathway relying on upstream caspases.³² The mechanisms by which 1,25-VD induces apoptosis remain to be further investigated.

In addition to its antiproliferative effect, 1,25-VD can inhibit prostate cancer cell invasion, adhesion, and migration through modulation of select cell surface adhesion molecules, such as integrins (alpha 4 and beta 4),³³ and by inhibition of metallo-proteases (MMP-2 and MMP-9).³⁴ These data suggest that 1,25-VD can not only inhibit the growth of the tumor cells, but can also stop tumor cell migration, penetration, and metastasis. Such data stimulated clinical trials of vitamin D as therapeutic agents for treatment of prostate cancer.^{35,36} In one of our most recent studies, we found that vitamin D prevents the prostate cancer cell invasion via modulation of selective proteinase activity. In our current study using the zymographic assay, we found that 1,25-VD inhibited MMP-9 activity and enhanced tissue inhibitors of metalloproteinase-1 (TIMP-1) activity, but had less of an effect on plasminogen activators and Cathepsin.³⁷ Real-time PCR analysis demonstrated that 1,25-VD inhibited MMP-9 and induced TIMP-1 transcript expression in a time dependent manner. The vitamin D effects on the TIMP-1 promoter activity further suggested a potential cross-talk between AP-1 and vitamin D/VDR signalings, which might contribute to the suppression of TIMP-1. In contrast, the regulation of MMP-9 by vitamin D did not seem to be regulated at the transcriptional level, suggesting some post-transcriptional modifications, or RNA stability might contribute to the suppression of MMP-9 activity by vitamin D.³⁷ From our study, we concluded that vitamin D modulates the activity of selective proteinases, such as MMP-9 and TIMP-1, to inhibit the prostate cancer cell invasion, and thus provides proof of the concept for treating advanced stage prostate cancer patients with vitamin D to stop disease progression.

Development of New Vitamin D Analogs and Their Use in Combination Therapy for Prostate Cancer

The beneficial effects of 1,25-VD against cancer cell proliferation have been supported by many *in vitro* studies, yet the therapeutic window of 1,25-VD usefulness is extremely narrow and effective doses cannot be administered without inducing hypercalcemia. The increase in calcium is achieved both by enhanced intestinal absorption and by liberation of calcium from the bone, eventually leading to decreased bone mass at higher doses. Therefore, much effort has been directed toward the identification of new analogs, which retain the favorable activities of 1,25-VD while avoiding the side effects. Several synthetic vitamin D analogs have been reported to exert promising anti-cancer effects with reduced hypercalcemia. Skowronski *et al.* showed that selected vitamin D analogs displayed reduced calcemic effects and that potency was even greater than 1,25-VD.³⁸ Among those vitamin D analogs, one of the most promising synthetic analogs is Seocalcitol (EB1089, Leo Pharmaceutical Products). A considerable number of *in vitro* studies have been carried out with EB1089 and show that the analog is more potent than 1,25-VD with respect to regulation of cancer cell growth and differentiation, and the effect of EB1089 on calcium metabolism *in vivo* is approximately 50% less than that of 1,25-VD. The anti-cancer effects of EB1089 without induction of hypercalcemia were also demonstrated *in vivo* in a rat model of mammary gland carcinoma. Similar effects were seen in an *in vivo* prostate cancer study where EB1089 inhibited prostate cancer cell proliferation and reduced tumorigenesis as well as tumor metastases. Several other vitamin D analogs or structural VDR activators, such as Maxacalcitol (OCT) (Chugai Pharmaceutical Co. Ltd.), 16-ene analogs (Hoffmann LaRoche, Inc.), 19-nor analogs (Hoffmann LaRoche, Inc.), 1 α -hydroxyvitamin D5.³⁹ LG190119 (Ligand Pharmaceuticals Inc.), and 1 α -hydroxyvitamin D2 have been developed and tested in clinical trials in the advanced prostate cancer patients.^{40,41} These compounds may have promise as therapeutic agents for cancer and other diseases, with fewer side effects than 1,25-VD.

Another strategy to preserve vitamin D anti-tumor effects while avoiding the side effects is vitamin D-based combination therapy in which other

agents that either promote vitamin D responsiveness or reduce the hypercalcemia are used. The regimens used for vitamin D combination therapy include the chemotherapy drug (Paclitaxel, Taxotere),^{42,43} anti-mitotic agents (Genistein),⁴⁴ differentiation agents (retinoic acid),^{45,46} and the agents that modulate vitamin D/VDR action (Dexamethasone).⁴⁷ All regimens were shown to potentiate the anti-tumor effects of vitamin D and reduce vitamin-D associated hypercalcemia in prostate cancer cells and animal models, yet detailed mechanisms are not yet known and more clinical trials need to be done.

Vitamin D-Based Clinical Trials

Evidence accumulated from both experimental and epidemiological studies supports the idea that vitamin D could be used as a therapeutic drug to control prostate cancer, and several vitamin D-based clinical trials have been conducted, mainly enlisting patients with advanced androgen-independent prostate cancer (AIPC). Pilot studies from small numbers of patients that took daily orally doses of calcitriol found that the PSA levels in those patients were significantly lower before calcitriol therapy. However, there is incidence of hypercalciuria or hypercalcemia, and the development of renal stones in some patients, which limits its clinical usefulness.³⁵ One Phase I clinical trial was conducted by injection of calcitriol subcutaneously every other day in the advanced malignancy patients and tested the tolerable toxicity via this route.⁴⁸ Then, different calcitriol-based clinical trials, with either modifications in the schedules and route of administration, or combinations with dexamethasone, paclitaxel have been developed.⁴⁹ In addition to the calcitriol, its analogs such as 1α -hydroxyvitamin D₂ have been tested in clinical trial Phase I and II in the AIPC patients.^{40,41} The results from all these clinical trials suggest further clinical investigation of this disease with vitamin D, its analogs, or in combination with other agents, such as chemotherapy, should be further pursued.

In the past, chemotherapy was considered ineffective, however, newer chemotherapeutic drugs and drug combinations are now demonstrating improved response rates. Among those chemotherapy drugs, taxotere, a semisynthetic taxane, is commonly used as a chemotherapeutic agent

by itself and in combination regimens in the treatment of hormone refractory prostate cancer (HRPC). 1,25-VD has been shown to sensitize prostate cancer cells to cytotoxic drugs, such as paclitaxel, platinum compounds, and Taxotere. The Phase II clinical trial of Taxotere[®] plus calcitriol has shown approximately twice the PSA decline response rate compared with taxotere alone,⁴³ which provide very promising strategies for treatment of AIPC. Quality of life (QOL) and pain relief were also checked during the calcitriol and taxotere combination therapy. Significant analgesic activity was demonstrated, yet worsening in several QOL parameters was also observed in patients experiencing relatively low pain intensity.⁵⁰

Loss of Vitamin D Anti-proliferative Responsiveness in Prostate Cancer

While current efforts focus on developing strategies to use vitamin D analogs to control prostate cancer, it is possible that prostate cancer cells could become resistant to the tumor suppressive effects of vitamin D. Some prostate cancer cells are already known to be resistant to growth inhibition by vitamin D. Analyses of experimental model systems reveal that prostate cancer cells become less sensitive to vitamin D through loss of vitamin D receptors, loss of signaling molecules that modulate vitamin D action, or through changes in metabolic enzymes, such as 1 α -hydroxylase and 24-hydroxylase, that sensitize or degrade vitamin D compounds.⁵¹ Such changes have been found in experimental models, yet whether these alterations occur in human prostate cancer tissues *in vivo*, and the associated frequencies of occurrences still need to be determined. One study suggested that VDR levels were decreased in the prostate after age 60, which might be linked with increased incidence of prostate cancer with age,⁵² and development of vitamin D resistance during the disease progression.

In one of our current studies, we identified a prostate cancer cell sub-line, CWR22R-2, which displays more aggressive behavior in the tumor invasiveness than its parental CWR22R cells. This aggressive CWR22R-2 line displays reduced vitamin D anti-proliferative effects compared to CWR22R cells, which provides evidence that prostate cancer cells might

develop resistance to vitamin D during disease progression. Further characterization of these two cell lines found reduced vitamin D transcriptional activity in CWR22R-2 cells, which might correlate with higher expression of VDR co-repressors such as SMRT and NCoR, and eventually result in reduced vitamin D responsiveness.⁵³ More evidence from other prostate cancer cell lines with different vitamin D responses and from prostate cancer patient samples is needed to confirm our findings.

Future Perspectives

With increasing numbers of people being diagnosed with prostate cancer each year, there is great interest in developing better strategies to treat, and even to prevent, prostate cancer. Vitamin D has been proven to inhibit prostate cancer growth, progression, and metastases in both *in vitro* and *in vivo* animal models. However, the therapeutic use of vitamin D is limited. Development of vitamin D-based treatment with improved therapeutic indices, so that desired activity can be maximized while the tendency toward hypercalcemia can be minimized, is necessary.

The Figure summarizes our studies and those of others regarding vitamin D action in prostate cancer. Vitamin D may work through multiple pathways to prevent prostate cancer growth and progression via direct or indirect modulation of intracellular signals or secreted proteins, which are involved in the tumor growth, invasion, and angiogenesis. Whether the vitamin D-mediated changes of gene profiles occur *in vivo* as they occur *in vitro*, remain to be answered. Development of the prostate cancer patient tissue array technique by combination of classical histochemical analyses with advanced molecular techniques might provide powerful tools to study prostate cancer progression and its correlation with the clinical features at the molecular level. Through analysis of the expression of those vitamin D responsive genes identified from *in vitro* studies using prostate cancer tissue array samples, we might be able to generate a vitamin D-response human database in prostate cancer and thus directly translate *in vitro* study results into clinical application. These will certainly help to predict outcomes of vitamin D-based treatment and enhance the treatment efficacy based on individual patients' biochemical and molecular profiles.

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FUNCTIONS OF ESTROGEN RECEPTOR IN PROSTATE AND PROSTATE CANCER

Shuyuan Yeh, Ming Chen, Jing Ni, Yi Yin, Eugene Chang,
Min Zhang and Xingqing Wen

*Departments of Urology and Pathology
University of Rochester Medical Center, Rochester, New York*

Introduction

It has long been known that the functions of estrogen and estrogen receptor (ER) are important for the female reproductive system, mammary gland development, and cancer progression.¹ Accumulated evidence from various studies indicated that ER is expressed and functions in the male reproductive system, including prostate. Earlier studies only identified one traditional ER, ERalpha (ER α), which was thought to be responsible for all the estrogen-mediated responses in prostate. Recently, ERbeta (ER β) was identified and shown to express in prostate epithelia.² Results from PCR, immunohistochemical staining and receptor knockout (KO) mice suggested that estrogens, ER α and ER β play significant roles in prostate development and the initiation and progression of prostate cancer. In this chapter, we will focus on the distribution of ER α and ER β , potential target genes, ER association proteins, their potential roles in the development of prostate, and the progression of prostate cancer based on the results from prostate tissues, cancer cell lines, and animal models, including α ERKO, β ERKO, $\alpha\beta$ ERKO, and hypogonadal (hpg) mice.

Distribution of ER α and ER β in Prostate Tissues, Cancer Specimens and Cancer Cell Lines

Estrogen has been used for the treatment of prostate cancer since the early 1940s.³ It is generally believed this action is indirectly mediated at the hypothalamic level to suppress the circulating androgens.⁴ However, in the early 1960s, a direct action of estrogen via their own receptors in the prostate was proposed by Mangan *et al.*⁵ Recently, the evidences that ER expressed in normal prostate, benign prostatic hyperplasia (BPH), prostate cancer specimens, and different prostate cancer cell lines, along with the demonstration of the stimulatory or inhibitory effects of estrogen on prostate cancer cells growth suggested estrogen may exert direct effects on prostate via their own receptors.⁶⁻¹¹ The following sections will discuss the distribution of ER α and ER β in prostate tissues, cancer specimens, and cancer cell lines.

Both ER α and ER β express in the human prostate tissue.^{2,12} In normal prostate tissue ER α is mostly expressed in stromal cells with occasional expression in the basal epithelial cells,^{8,11,13-15} whereas ER β is abundantly present in basal epithelial cells.^{8,11,13} The complementary location of both ER α and ER β might explain why some early studies reported ER α in both the stroma and epithelium of the normal prostate, which may be caused by cross reaction against both ER subtypes.¹⁶

In BPH, ER α immunostaining could not be detected in several studies.^{15,16} Using enzyme immunoassay, Mobbs *et al.* (1990) found high positivity of ER α in BPH.¹⁷ The latter immunostaining studies found ER α was stronger in BPH than in normal prostates and localized in both epithelial and stromal cells.⁸ Immunoexpression of ER β in BPH was also higher than in normal prostates and localized only in the epithelium.⁸

In prostate cancer specimens, the expressions of both ER α and ER β have been studied for years,^{6,8,13,18} however, the relative expression levels and location of ERs in prostate cancer are still controversial. Using methods of immunohistochemistry staining, loss or down-regulation of ER α in prostate cancer has been reported.^{19,20} It appears ER α gene is transcriptionally inactivated by DNA methylation in most prostate cancer cells lines and specimens.^{11,21} Conversely, several groups reported the higher ER α expression in the prostate cancer specimens than in BPH and normal prostate tissue, which is consistent with other reports that abundance of ER α is positively correlated with the malignancy of prostate cancer.^{7,17}

In terms of ER β expression, Royuela *et al.* (2001) reported the ER β has increasing epithelial staining in both BPH and prostate cancer, and some stromal cells acquire ER β in prostate cancer specimens, not in normal prostate and BPH. These findings suggest the involvement of ER β in prostate cancer. However, recent reports showed a frequent loss of ER β expression in prostate cancer samples relative to normal prostate tissue.^{13,18,22} In the primary prostate tumor sites, ER β was strongly expressed in low grade prostate carcinoma and was markedly diminished in higher grade tumor.^{18,22,23} These results suggested ER β may protect against abnormal prostate cell growth.^{11,13,22} Loss of ER β may cause prostate epithelial cells to escape the control of proliferation by ER β in prostate cancer. Further studies indicated ER β protein expression was significantly elevated in metastatic prostate tumor.¹³ Currently it is unclear why the ER β expression will be decreased in the higher grade prostate cancer and re-elevated in metastases. One hypothesis is that the failure to lose ER β may allow the cancer cells to gain the metastatic potential, and another could be the local environment in the distal metastatic sites may induce ER β expression. Although the controversy of ERs expression exists, the accumulating evidence shows the abnormality of ER signaling may contribute to the pathogenesis of prostate cancer.

The expression patterns of both ERs in different prostate cancer cell lines has been studied recently. The earlier studies on the effect of estrogens on prostate cancer cells growth showed the direct role of estrogen via their own receptors, but the specific ER has not been defined. Using RT-PCR, Lau *et al.* (1999) showed ER β , but not ER α , was found in normal prostate epithelial cells, along with pS2 and PR mRNA expression. Only ER β is detectable in LNCaP and DU145 cell lines, whereas PC-3 and BPH-1 expressed both ER α and ER β .¹¹ Currently, there are two ER β isoforms, ER β 1 and ER β 2. Only ER β 1 was detectable in PC-3, DU145, and LNCaP cell lines, whereas ER β 2 was absent.²⁴ Together, these studies suggested ER β could be the key player of estrogen-mediated effects in prostate cancer cell lines.

In rodents, it was found that ER α mRNA was the major type of ER expressed in two-week-old rat prostate and that as the rat aged ER β became the dominant expression.²⁵ In agreement with the results from human prostate, it was found that ER α protein is predominantly located in the stroma cells of ventral prostate of adult rodents, with over 90% of the epithelial cells stained positive for ER β .²⁶

Estrogen Regulated Genes in Prostate Cancer

Estrogens and ER have been shown to play important roles in prostate carcinogenesis.²⁷ The detailed mechanisms however, are not well known. Currently, some estrogen target genes have been identified, which may contribute to the diversified estrogen effect on prostate cancer. Those genes include telomerase,²⁷ endothelin-1,²⁸ eNOS,²⁹ E-cadherin,³⁰ metallothionein II,³¹ CGA gene,³² androgen receptor,²⁶ and glutathione-peroxidase.³³

In addition, PS2 and progesterone receptor (PR), have an estrogen responsive element (ERE) in the promoter regions, and are also expressed in the prostate, suggesting they can be regulated by estrogen in human prostate.¹¹ In mouse prostate, diethylstilbestrol (DES) treatment induces PR synthesis in secretory epithelial cells, which is usually low in the untreated prostate. Although a functional role for progesterone in regulating prostate growth is unknown, PR synthesis is an effective marker of estrogen action in the prostate. It was found that the acute DES exposure of WT male mice, the prostatic responses were similar, including induction of squamous metaplasia (SQM), and up-regulation of PR.³⁴ Table 1 lists the identified estrogen target genes in the prostate. In comparison, many other estrogen target genes have been identified in the breast or other estrogen target organs. For better understanding of the estrogen signal in prostate, ER α and ER β target genes and their roles in prostate need to be identified and characterized.

Table 1. Estrogen Regulated Genes in Prostate

Function	Genes	Up- or Down-regulation	Estrogen Response Element (ERE)
Carcinogenesis	Telomerase	↑	+
Angiogenesis	eNOS	↑	N/A
Metastasis	E-cadherin	↑	N/A
Metastasis	Endothelin-1	↓	N/A
Homeostasis	Metallothionein II	↑	N/A
Hormones	CGA gene	↑	N/A (link with ER α)
Oxidant enzyme	Glutathione-peroxidase	↑	N/A
Transcription factor	Androgen receptor	↓	N/A
Transcription factor	Progesterone receptor	↑	+
Unknown	PS2	↑	+

ER Coregulators in Prostate

ERs are ligand-dependent transcription factors which bind to the ERE to regulate the transcription of target genes. The transcription initiation is a complex process which involves the cooperative interaction between ERs and multiple factors at the promoter region of target genes. The occurrence of transcriptional interference between nuclear receptors in early transient receptor/reporter co-transfection assays suggested the existence of common rate limiting cofactors, other than general transcription factors (GTFs), required for ERs activation function.^{35,36} In this regard, coregulators were identified, and defined as coactivators or corepressors required for transcriptional regulation.³⁷ In this chapter, we will mainly focus on the expression pattern of some identified ER coactivators in the prostate tissue and prostate cancer cell lines.

To date, there are numerous ER coregulators that have been identified and the most well characterized ER coactivators belong to the p160 family, including SRC-1, SRC-2 (TIF2/GRIP1) and SRC-3 (AIB1/RAC3).³⁷ Among these identified ER coactivators, SRC-3/RAC3/AIB1 is the most well studied in prostate cancer cell lines. Using Western blotting, Gnanapragasam *et al.* showed that SRC3/RAC3/AIB1 protein has the highest expression in LNCaP cells, moderate expression in PC-3 cells, and low-level expression in DU145 cells.³⁸ In the human prostate tissue, the levels of SRC-3/RAC3/AIB1 expression is significantly correlated with tumor grade and stage of disease, but not with serum PSA levels.³⁸ SRC-1 is expressed as a major RNA transcript of 7.5 kb in many tissues, including prostate. The expression levels of SRC-1 were found to be elevated in the cancer specimens with a higher grade or poor response to endocrine therapy, than in those with a lower grade or good response to endocrine therapy.³⁹ In addition to SRC-1 and SRC-3, other ER co-regulators, CBP/p300, SMRT, and N-CoR, are also expressed in prostate.⁴⁰

Overall, most of ER co-regulators identified to date are not specific to ER, and can interact with other nuclear receptors, including AR.⁴¹ Therefore, how these co-regulators regulate ER function in prostate development and neoplastic transformation remains to be elucidated. In addition, whether any ER specific associated proteins exist in prostate needs further investigation.

Histological Changes in Prostates of α ERKO and β ERKO Mice

To understand the roles of estrogen, ER α and ER β in the development of prostate glands, the gene knockout strategy has been used. The adult male α ERKO mouse prostate shows normal development and histology, specifically in the ventral prostate (VP)^{42,43} and anterior prostate (AP).⁴⁴ With aging, the weights of α ERKO seminal vesicle (SV) and AP increased,⁴⁵ although they remain histologically indistinguishable from that of WT littermates.^{42,44}

The initial description of targeted disruption of the gene encoding ER β reported some evidence of epithelial hyperplasia in β ERKO prostates.⁴⁶ Weihua *et al.*²⁶ further reported that β ERKO mice had multiple hyperplastic foci in the peripheral and central zones of the VP at five months; by one year of age, eight of ten β ERKO VPs had multiple hyperplastic lesions, and most epithelial cells stained positively for the proliferation antigen Ki-67. However, other studies of the prostate phenotype in ER β -deficient mice,^{42,43,47} failed to corroborate the initial report of Krege *et al.*,⁴⁶ and no hyperplasia was seen in prostate lobes of β ERKO line at 12–20 months.^{42,43,47} The reason for discrepant results from mice with the same apparent genotype is unclear, yet other factors might affect the mice, such as diet or infection. In general, there is no significant histological changes in prostates of α ERKO mice, and with controversial observation in prostate of β ERKO Mice.

Estrogen Imprinting Effect on the Development of Prostate

Although estrogen levels are low or undetectable in adult male mice, administration of exogenous estrogens during development⁴⁸ dramatically affects prostate growth and function.^{49–54} The effects of estrogens vary according to timing and duration of exposure, in addition to the type and dose of estrogen administered. Furthermore, the individual lobes of the prostate exhibit varied degrees of response to estrogens and androgens.

The neonatal period after birth is very fundamental for the rodent prostate development in which the prostate involves branching morphogenesis followed by functional differentiation. In this period, brief exposure of male rats or mice to high-level estrogens will cause irreversible alterations in

development and function of the prostate gland and a reduced responsiveness to androgens during adulthood.^{50,55–57} The estrogen imprinting effect is associated with an aging-related prostatic lesion, which includes the hyperplasia of prostatic epithelial cells or severe dysplasia similar to high grade prostatic intra-epithelial neoplasia (PIN) and extensive immune cell infiltrate.^{51,58–61}

As early as 1978, Rajfer and Coffey reported that if high dose of 17 β -estradiol at 500 μ g per day, or estradiol benzoate at 250 μ g per day, or estradiol dipropionate at 100 μ g per day, is administered to intact male rats for 2 days during the 1st week after birth, the prostate, at adulthood, is diminutive in size and is inert to the action of exogenous androgens. If immature rodents are exposed to exogenous estrogen before puberty, the aging animals develop prostatic epithelial hyperplasia⁵⁰ or even dysplasia if androgen is given together with estrogen.⁶¹ A series of studies by McLachlan *et al.* demonstrated that perinatal exposure to the synthetic estrogen, DES, results in an assortment of apparently direct defects in the murine male reproductive tract, including undescended testes, epididymal cysts, aberrant expression of estrogen inducible genes, adenocarcinoma, and sterility.^{61–65}

In addition to morphological and histological changes, several groups characterized the other hallmarks of neonatal estrogen imprinting in the rat model as well as in different mouse strains. These included transient up-regulation of ER α , down-regulation of androgen receptor (AR), decreased ER β levels in adult prostate epithelium, lack of dorsal lateral prostate (DLP) secretory protein, up-regulated proto-oncogene c-fos, reduced TGF β type I receptor levels in the prostate epithelium, but not in stroma, whereas there was no effect on TGF β type II receptor. p21 (cip-1/waf-1) is a cyclin-dependent kinase inhibitor, and is known to be inducible by TGF β 1 in the prostate. Neonatal estrogenization prevented this transient expression of p21 (cip-1/waf-1) in the prostate epithelium cells. Those changes in molecular levels suggested they could be good markers for estrogenization in the prostate.^{66–72}

Estrogen Treatment of α ERKO, β ERKO, and Hypogonadal (*hpg*) Mouse Models

Intact adult α ERKO mice treated with DES showed no change in organ weight, no induction of SQM, or synthesis of CK10 or PR in any prostate

lobe.⁴⁴ In contrast, the intact adult β ERKO mouse treated with DES showed a full response, similar to that of WT mice.⁴⁴ In spite of normal ER β levels in the α ERKO prostate, these animals showed no morphological or molecular effects of estrogen in the prostate. These data suggest that these actions or effects of exogenous estrogen on the adult mouse prostate are predominantly mediated through ER α . ER α is thus the predominant receptor mediating mitogenic estrogen action in the prostate, and a role for ER β remains to be identified.

Because classic estrogen responses (SQM and up-regulation of PR) were seen in the β ERKO but not in the α ERKO mice, it is assumed that ER α mediates these responses in the adult prostate. A recent report involving tissue recombination examined the role of ER α in mediating estrogenic responses in prostate, using combinations of WT and α ERKO prostate epithelial and stromal cells.⁴⁴ The recombinant tissues were grafted under the kidney capsule of immunologically deprived nude mice exposed to DES treatment for 21 days. ER α is required for the induction of SQM, but not for the up-regulation of PR synthesis, which occurred in all recombinants, even though this response was absent after the *in vivo* DES treatment of α ERKO mice.⁴⁴ The functional significance of the separation of these two end points (SQM and PR synthesis) suggests that additional mechanisms are involved in mediating estrogen responses.

Exposure to estrogens during the neonatal period causes both acute and long-term effects on the prostate gland. Neonatally estrogenized WT animals show inhibited prostate growth during development and subsequently display evidence of dysplasia and an attenuated response to androgens.^{50,52–54,67} When estrogenized animals are castrated and then treated with androgen (testosterone) implants, the observed effects are not completely blocked, suggesting that much of the phenotype is the result of permanent alterations within the prostate tissue caused by direct estrogen action.⁶⁷ The neonatal studies described above were all conducted in a rat model system with high doses of DES. By contrast, a single report showed increased prostate size following low-dose maternal estrogen administration to male mouse fetuses.^{49,73} The discrepancies between rat and mouse models, and high- and low-dose estrogen, emphasize the compounding

influences of timing, duration, and dose of estrogen in similar studies. Recently, Prins *et al.*⁴² utilized α ERKO and β ERKO mice to demonstrate that both acute and chronic effects of neonatal DES treatment are predominantly mediated by ER α in mouse prostate.

Investigation of intact male mice with prostatic response to estrogen treatment requires analysis of both indirect and direct effects. Indirect actions occur via regulation of the hypothalamic-pituitary-gonadal (HPG) axis. They alter androgen production and the endocrine status of the mice. Direct responses to estrogen by the prostate differ and occur in response to local or intraprostatic changes in estrogen synthesis via aromatase enzyme activity or modulation of the ERs. More recently, the direct actions of estrogens *in vivo* were evaluated using the hypogonadal (*hpg*) mouse model⁷⁴ that is deficient in pituitary gonadotropin and sex steroid production. Mature male *hpg* mice were exposed to estradiol for 6 weeks, and proliferative changes were recorded in the prostate with specific effects observed in the stroma and epithelium; estradiol administration stimulated growth and expansion of the stromal, epithelial and luminal compartments of the mouse prostate lobes.⁷⁵ The epithelial cells became multi-layered and squamous, showing immunoeexpression of high molecular weight cytokeratins and cytokeratin 10 (CK10) that is characteristic of the pathology known as squamous metaplasia (SQM).^{44,76} The stromal response was characterized by an increase in fibroblastic stroma that penetrated the smooth muscle layer, accompanied by a reduction and disorganization in α -actin-positive smooth muscle cells surrounding the glandular ducts. Additionally, neutrophils were identified in the stroma and were shown to migrate through the epithelium to the lumen. Although secretory activity of prostate epithelial cells was significantly reduced by estradiol exposure, the lumen was distended as a result of accumulated cellular debris comprising epithelial cells, inflammatory cells, and anuclear keratinised deposits.⁷⁵

Overall, results from these genomic-manipulated mice suggested that ER α is the dominant ER form mediating the developmental estrogenization of the prostate gland. Estrogens induced direct proliferative changes in the prostate of male *hpg mice*, which do not have physiological levels of androgen.

Estrogen Effect on Initiation, Growth, and Progression of Prostate Cancer

While androgens play essential roles in the development and growth of prostate and pathogenesis of prostate cancer, extensive *in vivo* and *in vitro* studies suggested that estrogens are required for carcinogenesis of prostate cancer. It was demonstrated that treatment with both estrogen and testosterone will induce 100% dorsolateral prostate carcinoma in rats.⁷⁷ In human, direct estrogen effects and the balance of androgens and estrogens may contribute to these estrogen activities. Men synthesize both estrogens and androgens and as men age, the plasma levels of androgen decreases while estrogen remains constant. However, the expression pattern and activity of aromatase, the enzyme that turns testosterone into estrogen, suggests that local synthesized estrogen may have significant consequences in tumorigenesis of prostate.^{78,79} Here we will focus on estrogen effects on prostate cancer cells from *in vitro* and *in vivo* approaches. While this approach provides some insights to explain the *in vivo* observation from clinical and mouse study, many unclear and controversial data remain.

In prostate, estrogen induced cancerous transformation may be partially due to their genotoxic metabolites, including 2-hydroxy-estrogens, 4-hydroxy-estrogens, quinone, and semiquinone intermediates. These metabolites may directly induce genomic damage or function via formation of reactive oxygen species (ROS).^{80,81} In addition, the enzymes responsible for the formation or inactivation of these estrogen metabolites may also play critical roles in the estrogen mediated transformation in prostate. These enzymes include (1) cytochrome p4501A1, which can convert estrogen into 2-hydroxy-estrogens, (2) cytochrome p4501B1, which can convert estrogens to 4-hydroxy-estrogens, (3) catechol-O-methyl-transferase, which can inactivate 2- and 4-hydroxy-estrogens, and (4) glutathione-S-transferases that detoxify ROS.⁸¹⁻⁸³ Although these may be important factors for estrogen mediated effects in prostate, few of them have been investigated for their contribution in prostate cancer.

As the aromatase can convert androgens into estrogens, thus it could be one of the risk factor of prostate cancer.^{84,85} However, the aromatase transgenic mice and knockout mice have numerous endocrine defects,^{34,86} thus

these mice cannot be used as good models to study the relationship between estrogen formation and prostate cancer risk. The studies will be advanced by producing mice with prostate specific expression or knock-out of aromatase in the future.

In adult WT mice, administration of high-dose DES, a potent synthetic estrogen, causes regression of the prostate and induces SQM in the epithelium. The reduced organ size is associated with declining androgen levels, whereas SQM is considered to be a direct, ER α mediated response to estrogen.⁴⁴ Prostatic SQM is characterized by proliferation of basal cells, leading to formation of stratified squamous epithelium and altered CK10 synthesis. In mouse prostate, DES treatment induces PR synthesis in secretory epithelial cells, which is usually low in the untreated prostate.

Although studies suggested that estrogens are required for carcinogenesis of prostate cancer, yet extensive *evidences also* suggest that estrogens can suppress prostate cancer growth. It has been found that estrogen inhibits the cell growth of PC-3, an androgen independent prostate cancer cell line,⁹ while it can stimulate the growth of LNCaP, an androgen responsive prostate cancer cell line.¹⁰ This difference may be due to different ER forms and ER levels in these two cell lines^{85,86} and other mechanisms. Although estrogen stimulates both ER α and ER β , it is generally believed that ER α stimulates cell proliferation whereas ER β counteracts ER α activity. In the prostate, ER α is exclusively expressed in stroma, while ER β is largely expressed in epithelial cells. In tumors, ER α is expressed in both stroma and epithelial. Many studies support the expression pattern of ER α and ER β , and the interaction between stroma and epithelial of prostate largely contributes to the different estrogen effects on the prostate cancer cells growth *in vivo* and *in vitro*.^{11,87–89} However, the expression pattern of ER α and ER β in normal and tumor prostate tissue, and prostate cancer cell lines are different. More studies need to be elucidated.

The effect of estrogen regulation of prostate cancer cell growth was also tested with the estrogen metabolite, 2-methoxyestradiol (2-ME), which can arrest the cell cycle and induce apoptosis.^{90,91} However, the 10 μ M dose of 2-ME used in the study is much higher than physiological concentration. Cellular component concentrations are another important factor for the cellular growth or signal. Homeostatic changes might also

contribute to malignant growth. Estrogen can increase intracellular Ca (2+) in PC-3 cells.⁹² Estrogen also reduces the uptake of rubidium chloride, suggesting an estrogen effect on ion transport and change of cellular membrane permeability.⁹³

Although estrogen effects on prostate cancer cell growth are somewhat controversial, the fact that estrogen is required for prostate carcinogenesis is consistent. It was reported that a low ratio of androgen to estrogen results in a higher risk for the prostate cancer. *In vivo* animal studies also supported that estrogen, in a milieu of decreasing androgen, contributes significantly to the prostate hyperplasia, prostate dysplasia, and prostate cancer. β ERKO mice cannot be induced by estrogen treatment to grow prostate cancer.^{76,94} Therefore, estrogens and ERs, together with androgen, are required for the pathogenesis of prostate cancer. Recently, telomerase, whose activity increased during tumorigenesis, has been shown to be stimulated by estrogen in primary cultured cells from BPH and normal prostate tissue. Telomerase contains ERE in its promoter, thus could be a target gene of ER.²⁷ This may be one of the mechanisms by which estrogens stimulate carcinogenesis. Antiestrogen treatment therefore may prevent prostate cancer.

It is very significant that in the transgenic adenocarcinoma mouse prostate (TRAMP) mouse model of adenocarcinoma of the ventral prostate,⁹⁵ the selective estrogen receptor modulator (SERM), toremifene,⁹⁶ and the phytoestrogen genistein,⁹⁷ prevent the development of cancer. Although it has been suggested that this protection results from inhibition of ER α in the prostate stroma, an equally acceptable explanation is that these agents, acting as ER β agonists, prevent proliferation of the prostate epithelium. Such a mechanism is likely in view of the role of ER β in preventing proliferation of the ventral prostate epithelium in the mouse and strongly suggests a role for ER β agonists in the treatment and/or prevention of prostate cancer.

Implantation of prostate cancer xenografts (LuCaP 35, LuCaP 49, LuCaP 58, LuCaP 73, PC-3, and LNCaP) into intact and ovariectomized female mice was done by Corey *et al.* to characterize growth and uptake rates in the absence of androgens.⁹⁸ Significant inhibition of prostate cancer growth in intact *vs.* ovariectomized female animals was observed in five of six prostate cancer xenograft lines (except for PC-3). E2 supplements given to ovariectomized female mice led to inhibition of tumor establishment and

diminished growth of LuCaP 35, similar to that observed in intact female mice. RT-PCR showed that these xenografts express the ER β message. Two hypothetical mechanisms may be suggested to explain how E2 can affect prostate cancer tumor growth in female mice in the absence of androgens: (1) E2 exerts direct inhibitory effects via ER expressed on prostate cancer cells or via other, as yet unidentified, mechanisms; and (2) E2 exerts effects on other cells, which then secrete signaling molecules that inhibit prostate cancer growth.

Together, these *in vivo* and *in vitro* studies suggested that estrogens may have dual roles: (1) estrogens are required for tumorigenicity of prostate cancer; and (2) Estrogens may suppress growth of prostate cancer.

Conclusion

To date, results from various studies clearly demonstrated that estrogens and ERs play important roles in prostate development and tumorigenesis. However, the roles are still not completely elucidated. Results from α ERKO male mice suggest that ER α plays roles in the initiation of BPH and SQM.⁸⁶ Results from β ERKO male mice indicated that when mice aged they may develop hypertrophy²⁶ suggesting that ER β plays a negative role or is a factor controlling prostatic cell growth. However, results from other groups are not entirely consistent with that conclusion.⁹⁹ It is currently unclear whether nutritional and environmental factors contribute to the discrepancy. Furthermore, the number and function of different isoforms of ER β that exist in the prostate have not been conclusively determined.

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14

EPIDEMIOLOGY OF PROSTATE CANCER

Ann W. Hsing

*Division of Cancer Epidemiology and Genetics
National Cancer Institute,
Bethesda, Maryland 20852-7234, USA*

Anand P. Chokkalingam

*Celera Diagnostics, LLC
Alameda, California 94502, USA*

Introduction

Prostate cancer is the most common non-skin cancer in most western populations, and although worldwide incidence rates were on the rise through the 1990s,¹ they now appear to be declining slightly.² In western countries, the rise in incidence in the late 1970s and early 1980s was due, in part, to the increased use of transurethral resection of the prostate for benign prostatic hyperplasia (BPH).³ However, the increase in incidence between 1986 and 1992 was largely due to the increasing use of prostate specific antigen (PSA) testing for early detection of prostate cancer.⁴ Although incidence rates in Asian countries are low, their recent relative increases are larger than those of western countries and have been attributed to increased westernization.¹

Despite prostate cancer's high morbidity, its etiology remains obscure. The only established risk factors are age, race and a family history of prostate cancer. Many putative factors, such as hormones, diet, obesity, physical inactivity, occupation, vasectomy, smoking, sexual factors, and genetic susceptibility, have been implicated, but the epidemiologic evidence is inconclusive. An overview of these factors is presented below.

Table 1. Summary of Epidemiologic Risk Factors for Prostate Cancer

	Observation	Evidence	Implications
Established Factors			
Age	Incidence rises with age	Consistent	Latency is long and progression is slow
Race	African-Americans have the highest reported rates in the world, while Chinese men living in China have the lowest reported rates.	Consistent	Suggests both environmental and genetic factors may have a role in prostate cancer
	Migrants have much higher risk than their counterparts in ancestral countries	Consistent	Suggests a role of environmental factors Suggests westernization may be related to an increased risk
Family history of prostate cancer	Familial aggregation	Consistent	Suggests a role of genetic predisposition
Probable Factors			
Diet	Animal fat and red meat intake is associated with an increased risk	Somewhat consistent	Suggests fat or other constituents in meat may contribute to prostate carcinogenesis
	Selenium and vitamin E are associated with a reduced risk	Somewhat consistent	Suggests anti-carcinogenic effect of these compounds Chemoprevention trials are underway to evaluate these effects
	Consumption of tomato products is associated with a decreased risk	Somewhat consistent	Lycopene may protect against prostate cancer

Table 1 (Continued)

	Intake of cruciferous vegetables may be associated with decreased risk	Suggestive	Suggests intake of broccoli, cauliflower, Brussels sprouts and other cruciferous vegetables may protect against prostate cancer
	Allium vegetable intake may be associated with decreased risk	Needs confirmation	
	Intake of fish and marine fats may be associated with a decreased risk	Needs confirmation	
	Calcium may be associated with increased risk	Inconsistent	
	Intake of total vegetables may be associated with decreased risk	Inconsistent	
IGFs	Higher serum/plasma levels of IGF-I and lower levels of IGFBP-3 may be related to an increased risk	Somewhat consistent	Suggests IGFs may be related to the progression of prostate cancer Clinical utility of IGFs is under evaluation
Occupation	Farmers have ~10% excess risk	Consistent	Suggests exposures to herbicides or pesticides or lifestyles among farmers may be related to prostate cancer risk
	Workers in heavy metal and rubber industries may have an increased risk	Suggestive	Suggests exposures to certain chemicals may increase prostate cancer risk
Androgens	Higher serum levels of androgens may be associated with an increased risk	Suggestive	Suggests androgenic action is involved in prostate carcinogenesis
Obesity	Abdominal obesity may be related to an increased risk	Suggestive	Suggests that alteration of hormone synthesis or metabolism may have a role in prostate cancer etiology

Table 1 (Continued)

	Observation	Evidence	Implications
Chronic inflammation	Inflammation is found in prostate biopsies and resected prostate tissue, and pro-inflammatory markers are associated with increased risk	Suggestive	Suggests that factors contributing to inflammatory states may have a role in prostate cancer initiation or promotion
Vitamin D	Higher serum levels of vitamin D may be associated with a reduced risk	Inconsistent	
Sexual factors	Sexual factors, especially sexually transmitted infections such as HPV infection and syphilis, may be related to an increased risk	Inconsistent	
Vasectomy	Vasectomy may be associated with an increased risk	Inconsistent	
Physical activity	Long-term physical activity may be associated with a reduced risk of prostate cancer	Inconsistent	
Liver cirrhosis	Patients with liver cirrhosis may have a lower risk	Inconsistent	
Diabetes	Diabetic patients may have a lower risk	Inconsistent	
Smoking	Smoking may be associated with an increased risk	Inconsistent	

Table 2. Summary of Epidemiologic Studies of Rare, High Penetrance Genes, and Prostate Cancer

Region	Gene	Markers	Studies (Ref.), No. of cases Studied, Population	Results
1q24-25	<i>RNASEL</i> (HPC1)	E265X, R462Q, D541E, I97L	Rokman <i>et al.</i> (2002), ⁷⁵ N=116 HPC* cases, Finns	Positive association
			Nakazato <i>et al.</i> (2003), ⁷⁶ N=101 HPC cases, Japanese	Positive association
			Wang <i>et al.</i> (2002), ⁷⁷ N=438 HPC cases, US Caucasians	Positive association
			Casey <i>et al.</i> (2002), ⁷⁸ N=423 HPC cases, US subjects	Positive association
				Overall, consistent positive association
17p11	<i>ELAC2</i> (HPC2)	A541T, S217L	Rebbeck <i>et al.</i> (2000), ⁷⁹ N = 359 cases, US subjects	Positive association
			Suarez <i>et al.</i> (2001), ⁸⁰ N = 257 HPC cases, US Caucasians	Positive association
			Tavtigian <i>et al.</i> (2001), ⁸¹ N = 429 HPC cases, US Caucasians	Positive association
			Vesprini <i>et al.</i> (2001), ⁸² N = 431 cases, Canadians	No association
			Wang <i>et al.</i> (2001), ⁸³ N = 446 HPC cases, US Caucasians	No association
			Xu <i>et al.</i> (2001), ⁸⁴ N = 249 cases, 159 HPC cases, US Caucasians	No association
			Rokman <i>et al.</i> 2001, ⁸⁵ N = 467 cases, 107 HPC cases, Finns	No association
			Meitz <i>et al.</i> (2002), ⁸⁶ N = 432 cases, UK subjects	No association
			Adler <i>et al.</i> (2003), ⁸⁷ N = 199 cases, Canadians	Positive association

*HPC: Hereditary prostate cancer.

Table 2 (Continued)

Region	Gene	Markers	Studies (Ref.), No. of cases Studied, Population	Results
			Stanford <i>et al.</i> (2003), ⁸⁸ N = 591 cases, US subjects	Positive association
			Takahashi <i>et al.</i> (2003), ⁸⁹ N = 98 cases (BPH controls), Japanese	Positive association
			Severi <i>et al.</i> (2003), ⁹⁰ N = 825 cases, Australians	No association
			<i>Meta-analysis</i> : Camp and Tavitgian (2002) ⁹¹	Association only for HPC Overall, weak, inconsistent associations May be associated only with HPC, not sporadic disease
xq27-28	None (HPCX)		Linkage studies	AR (also on X chromosome) unlikely to be HPCX susceptibility gene
20q13	None (HPC20)		Linkage studies	Linkage studies need further confirmation
1p36	None (CAPB)		Linkage studies	Most consistent linkage to strong family history with early onset disease
1q42.2-43	PCTA-1 (PCAP)		Linkage studies	PCTA is possible candidate gene, but no functional markers
8p22-23	MSR1	PRO3, P275A, D174Y, IVS5-59, R293X	Xu <i>et al.</i> (2003), ⁹² N = 301 cases, US Caucasians Miller <i>et al.</i> , 2003, ⁹³ N = 134 cases, African-Americans	Positive association Positive association

Table 2 (Continued)

Region	Gene	Markers	Studies (Ref.), No. of cases Studied, Population	Results
			Wang <i>et al.</i> (2003), ⁹⁴ N = 499 cases, 438 HPC cases, US Caucasians	Null association
			Seppala <i>et al.</i> (2003), ⁹⁵ N = 537 cases, Finns	Null association
				Overall, weak results, with larger studies showing null associations even for HPC

Rates and Patterns

Incidence

There is considerable variation in reported incidence rates of prostate cancer worldwide.^{5,6} Age-adjusted prostate cancer incidence rates among African-Americans are the highest in the world (185.4 per 100,000 person-years), and rates among Caucasian-Americans are second (107.8 per 100,000 person-years) (Fig. 1). Reported rates in the Caribbean and in Brazil, where there are large populations of African descent (92–96 per 100,000 person-years), are comparable to the high rates among Caucasian-Americans. In contrast, in Central America and other parts of South America, rates are much lower (28–42 per 100,000 person-years). Rates within Europe vary almost seven-fold (from 15–100 per 100,000 person-years), with Austria having the highest reported rates. Although rates in Canada, Oceania (including Australia and New Zealand), Western Europe and Scandinavia (50–103 per 100,000 person-years) are generally not as high as the rates reported in the US, they are 2–3 times higher than rates in Eastern Europe (15–36 per 100,000 person-years). Within Asia, where the rates are the lowest, there is also considerable variation in reported incidence, with more westernized Asian countries such as Israel and the Philippines (22–47 per 100,000 person-years) showing markedly

Table 3. Summary of Epidemiologic Studies of Common, Low Penetrance Genes and Prostate Cancer

Gene	Marker	Studies (Ref.), No. of Cases Studied, Population	Results and Comments
Androgen Biosynthesis/Metabolism Pathway			
<i>CYP17</i>	MspA1	Lunn <i>et al.</i> (1999), ¹²⁰ N = 108 cases, US subjects	Positive association for Caucasians, null for African-Americans
		Wadelius <i>et al.</i> (1999), ¹³⁴ N = 178 cases, Swedish Caucasians	Positive association
		Gsur <i>et al.</i> (2000), ¹³⁵ N = 63 cases, Austrians	Positive association
		Habuchi <i>et al.</i> (2000), ¹³⁶ N = 252 cases, Japanese	Positive association
		Haiman <i>et al.</i> (2001), ¹³⁷ N = 600 cases, US Caucasians	Null association
		Yamada <i>et al.</i> (2001), ¹²⁵ N = 105 cases, Japanese	Positive association
		Kittles <i>et al.</i> (2001), ¹³⁸ N = 71 cases, African-Americans	Positive association
		Latil <i>et al.</i> (2001), ¹¹⁰ N = 226 cases, French Caucasians	Null association
		Chang <i>et al.</i> (2001), ¹³⁹ N = 225 cases, US Caucasians	Null association
		Stanford <i>et al.</i> (2002), ¹⁴⁰ N = 596 cases, US Caucasians and African-Americans	Null association overall, positive association among Caucasians with family history
		Madigan <i>et al.</i> (2003), ¹⁴¹ N = 174 cases, Chinese	Null association
		Lin <i>et al.</i> (2003), ¹⁴² N = 93 cases, Taiwanese	Null association
		Nam <i>et al.</i> (2003), ¹¹⁹ N = 483 cases, Canadians	Null association
		Review: Ntais <i>et al.</i> (2003) ⁹⁸	Meta-analysis indicates no overall association, but A2 allele may be associated with risk in African-Americans. ⁹⁸ A1 is reported to be risk allele in Asians.

Table 3 (Continued)

<i>CYP19</i>	TTTA repeats, N264C	Latil <i>et al.</i> (2001), ¹¹⁰ N = 226 cases, French Caucasians	Positive association
		Modugno <i>et al.</i> (2001), ¹¹¹ N = 88 cases, US Caucasians	Positive association
		Suzuki <i>et al.</i> (2003), ¹⁴³ N = 99 HPC* cases, Japanese	Positive association
		Overall, suggestive but mixed results — longer TTTA alleles associated with higher risk in Caucasians, but lower risk in Asians. Further investigation needed.	
<i>CYP11A1</i>	2455A>G 3801T>C 2453C>A	Murata <i>et al.</i> (1998), ¹⁴⁴ N = 115 cases, Japanese	Positive association
		Suzuki <i>et al.</i> (2003), ¹⁴⁵ N = 81 HPC cases, Japanese	Positive association
		Chang <i>et al.</i> (2003), ¹⁴⁶ N = 245 cases, US Caucasians	Positive association
<i>CYP3A4</i>	5' promoter variant	Rebbeck <i>et al.</i> (1998), ¹⁴⁷ N = 230 cases, US Caucasians	Positive association
		Paris <i>et al.</i> (1999), ¹⁴⁸ N = 174 cases, African-Americans	Positive association
		Nam <i>et al.</i> (2003), ¹¹⁹ N = 483 cases, Canadians	Null association
<i>SRD5A2</i>	V89L, A49T, R227Q, TA repeats	Lunn <i>et al.</i> 1999, ¹²⁰ N = 108 cases, US Caucasians and African-Americans	Null association
		Kantoff <i>et al.</i> (1997), ¹²¹ N = 590 cases, US Caucasians	Null association
		Febbo <i>et al.</i> (1999), ¹²² N = 592 cases, US Caucasians	Null association
		Makridakis <i>et al.</i> (1999), ¹²³ N = 388 cases, US Hispanics and African-Americans	Positive association

*HPC: Hereditary prostate cancer.

Table 3 (Continued)

Gene	Marker	Studies (Ref.), No. of Cases Studied, Population	Results and Comments
		Margiotti <i>et al.</i> (2000), ¹²⁴ N = 108 cases, Italians	Positive association
		Yamada <i>et al.</i> (2001), ¹²⁵ N = 105 cases, Japanese	Null association
		Nam <i>et al.</i> (2001), ¹²⁶ N = 158 cases, Canadians	Positive association
		Latil <i>et al.</i> (2001), ¹¹⁰ 226 cases, French	Null association
		Mononen <i>et al.</i> (2001), ¹²⁷ N = 449 cases, Finns	Null association
		Hsing <i>et al.</i> (2001), ¹²⁸ N = 191 cases, Chinese	Null association
		Pearce <i>et al.</i> (2002), ¹²⁹ N = 921 cases, US subjects	Null association
		Soderstrom <i>et al.</i> (2002), ¹³⁰ N = 176 cases, Swedes	Null association
		Lamharzi <i>et al.</i> (2003), ¹³¹ N = 300 cases, US subjects	Positive association
		Chang <i>et al.</i> (2003), ¹³² N = 245 cases, 159 HPC cases, US Caucasians	Null association
		Li <i>et al.</i> (2003), ¹³³ N = 302 cases, Japanese	Positive association
		Nam <i>et al.</i> (2003), ¹¹⁹ N = 483 cases, Canadians	Null association
		Review: Ntais <i>et al.</i> (2003) ⁹⁶	Overall, the T allele of A49T (associated with higher enzymatic activity) and shorter TA repeats may be associated with a modest increase in risk. ⁹⁶ While results are mixed, the V89L marker's LL genotype, which is associated with lower serum levels of androgens, may be associated with a reduced risk. R227Q is very rare, observed only in Asians.

Table 3 (Continued)

AR	CAG repeats, GGN repeats	Ingles <i>et al.</i> (1997), ¹⁰⁰ N = 57 cases, US Caucasians	Positive association
		Stanford <i>et al.</i> (1997), ¹⁰¹ N = 301 cases, US Caucasians	Positive association
		Giovannucci <i>et al.</i> (1997), ¹⁰² N = 587 cases, US Caucasians (and Platz <i>et al.</i> (1998), ¹⁰³ N = 582 cases)	Positive association
		Correa-Cerro <i>et al.</i> (1999), ¹⁰⁴ N = 132 cases, French and Germans	Null association
		Ekman <i>et al.</i> (1999), ¹⁰⁵ N = 93 cases, 59 HPC cases, Swedes and Japanese	Positive association
		Edwards <i>et al.</i> (1999), ¹⁰⁶ N = 178 cases, U.K. Caucasians	Positive association
		Hsing <i>et al.</i> (2000), ¹⁰⁷ N = 190 cases, Chinese	Positive association
		Miller <i>et al.</i> (2001), ¹⁰⁸ N = 140 cases, US subjects	Null association
		Beilin <i>et al.</i> (2001), ¹⁰⁹ N = 445 cases, Australians	Null association
		Latil <i>et al.</i> (2001), ¹¹⁰ N = 226 cases, French	Null association
		Modugno <i>et al.</i> (2001), ¹¹¹ N = 88 cases, US Caucasians	Positive association
		Chang <i>et al.</i> (2002), ¹¹² N = 245 cases, 159 HPC cases	Positive association
		Mononen <i>et al.</i> (2002), ¹¹³ N = 449 cases, Finns	Positive association
		Gsur <i>et al.</i> (2002), ¹¹⁴ N = 190 cases, Austrians	Null association
		Chen <i>et al.</i> (2002), ¹¹⁵ N = 300 cases, US subjects	Null association
		Balic <i>et al.</i> (2002), ¹¹⁶ N = 82 cases, Hispanics	Positive association
		Santos <i>et al.</i> (2003), ¹¹⁷ N = 133 cases, Brazilians	Null association
		Huang <i>et al.</i> (2003), ¹¹⁸ N = 66 cases, Taiwanese	Null association
		Nam <i>et al.</i> (2003), ¹¹⁹ N = 483 cases, Canadians	Null association

Table 3 (Continued)

Gene	Marker	Studies (Ref.), No. of Cases Studied, Population	Results and Comments
<i>HSD3B1</i>	N367T, c7062t	Chang <i>et al.</i> (2002), ¹⁴⁹ N = 245 cases, 159 HPC cases, US Caucasians	Positive association
<i>HSD3B2</i>	c7159g, c7474t	Chang <i>et al.</i> (2002), ¹⁴⁹ N = 245 cases, 159 HPC cases, US Caucasians	Null association
<i>HSD17B3</i>	G289S	Margiotti <i>et al.</i> (2002), ¹⁵⁰ N = 103 cases, Italians	Positive association
Growth Factors and Non-androgenic Hormone Pathways			
<i>VDR</i>	BsmI, TaqI, polyA, ApaI, FokI	Taylor <i>et al.</i> (1996), ¹⁵⁴ N = 108 cases, US Caucasians Ingles <i>et al.</i> (1997), ¹⁰⁰ N = 57 cases, US Caucasians Ingles <i>et al.</i> (1998), ¹⁵⁵ N = 151 cases, African-Americans Ma <i>et al.</i> (1998), ¹⁵⁶ N = 372 cases, US Caucasians Correa-Cerro <i>et al.</i> (1999), ¹⁵⁷ N = 131 cases, Europeans Habuchi <i>et al.</i> (2000), ¹⁵⁸ N = 222 cases, Japanese Furuya <i>et al.</i> (1999), ¹⁵⁹ N = 66 cases, Japanese Watanabe <i>et al.</i> (1999), ¹⁶⁰ N = 100 cases, Japanese Blazer <i>et al.</i> (2000), ¹⁶¹ N = 77 cases, US Caucasians Chokkalingam <i>et al.</i> (2001), ¹⁶² N = 191 cases, Chinese Gsur <i>et al.</i> (2002), ¹⁶³ N = 190 cases, Austrians Hamasaki <i>et al.</i> (2002), ¹⁶⁴ N = 110 cases, Japanese Medieros <i>et al.</i> (2002), ¹⁶⁵ N = 163 cases, Portugese Suzuki <i>et al.</i> (2003), ¹⁶⁶ N = 81 HPC cases, Japanese Nam <i>et al.</i> (2003), ¹¹⁹ N = 483 cases, Canadians	Positive association Positive association Null association Null association Null association Positive association Null association Null association Null association Null association Null association Null association Null association Null association Null association Positive association for aggressive disease Positive association for late-onset disease Null association Null association

Table 3 (Continued)

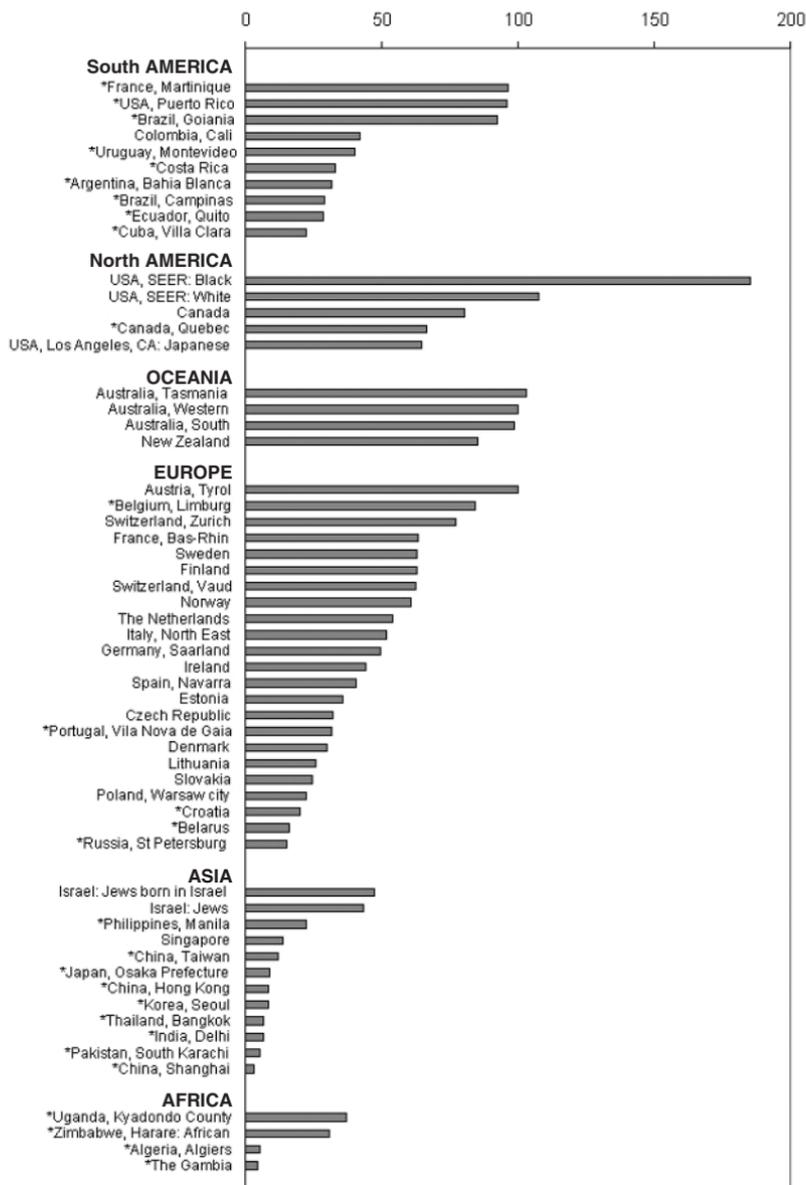
		<i>Review: Ntais et al. (2003)</i> ⁹⁷	Overall, meta-analysis ⁹⁷ shows null association for all markers. 3' markers (BsmI, Taq1, ApaI and polyA) are non-functional, 5' FokI marker is functional.
<i>INS</i>	+1127PstI	Ho <i>et al.</i> (2003), ¹⁵³ N = 126 cases, US subjects	Positive association
<i>TH</i>	-4217PstI	Ho <i>et al.</i> (2003), ¹⁵³ N = 126 cases, US subjects	Null association
<i>IGF-1</i>	CA repeats	Nam <i>et al.</i> (2003), ¹¹⁹ N = 483 cases, Canadians	Positive association
<i>IGF-2</i>	MspI	Ho <i>et al.</i> (2003), ¹⁵³ N = 126, US subjects	Null association
<i>IGFBP-3</i>	-202A/C	Wang <i>et al.</i> (2003), ⁹⁴ N = 307 cases, Japanese	Null association
		Nam <i>et al.</i> (2003), ¹¹⁹ N = 483 cases, Canadians	Null association
Carcinogen Metabolism Pathway			
<i>GSTT1</i>	Deletion	Medeiros <i>et al.</i> (2004), ¹⁶⁷ N = 150 cases, Portugese	Null association
		Nakazato <i>et al.</i> (2003), ⁷⁶ N = 81 cases, Japanese	Null association
		Kidd <i>et al.</i> (2003), ¹⁶⁸ N = 206 cases, Finns	Null association
		Kote-Jarai <i>et al.</i> (2001), ¹⁶⁹ N = 275 cases, U.K. Caucasians	Null association
		Gsur <i>et al.</i> (2001), ¹⁷⁰ N = 166 cases, Austrians	Null association
		Murata <i>et al.</i> (2001), ¹⁷¹ N = 115 cases, Japanese	Null association
		Steinhoff <i>et al.</i> (2000), ¹⁷² N = 91 cases, Germans	Positive association
		Astrup <i>et al.</i> (1999), ¹⁷³ N = 153 cases, Dutch subjects	Null association
		Nam <i>et al.</i> (2003), ¹¹⁹ N = 483 cases, Canadians	Positive association
		Kelada <i>et al.</i> (2000), ¹⁷⁴ N = 276 cases, US subjects	Positive association
<i>GSTM1</i>	Deletion	Medeiros <i>et al.</i> (2004), ¹⁶⁷ N = 150 cases, Portugese	Null association
		Nakazato <i>et al.</i> (2003), ⁷⁶ N = 81 cases, Japanese	Null association
		Kidd <i>et al.</i> (2003), ¹⁶⁸ N = 206 cases, Finns	Positive association
		Kote-Jarai <i>et al.</i> (2001), ¹⁶⁹ N = 275 cases, U.K. Caucasians	Null association

Table 3 (Continued)

Gene	Marker	Studies (Ref.), No. of Cases Studied, Population	Results and Comments
<i>GSTM3</i> <i>GSTP1</i>	I105V	Gsur <i>et al.</i> (2001), ¹⁷⁰ N = 166 cases, Austrians	Null association
		Murata <i>et al.</i> (2001), ¹⁷¹ N = 115 cases, Japanese	Positive association
		Steinhoff <i>et al.</i> (2000), ¹⁷² N = 91 cases, Germans	Null association
		Astrup <i>et al.</i> , 1999, ¹⁷³ N = 153 cases, Dutch subjects	Null association
		Nam <i>et al.</i> (2003), ¹¹⁹ N = 483 cases, Canadians	Null association
		Kelada <i>et al.</i> (2000), ¹⁷⁴ N = 276 cases, US subjects	Null association
		Medeiros <i>et al.</i> (2004), ¹⁶⁷ N = 150 cases, Portugese	Positive association
		Nakazato <i>et al.</i> (2003), ⁷⁶ N = 81 cases, Japanese	Positive association
		Kidd <i>et al.</i> (2003), ¹⁶⁸ N = 206 cases, Finns	Null association
		Kote-Jarai <i>et al.</i> (2001), ¹⁶⁹ N = 275 cases, U.K. Caucasians	Positive association
		Gsur <i>et al.</i> (2001), ¹⁷⁰ N = 166 cases, Austrians	Positive association
		Steinhoff <i>et al.</i> (2000), ¹⁷² N = 91 cases, Germans	Null association
		Shepard <i>et al.</i> (2000), ¹⁷⁵ N = 590 cases, US Caucasians	Null association
		Astrup <i>et al.</i> (1999), ¹⁷³ N = 153 cases, Dutch	Null association
<i>NAT2</i>		Wadelius <i>et al.</i> (1999), ¹³⁴ N = 850 subjects, Swedes and Danes	Null association
		Nam <i>et al.</i> (2003), ¹¹⁹ N = 483 cases, Canadians	Null association
		Wadelius <i>et al.</i> (1999), ¹³⁴ N = 850 subjects, Swedes and Danes	Null association
DNA Repair Pathway			
<i>XRCC1</i>	R399Q, R194W, R280H	Rybicki <i>et al.</i> (2004), ¹⁷⁷ N = 637 cases, US Caucasians	Null association
		van Gils <i>et al.</i> (2002), ¹⁷⁸ N = 77 cases, US subjects	Positive association

Table 3 (Continued)

<i>XPD</i>	D312N, K751Q	Rybicki <i>et al.</i> (2004), ¹⁷⁷ N = 637 cases, US Caucasians	Positive association, needs further investigation
<i>hOGG1</i>	S326C, +11657A/G	Xu <i>et al.</i> (2002), ¹⁷⁹ N = 245 cases, US Caucasians Chen <i>et al.</i> (2003), ¹⁸⁰ N = 84 cases, US Caucasians	Positive association Positive association
Inflammation/Angiogenesis/Cytokine Pathways			
<i>VEGF</i>	VEGF-1154, VEGF-460	McCarron <i>et al.</i> (2002), ¹⁸³ N = 247 cases, U.K. Caucasians Lin <i>et al.</i> (2003), ¹⁴² N = 96 cases, Taiwanese	Positive association Positive association
<i>TNF-α</i>	TNF- α -308	McCarron <i>et al.</i> (2002), ¹⁸³ N = 247 cases, U.K. Caucasians	Null association
<i>IL-1-β</i>	IL-1 β -511	McCarron <i>et al.</i> (2002), ¹⁸³ N = 247 cases, U.K. Caucasians	Null association
<i>IL-8</i>	IL-8-251	McCarron <i>et al.</i> (2002), ¹⁸³ N = 247 cases, U.K. Caucasians	Positive association
<i>IL-10</i>	IL-10-1082	McCarron <i>et al.</i> (2002), ¹⁸³ N = 247 cases, U.K. Caucasians	Positive association
<i>PPAR-γ</i>	P12A	Paltoo <i>et al.</i> (2003), ¹⁸⁴ N = 193 cases, Finns	Null association
<i>TGF-β</i>	L10P	Li <i>et al.</i> (2004), ¹⁸¹ N = 351 cases, Japanese	Positive association
<i>COX-2</i>	-1285A/G -1265G/A -899G/C -297C/G	Panguluri <i>et al.</i> (2004), ¹⁸² N = 288, 264 and 184 cases, African-Americans, Nigerians, and US Caucasians	Positive association in all ethnic groups



Source: Parkin DM, Whelan SL, Ferlay J, Teppo L, and Thomas DB. Cancer Incidence in Five Continents, Vol VIII, IARC Sci Publ 155, 2003.

Fig. 1. Age-adjusted incidence rates (per 100,000 person-years) for prostate cancer in 48 countries, 1993–1997.

higher rates than Thailand, India, Pakistan and Shanghai, China (3–7 per 100,000 person-years). There are few data on incidence in Africa, with only four registries included in the IARC report.⁶ The rates within the African continent vary widely, from 5–37 per 100,000 person-years. Part of the difference in incidence rates in various countries is related to the extent of prostate cancer screening, especially the use of prostate-specific antigen (PSA) testing. However, since screening is less common in developing countries, it is not likely to explain the nearly 60-fold difference in prostate cancer risk between high- and low-risk populations.

Mortality

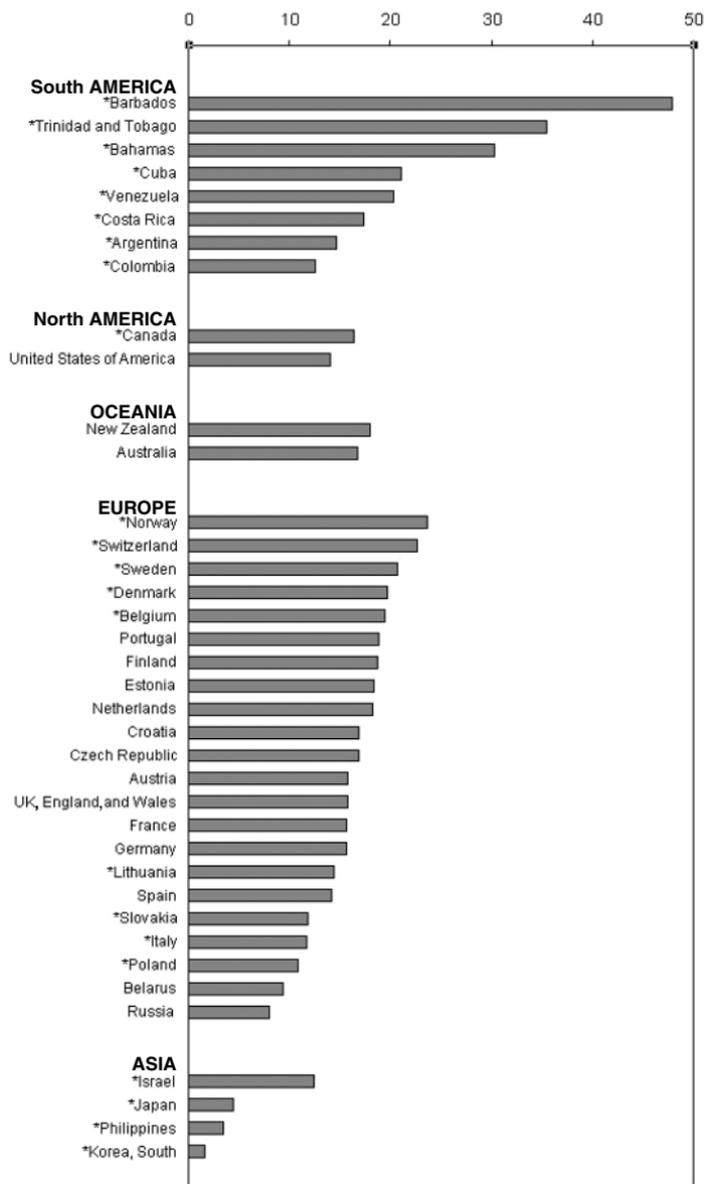
In the US, only one in six men diagnosed with prostate cancer will eventually die from it. Nevertheless, 29,900 prostate cancer deaths are expected in 2004, making prostate cancer the second leading cause of cancer death among US men after lung cancer.⁷ Age-adjusted prostate cancer mortality rates from 38 countries in 1998 are shown in Fig. 2. Overall, mortality patterns mimic those of incidence in various countries, although mortality rates show less diversity worldwide than do incidence rates, but are still higher in Western nations than in lower-risk, Asian countries (Fig. 2). Of special interest is the observation that the Caribbean nations of Barbados, the Bahamas and Trinidad and Tobago, where there are large populations of men of African descent, had the world's highest mortality rates (30.3 to 47.9 per 100,000 person-years). Mortality was higher in Scandinavian countries and parts of northern Europe than in the US (18.7–23.6 versus 14.0 per 100,000 person-years), and lowest of all in the Asian countries of South Korea, Philippines and Japan (1.6–4.4 per 100,000 person-years).

Risk Factors

Demographic Factors

Age

Over 80% of prostate tumors in the US are diagnosed among men over age 65,⁸ and the incidence of prostate cancer increases exponentially



Source: <http://www-depdb.iarc.fr/who/menu.htm>

* Rates are from 1994

Fig. 2. Age-adjusted mortality rates (per 100,000 person-years) for prostate cancer in 38 countries, 1998.

with advancing age — an increase that is faster than that for any other malignancy (Table 1). Estimates from the Surveillance, Epidemiology, and End Results (SEER) program from 1996–2000 indicate that for US men under 65 years of age and 65 years and over, age-adjusted prostate cancer incidence rates were 56.8 and 974.7 per 100,000 person-years, respectively.²

Racial/Ethnic Variation

Another consistently observed but poorly understood risk factor is ethnicity. African-Americans have the highest incidence rate in the world, roughly 60 times that of the ethnic group with the world's lowest rates, in Shanghai, China¹ (Fig. 1).

Adjustment of incidence rates for prevalence of latent disease at autopsy and proportion of localized tumors among all cancers of the prostate revealed that Japanese men still experience a markedly lower incidence than Americans, indicating that the international variation cannot be explained by differences in detection alone.⁹ This bolsters the results of migrant studies suggesting that ethnic factors, including genetic, lifestyle, and environmental factors, may affect prostate cancer risk and explain many of the differences in risk between high- and low-risk populations.^{9,10}

Hormonal, Behavioral and Lifestyle Factors

Hormones and Growth Factors

Androgens play a key role in the development and maintenance of the prostate gland; however, the precise role of androgens in the etiology of prostate cancer is unclear. Prostate cancer is notably absent in castrated men, and laboratory studies show that administration of testosterone induces prostate cancer in rats and that androgens promote cell proliferation and inhibit prostate cell death.^{11–13} However, epidemiologic data supporting a role of androgens are inconclusive.^{14–16} To date, over 13 prospective studies have investigated the role of circulating androgens, and only one was able to show that men with higher serum testosterone

levels have a higher risk of prostate cancer.¹⁷ More comprehensive reviews of this topic are reported elsewhere.^{14–16} Studies of genetic markers involved in the androgen pathways offer further insight into this avenue of research, and are reviewed later in this chapter.

In addition to androgens, insulin-like growth factors (IGFs), insulin and vitamin D have been implicated in prostate cancer. IGF-I and IGF-II are polypeptides that function as both tissue growth factors and endocrine hormones with mitogenic and anti-apoptotic effects on prostate epithelial cells. There are at least six known IGF binding proteins (IGFBPs) that can bind to IGFs and thus prevent activation of the IGF receptor, which mediates IGF effects. At least nine epidemiologic studies have evaluated the roles of the IGF axis in prostate cancer, and most have reported a positive association with IGF-I and an inverse association with IGFBP3.^{18,19} However, the role of IGF-II is less clear.

Vitamin D is a steroid hormone obtained primarily from dermal synthesis in response to sunlight exposure. Vitamin D and its analogs have potent anti-proliferative, pro-differentiative, and pro-apoptotic effects on prostate cancer cells. In addition, vitamin D inhibits prostate tumor growth *in vivo*. In general, laboratory data are consistent and support the hypothesis that vitamin D may protect against prostate cancer. However, results from epidemiologic studies investigating serum vitamin D levels have been inconsistent.²⁰ The reasons for these conflicting results are unclear.

Diet

Ecologic studies have shown a strong correlation between the incidence of prostate cancer and dietary fat intake.²¹ A western diet has been linked to a higher risk of prostate cancer, and it has been suggested that the western diet, high in fat, increases production and availability of both androgen and estrogen, while Asian (low-fat, high-fiber) and vegetarian diets lead to lower circulating levels of these hormones.²¹

Fat is the most studied dietary factor in relation to prostate cancer. Most epidemiologic studies have investigated the role of total, saturated, and/or animal fat. Findings from these studies suggest a possible positive association with monounsaturated, animal and saturated fats, and an inverse association with omega-3 fat. The results for polyunsaturated fat are

less consistent.^{22,23} Consumption of meat, particularly red meat, is also consistently linked to an increased risk of prostate cancer. However, it is unclear whether the excess risk is due to the fat content in red meat, mutagens such as heterocyclic amines that are induced during high-temperature cooking of meat products, animal proteins, or other unidentified factors.²⁴

Several epidemiologic studies have also investigated whether intake of fatty fish, rich in potentially tumor-inhibitory marine fatty acids, is associated with reduced prostate cancer risk. However, a recent review of 17 studies, including eight prospective studies, found suggestive but inconsistent results, possibly due to inadequate assessment of fish intake or lack of information on specific marine fatty acids, particularly the polyunsaturated fatty acids eicosapentaenoic and docosahexaenoic acids,²⁵ in these studies.

Although consumption of fruits and vegetables is associated with a reduced risk of several cancers, their role in prostate cancer is less clear. The only consistent finding is an inverse association with consumption of tomatoes and tomato paste, which has been largely attributed to the antioxidant effect of lycopene.²⁶ Cruciferous and allium vegetables have been implicated. A recent review concluded that there is modest evidence that intake of cruciferous vegetables, including broccoli, cabbage, cauliflower, and Brussels sprouts, is inversely associated with prostate cancer risk, possibly due to their content of isothiocyanates.²⁷ Intake of allium vegetables, including onions, garlic, and chives, was associated with a reduced risk in a case-control study in China.²⁸ This protective effect may be due to the tumor inhibitory properties of organosulfur compounds.

Dietary calcium, from either dairy intake or supplements, has also been linked to prostate cancer. Because of its role in regulation of vitamin D synthesis, calcium may down-regulate vitamin D's anti-proliferative effects on prostate cancer. However, the epidemiologic evidence for calcium is as yet unclear, complicated by differences in assessment of calcium (dietary intake versus circulating levels).²⁹ Recent data suggest a threshold effect in that only very high calcium intake (≥ 2000 mg/day) appears to be associated with disease.³⁰

Chronic excess of zinc, another mineral obtained largely through dietary supplements, may be positively associated with prostate cancer risk, although *in vitro* studies demonstrating mitogenic effects of zinc on prostate cancer suggest that it may reduce risk.³¹

A large body of epidemiological evidence, including observational, case-control, cohort and randomized controlled clinical trials, supports the hypothesis that selenium may prevent prostate cancer in humans.³² Molecular data show that selenium prevents clonal expansion of tumors by causing cell cycle arrest, promoting apoptosis, and modulating p53-dependent DNA repair mechanisms. Clinical trials have also shown that vitamin E supplementation is associated with a reduced risk of prostate cancer.^{33,34} Currently a clinical trial is under way to test the chemopreventive efficacy of these two compounds.³⁵

Obesity

In epidemiologic studies, overall obesity is usually measured by body mass index (weight in kg divided by the square of height in meters, kg/m²) and abdominal obesity by the ratio of waist to hip circumference. The findings on overall obesity are mixed. However, recent data suggest that abdominal obesity may be associated with an increased risk of prostate cancer even in relatively lean men.^{36,37} In addition, higher serum levels of insulin were associated with an increased risk of prostate cancer in China,³⁸ and higher serum levels of leptin were associated with larger tumor volume (> 5 cm³).³⁹ Although the role of obesity in prostate cancer is not clearly defined, future studies should attempt to clarify it further because obesity is linked to numerous putative risk factors for prostate cancer, including high intakes of meat and fat intake, hormone metabolism, and serum level IGFs and insulin. Furthermore, the prevalence of obesity correlates with prostate cancer risk across populations. It is likely that obesity may thus provide a link between westernization and increased prostate cancer risk. With the epidemic of obesity in both developed and developing countries, the role of obesity needs to be clarified further.

Physical Activity

Physical activity may decrease levels of total and free testosterone, reduce obesity, and enhance immune function,⁴⁰ all of which may lead to protection from prostate cancer. However, perhaps due to challenges in classifying physical activity and/or identifying the age/time period at which

activity may be most protective, results from numerous epidemiologic studies are equivocal.^{40,41}

Occupation

Occupation is highly correlated with socioeconomic status and lifestyle factors. There is a large body of literature on prostate cancer and occupation, and one consistent result from these studies is that farmers and other agricultural workers have a 7–12% increased risk.^{42,43} While this excess could reflect lifestyle factors such as increased intake of meat and fats, chemical exposures may also play a role. These chemicals, which have a wide variety of poorly characterized effects, may include fertilizers, solvents, pesticides and herbicides.⁴⁴ Organochlorines present in many pesticides and herbicides can affect circulating hormone levels; however the epidemiologic evidence linking specific pesticide or herbicide exposures to prostate cancer is weak. In addition to agriculture, workers in the heavy metals industry, rubber manufacturing, and newspaper printing may be at elevated risk,⁴² suggesting that exposure to certain chemicals common in these work environments may increase the risk of prostate cancer.

Vasectomy

Several, but not all, studies investigating the association between vasectomy and prostate cancer risk suggest a modest positive association. The role of vasectomy remains controversial, however, since most studies are unable to exclude the possible effect of detection bias: men undergoing vasectomies are more likely to have prostate cancer detected than men who do not. Vasectomy is linked to elevations in anti-spermatozoa antibodies, decreased seminal hormone concentrations and decreased prostatic secretion.⁴⁵ Whether these conditions can influence prostate carcinogenesis needs to be clarified.

Chronic Inflammation

Evidence for chronic inflammation and prostate cancer is just emerging,⁴⁶ but an association of prostate cancer with chronic inflammation of the prostate (chronic prostatitis) has long been suspected. Inflammation is

frequently found in prostate biopsy specimens obtained from both radical prostatectomy and surgical treatment for BPH,^{47,48} however, epidemiologic findings have been mixed. A recent meta-analysis of 11 studies of prostatitis and prostate cancer reported an overall relative risk of 1.6.⁴⁹

Results from pathologic and molecular surveys suggest that the earliest stages of prostate cancer may develop in lesions generally associated with chronic inflammation.^{50,51} De Marzo *et al.* showed that almost all forms of focal prostatic glandular atrophy, thought to be precursors of prostatic adenocarcinoma, are proliferative, and that such proliferative inflammatory atrophy (PIA) lesions often contain inflammatory infiltrates and are frequently found adjacent to or near high-grade prostatic intraepithelial neoplasia (PIN).^{50,51} Inflammation may lead to tumorigenesis by stimulating angiogenesis, enhancing cell proliferation, and damaging DNA through radical oxygen species such as nitric oxide.

Additional support for a role for chronic inflammation in prostate cancer comes from the observation that a higher intake of fish and use of aspirin and other non-steroidal anti-inflammation drugs (NSAIDs) has been associated with reduced prostate cancer risk.⁵² In two large prospective studies, higher intake of fish was associated with a lower risk of total prostate cancer and metastatic prostate cancer.^{53,54} Abundant in fatty fish, omega-3 fatty acids are known antagonists of arachidonic acid and suppress the production of pro-inflammatory cytokines.⁵⁵ In addition, use of anti-inflammatory agents, especially NSAIDs such as ibuprofen or aspirin, has been related to lower prostate cancer risk in epidemiologic studies,⁵⁶⁻⁵⁸ and a recent meta-analysis of 12 of these studies concluded that aspirin use was associated with a 15% reduction in prostate cancer risk.⁵⁹ Taken together, these data suggest chronic inflammation may increase the risk of prostate cancer. However, there are few epidemiologic studies investigating this directly, possibly due to the difficulty in diagnosing chronic prostatitis and in measuring cytokine levels reliably in serum samples. This is likely to be a fruitful area for future research.

Sexually Transmitted Diseases

Chronic inflammation induced by bacterial or viral agents has been implicated as a potential underlying mechanism for the link between STDs and

prostate cancer. One recent large, population-based study showed two- to three-fold increased prostate cancer risks associated with STDs, particularly syphilis and recurrent gonorrhea infections.⁶⁰ Other studies reported associations of human papillomavirus-16, -18 and -33 serology with an increased risk of prostate cancer.^{61,62} In addition, epidemiological data are accumulating to suggest that sexual history may be associated with prostate cancer risk,⁶³ and a recent meta-analysis of 17 studies concluded that increased sexual frequency and number of partners are associated with increased prostate cancer risk.⁴⁹

Benign Prostatic Hyperplasia

The relationship between BPH and prostate cancer is not well established. BPH is currently not considered a precursor to prostate cancer, since prostate cancer occurs mostly in the peripheral zone of the prostate and BPH is more common in the transition and periurethral zones. However, because both conditions are common in elderly men, and because they may coexist within the prostate, they appear to share risk profiles, making it difficult to elucidate the independent role, if any, of BPH in prostate cancer etiology. Detection bias also complicates investigation: excess prostate cancer risk in men who are symptomatic for BPH may be simply a reflection of the increased intensity of evaluation and medical surveillance in such patients. In addition, in most epidemiologic studies, it has been difficult to completely rule out the presence of BPH in control populations, since the prevalence of BPH is very common in elderly men. Due in part to these limitations, the epidemiologic evidence for BPH as a risk factor for prostate cancer remains weak and inconsistent,⁶⁴ with the largest study to date (over 85,000 BPH patients) showing only a marginally elevated risk of prostate cancer versus the general population (<2% in 10 years).⁶⁵

Other Factors

Several other risk factors, such as smoking, use of alcohol, diabetes and liver cirrhosis, have been investigated, but their roles in prostate cancer are weak or unclear based on data in the current literature.⁶⁶⁻⁶⁸

Genetic Factors

Family History of Cancer

Prostate cancer etiology has a hereditary component. Numerous studies have consistently reported familial aggregation of prostate cancer, showing a two- to three-fold increased risk of prostate cancer among men who have a first-degree male relative (father, brother, son) with a history of prostate cancer.⁶⁹ Recent data from a large twin study suggests that as much as 42% (95% CI 29–50%) of the risk of prostate cancer may be accounted for by genetic factors.⁷⁰ Genetic factors involved in prostate cancer include individual and combined effects of rare, highly penetrant genes, more common weakly penetrant genes and genes acting in concert with each other.

High-Penetrance Markers

Segregation and linkage analyses have shown that certain early-onset prostate cancers may be inherited in an autosomal dominant fashion,⁷¹ and it is estimated that such hereditary prostate cancers (HPCs) due to highly penetrant genes may account for about 10% of all prostate cancer cases.⁷⁰ Several family studies are currently underway to identify hereditary prostate cancer candidate genes. However, these investigations have proven to be difficult for several reasons.⁷² One is that, due to the high incidence of prostate cancer and the heterogeneity of tumors, it is possible that sporadic cases are included in HPC families, thereby reducing the statistical power to detect genes for HPC. In addition, because prostate cancer is generally diagnosed at a late age, it is often impossible to obtain DNA specimens from fathers of HPC cases, and sons of HPC cases are often too young to have developed prostate cancer. Therefore, studies of HPC families are often unable to include more than one generation. Finally, the genetic heterogeneity of prostate cancer makes it difficult to devise appropriate statistical transmission models that also account for multiple susceptibility genes, many of which may be at only moderate penetrance. Despite these challenges, seven loci have been described to date, including *HPC1*, *ELAC2*, *HPCX*, *HPC20*, *CAPB*, *PCAP*, and an unnamed locus at 8p22-23 (Table 2), and fine mapping has led to the identification of a

number of candidate genes, including *RNASEL*, *ELAC2* and *MSR-1*.^{73,74} The results of studies of these loci,⁷⁵⁻⁹⁵ which have been extensively reviewed elsewhere,⁷³ have largely been mixed, with subsequent studies failing to replicate promising earlier findings. The absence of strong, consistent results for high penetrance markers strongly suggests that the heritable component of prostate cancer largely comprises effects of multiple factors, including common, weakly penetrant markers, possibly interacting with one another and with environmental factors.

Common Low-Penetrance Markers

Results of epidemiologic studies of common polymorphisms are summarized below and in Table 3 by biological pathway; several of these markers have been reviewed elsewhere.^{73,96-99} In reviewing these results, it is important to note that, as with any other epidemiologic exposure, replication of findings is critical to establishing causality. This is particularly true of genetic association studies, because the recent explosion of genetic data has increased the potential for publication bias as investigators and publishers become more selective about writing up and publishing findings.

Androgen Biosynthesis and Metabolism Pathway

Because prostate cancer is an androgen-dependent tumor, it is likely that markers in genes whose gene products are involved in androgen biosynthesis and metabolism (Fig. 3) may be associated with disease. Recent epidemiologic studies have investigated the role of polymorphisms of over 10 genes involved in androgen biosynthesis, metabolism, transport, and regulation. These data are promising and accumulating at a remarkable pace but still are too sparse to support a role for any particular gene.

Results for the androgen receptor (*AR*), which is involved in androgen binding and transport, are fairly consistent, showing that shorter CAG repeat lengths are associated with increased risk in most, but not all, populations.¹⁰⁰⁻¹¹⁹ For the type II steroid 5 α -reductase (*SRD5A2*), which converts testosterone to the more active androgen dihydrotestosterone, the results are mixed,^{110,119-133} with a recent meta-analysis showing modest

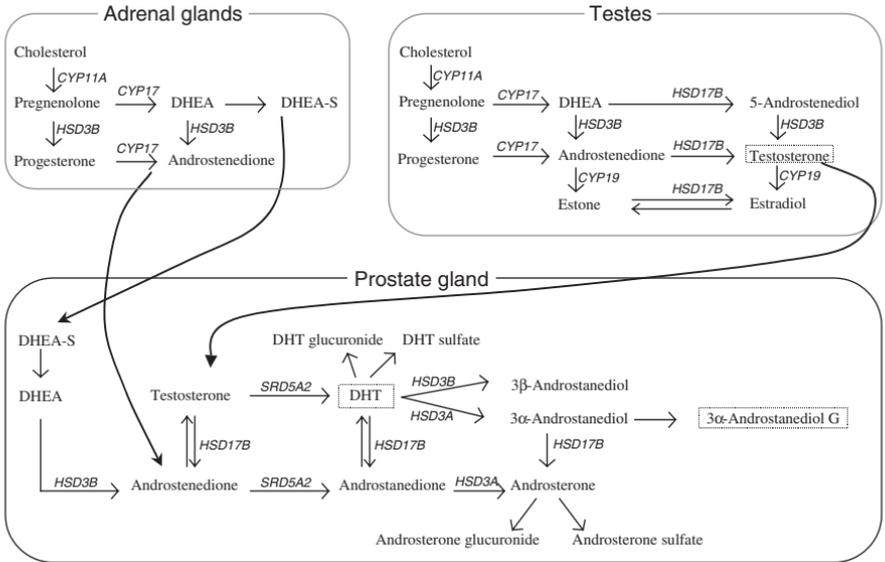


Fig. 3. Androgen biosynthesis and metabolism pathway.

risk increases associated with shorter TA repeats and the T allele of the A49T marker, but not for other studied markers.⁹⁶ Markers in several other genes, including cytochrome p450-17 (*CYP17*), cytochrome p450-19 aromatase (*CYP19*), cytochrome p450-1A1 (*CYP1A1*) and cytochrome p450-3A4 (*CYP3A4*) have shown promising initial results that often cannot be replicated.^{110,111,119,120,125,134-148} Furthermore, recent initial studies of 17β-hydroxysteroid dehydrogenase 3 (*HSD17B3*) and 3β-hydroxysteroid dehydrogenase 1 (*HSD3B1*) have shown promising results,^{149,150} but further study is needed to elucidate the role these may play in prostate cancer.

The totality of current data suggests that racial/ethnic variation exists in polymorphisms of genes involved in the androgen pathways.^{151,152} However, their role in prostate cancer needs to be clarified further.

Growth Factor and Non-Androgenic Hormone Pathways

Due to serological evidence linking them to prostate cancer, a number of studies have explored the prostate cancer risk associated with polymorphic markers in genes involved in the insulin and insulin-like growth factor (IGF) signaling pathway. However, while the only study of the insulin gene (*INS*)

has shown promising results, early studies of markers in the IGF-II and IGF binding protein-3 (IGFBP-3) genes have shown null results.^{94,119,153}

Strong laboratory evidence showing chemoprotection of vitamin D against prostate cancer, in addition to suggestive but inconsistent sero-epidemiological studies, has led to numerous studies of the vitamin D receptor gene (*VDR*).^{100,119,154–166} However, despite promising early studies, a recent comprehensive meta-analysis showed no overall associations and concluded that markers in the *VDR* gene are unlikely to be major genetic determinants of prostate cancer risk.⁹⁷

Carcinogen Metabolism Pathway

Genes encoding enzymes that metabolize carcinogens and other toxins may play a role in prostate cancer. However, results from several studies of markers in different glutathione-S-transferases (GSTs), including *GSTT1*, *GSTP1* and *GSTM1*, have mostly been null.^{76,119,134,167–175} Recent initial epidemiologic studies of other genes in these pathways, including *GSTM3* and *N*-acetyl transferase 2 (*NAT2*), have been positive but require confirmation.^{134,167}

DNA Repair Pathway

The DNA repair pathway serves to prevent disruptions in DNA integrity that might otherwise lead to gene rearrangements, translocations, amplifications and deletions that may contribute to cancer development.¹⁷⁶ Initial reports of markers in genes encoding DNA repair enzymes, including the X-ray repair cross-complementing group (*XRCC1*), human 8-oxoguanine glycosylase I (*hOGGI*) and the xeroderma pigmentosum group D (*XPB*), show promising results.^{177–180} These results, combined with strong biological plausibility, suggest that this may be a fruitful area for further research.

Chronic Inflammation Pathway

Several lines of evidence point to a role of inflammation in prostate cancer etiology, and studies of markers in the genes involved in inflammation are emerging.⁴⁶ Initial studies show positive results for transforming

growth factor- β (*TGF- β*) and COX-2^{181,182} and negative results for tumor necrosis factor- α -308 (*TNF- α -308*), interleukin-1 β (*IL-1 β*) and peroxisome proliferator-activated receptor- γ (*PPAR- γ*).^{183,184} Evidence for a role of inflammation markers in prostate cancer is increasing. Given the biological plausibility of this hypothesis, this should be a fruitful area for future research.

Angiogenesis Pathways

The need for increased vasculature to support cancer growth is an area of research that is currently gaining momentum. Genetic investigations of angiogenesis in prostate cancer have thus far involved the vascular endothelial growth factor (*VEGF*) gene as well as the genes for *IL-8* and *IL-10*, and the handful of studies conducted to date have shown positive results.^{142,183} These findings await further confirmation and support the notion that angiogenesis may indeed be involved in prostate cancer.

Biological Pathways Related to Dietary Factors

It is clear that genetic susceptibility to both Phase I and II enzymes (cytochrome p450) affects the association between certain dietary factors and prostate cancer risk. For example, the effect of cruciferous vegetables is related to both their high glycosinolate content and functional variations in enzymes, particularly *GSTM1* and *GSTT1*, that metabolize glycosinolates to isothiocyanates (ITCs).²⁷ Thus, to better assess the role of ITCs in prostate cancer, studies with both comprehensive and reliable assessment of cruciferous vegetable intake and genetic polymorphisms in *GSTM1* and *GSTT1* will be required. Moreover, genetic polymorphisms in receptors and transcription factors that interact with these compounds may contribute to variations in response to cruciferous vegetable intake. With sufficiently large sample size and careful assessment of diet and genetic factors, this important area should be investigated further.

Challenges of Studies with Common Polymorphisms

Currently, the totality of data suggests that racial/ethnic variation exists in common polymorphisms of certain genes, such as the *SRD5A2*, *AR*, and

ELAC2/HPC2, but few variants or genes have been firmly shown to contribute to prostate cancer susceptibility. Challenges in molecular epidemiology studies of common polymorphisms include the selection of relevant single nucleotide polymorphisms (SNPs) for genotyping and the difficulty in replicating results. The difficulty in replicating earlier findings in subsequent association studies is due, in part, to (1) the relatively small to modest effects of most common polymorphisms, ranging from 10 to 80%, (2) the relatively small sample size in most previous studies, ranging from 100 to 500 cases, and the limited power of these studies to detect a modest effect on the order of 10 to 50%, (3) the tendency of small studies to produce false positive findings, and (4) differences in study design and populations, including differences in the severity of cases. Thus, studies with large sample size (>1000 cases) are needed to clarify further the role of these polymorphic markers. In addition, it is becoming clear that a single gene or SNP alone is unlikely to explain most of the variation in prostate cancer susceptibility, thereby requiring even larger sample sizes (>3000 cases) to evaluate the effect of multiple variants.

Another challenge in epidemiologic studies investigating the role of genetic variants in complex disease (e.g., prostate cancer) is the limited ability to identify "causal SNPs" through association studies. This is partly related to two factors, (1) the difficulty in selecting biologically relevant SNPs for genotyping and (2) the inability to tease out causal SNPs from blocks of SNPs that are in high linkage disequilibrium (LD). For each gene of interest, there may be a dozen to a few hundred SNPs. The conventional approach is to choose SNPs with functional significance for genotyping. This is a difficult task in practice, given the very large pool of known SNPs and the limited information on the functional significance of many SNPs. In some studies, a haplotype-tagging approach has been used to identify informative SNPs by exploiting blocks of SNPs that are in high LD.¹⁸⁵⁻¹⁸⁷

Rapid progress in molecular epidemiology during the next few years is likely to hinge upon several factors, including the availability of large well-designed interdisciplinary epidemiologic studies, development of novel approaches, and statistical methods to deal with the vast amount of data, and innovative laboratory methods, such as DNA pooling¹⁸⁸ or whole genome scans, that permit typing multiple genetic markers at a much lower cost with higher throughput.

It is clear that prostate cancer etiology involves an intricate interplay between lifestyle and genetic factors. To fully explore the complexity of interrelationships between the numerous elements in these pathways will require large cohort studies in which blood is sampled prior to diagnosis. Such studies will be important for identifying which modifiable aspects of lifestyle (such as diet, obesity, and physical activity) can be targeted for prevention and risk reduction. To this end, studies such as the Cohort Consortium, a collaborative agreement launched in 2003 involving over 10 large, prospective cohorts with a combined total of over 7000 incident prostate cancer cases, have been organized to provide unique opportunities to evaluate the complex relationships between lifestyle and genetics in prostate cancer etiology with sufficient statistical power.

The widespread use of PSA testing in western populations has changed the characteristics of cases included in epidemiologic studies.¹⁸⁹ Prostate cancer cases diagnosed in the PSA era are more likely to have early lesions, which may differ in etiology from advanced lesions and more aggressive tumors. This is frequently reflected in recent epidemiologic investigations that include a large number of cases with both early and advanced lesions, which frequently show positive associations for advanced stage or more aggressive tumors but not for early stage or localized tumors. It is important that future studies include prostate tumor subclassification, such as methods of detection, markers of biological aggressiveness, and genetic changes, in order to provide more accurate risk estimates related to specific risk factors.

Summary

Epidemiologic observations provide important clues to the etiology of prostate cancer. Although the causes of prostate cancer remain unclear, there are many intriguing leads, including both environmental and genetic factors. The pathogenesis of prostate cancer reflects complex interactions between several environmental and genetic factors. With newly available tools in molecular biology and genomics, a new generation of large-scale multidisciplinary population-based studies is beginning to investigate the individual and combined effects of environmental and genetic factors. These studies are likely to provide unique information on risk factors and

help identify subsets of the population that are more susceptible to prostate cancer through certain environmental insults.

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PROFILING GENE EXPRESSION CHANGES IN PROSTATE CARCINOMA

Peter S. Nelson

*Fred Hutchinson Cancer Research Center
Seattle, Washington, USA*

Introduction

Prostate carcinogenesis represents a complex process involving a series of overlapping molecular events that occur in the transformation from a normal to a malignant cell. The accumulation of mutations and epigenetic modifications in critical genes involved in growth regulatory pathways result in sporadic cancers. This process can be accelerated with inherited predispositions resulting from the transmission of one or more of the necessary genetic alterations to successive generations. Despite great advances over the last several decades in identifying and characterizing individual molecular alterations mediating various aspects of prostate cancer growth and invasion, it is now clear that a more comprehensive approach may be required to fully understand the complex network of genes, their protein products and the various intra- and inter-cellular interactions that together comprise the neoplastic phenotype. Advances brought about by the Human Genome Project now allow for comprehensive analyses of DNA, transcripts, and proteins such that the complexities of the normal cellular machinery and the attendant perturbations in neoplasia, can at least be thoroughly identified. This knowledge provides a starting point for understanding how molecular alterations actually dysregulate cellular controls governing growth, death, invasion, and ultimately, responses to therapy.

Methods for Profiling Gene Expression Alterations

Several methods have been developed for thoroughly assessing and comparing gene expression between two or more tissues or cell types. Most approaches have centered on studies of messenger RNA (mRNA) molecules or transcripts, due to the versatility and stability offered by the conversion of mRNA into complementary DNA (cDNA) and the ability to use hybridization and amplification strategies to evaluate RNA extracted from small quantities of cells and tissues. One approach, termed *subtractive hybridization*, relies on the molecular binding of complementary nucleic acids to remove transcripts in common between tumor cells and normal cells, leaving behind for analyses those transcripts differing in abundance between the two cell states.¹ The method of *differential display* utilizes the polymerase chain reaction to amplify transcripts comprised of regions complementary to combinations of primer sequences such that mRNAs expressed in different quantities between cells of interest can be visualized as bands following separation by polyacrylamide gel electrophoresis.² Several genes with altered levels in prostate carcinoma have been identified using this technique including *Hevin*,³ *dd3*,⁴ *PCGEM*,⁵ and *COSVIC*.⁶ The large scale partial sequencing of clones from cDNA libraries constructed from normal and neoplastic tissues generated *expressed sequence tags* (ESTs) that can be assessed to provide qualitative and quantitative data on gene expression.⁷ Statistical algorithms allow for computational *in silico* comparisons of EST numbers between multiple tissue types, termed virtual Northern or Digital Differential Display comparisons.⁸ Using EST profiles generated through the Cancer Genome Anatomy Project (CGAP), several genes such as *PATE*,⁹ *PRAC*¹⁰ and *GDEP*¹¹ have been determined to be specifically expressed in prostate epithelium, or differentially expressed between normal prostate and prostate carcinomas. An elegant enhancement of the EST approach involves the truncation of cDNAs by restriction enzymes, followed by concatenation and amplification such that multiple transcript tags can be identified and quantitated with a single sequence analysis.¹² This method, termed the Serial Analysis of Gene Expression (SAGE) has been used to profile the genes expressed by normal and neoplastic prostate tissues; identifying PMEPA1¹³ and E2F4¹⁴ as up-regulated in malignancy. While each of these approaches can provide a deep sampling of a tissue or

cellular gene expression repertoire, they remain poorly suited for the analysis and comparisons of multiple different tissue samples due to the labor entailed in conducting the procedures.

The current method of choice for high-throughput comprehensive studies of gene expression involves the analyses of microarrays: wafers, chips, or slides with spatially-defined immobilized DNA fragments representing genes of interest.^{15,16} This technique combines the proven chemistry of nucleic acid hybridization with advanced automation and image-analysis technology to quantitatively monitor changes in gene expression patterns. Briefly, individual cDNAs or oligonucleotides representing known or unknown genes are spotted or synthesized on a solid support such as a glass slide. Replicates are made of the arrays using high-precision robotics. Complex tissue probes are constructed with radioactive or fluorescent labels and hybridized to the arrays. Individual or groups of transcripts with differential expression signals are identified using quantitative image analysis software. This methodology allows the simultaneous assessment of expression levels of thousands of genes represented at 0.01%–0.001% abundance in a population, making it ideally suited for the identification of the numerous molecular alterations that occur in the development and progression of human cancers (see Fig. 1).

Microarray Studies of Gene Expression in Prostate Carcinoma

Microarray analysis has been used in several published studies designed to profile gene expression alterations in prostate carcinoma (Table 1). Importantly, although all of these experiments used microarrays, there are important differences that preclude a simple comparison of the reported results. These include the use of different patient samples, microarrays with different genes represented and variations in experimental and analytical approaches. Magee *et al.* used oligonucleotide arrays to characterize the expression of 4712 genes in four benign and 11 prostate cancer samples.¹⁷ Most of the neoplastic samples represented primary tumors of various Gleason grades, though two metastatic lesions were also characterized. The samples were enriched for epithelial cells using macrodissection procedures. Analyses of the gene-expression profiles identified four genes with significant changes associated with carcinoma, including Hepsin, a type-II membrane-bound serine protease. Luo *et al.* used microarrays comprised

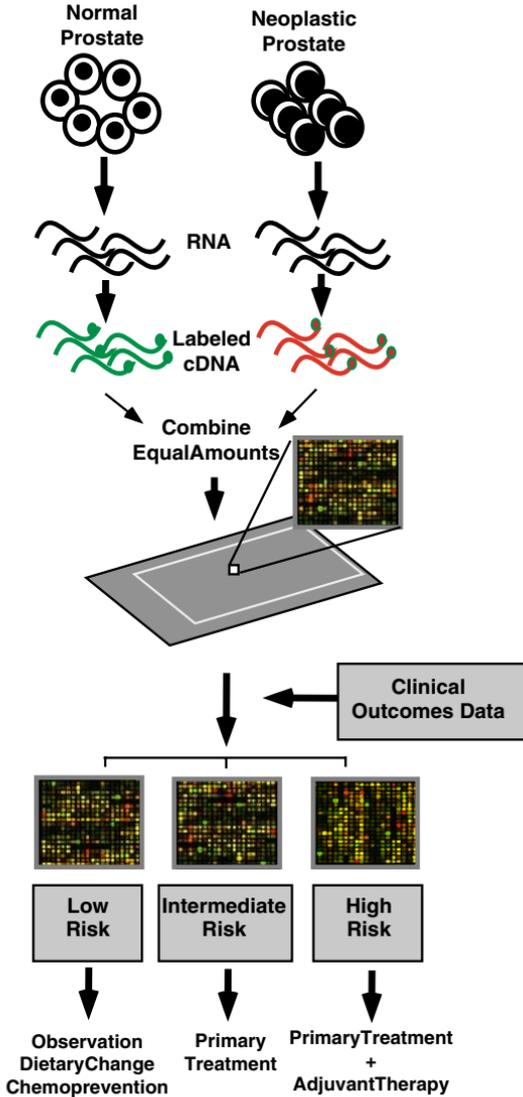


Fig. 1. Prostate cancer outcomes determined by microarray expression profiles. Messenger RNA (mRNA) is isolated separately from normal and neoplastic prostate tissues, preferably from specific microdissected cell types. RNA samples are either labeled directly with fluorescent probes or first converted to complementary cDNA followed by hybridization to microarrays of spotted or synthesized DNAs representing genes of interest. Gene expression measurements are determined by analyzing the fluorescent signal intensity for each gene on the microarray and subsequently comparing the signal levels between normal

and neoplastic cell types and neoplastic cell types representing different clinical outcomes. Expression data are correlated with known clinical outcomes to determine a profile or fingerprint capable of predicting the risk of local and distant cancer progression. Patients with a minimal risk of progression (i.e. with indolent disease) would receive no primary intervention but could be considered for dietary alteration or chemoprevention trials. Patients at intermediate risk would receive primary curative therapy such as radical prostatectomy or radiation therapy. Patients at high risk would receive primary therapy with the addition of systemic adjuvant or neoadjuvant therapy.

of 6500 spotted cDNAs to profile gene expression in nine benign prostatic hyperplasia (BPH) specimens and 16 prostate cancer samples.¹⁸ A common reference standard was used to facilitate comparisons and clearly discernable patterns that discriminated prostate cancer and BPH was evident. The study identified 210 genes with statistically significant expression differences between the two tissue types. This study also identified Hepsin overexpression in cancer epithelium relative to benign epithelium. A study by Dhanasekaran *et al.* characterized the gene expression signatures of more than 50 normal and cancerous prostate specimens and three prostate cancer cell lines using arrays comprised of 9984 cDNAs.¹⁹ Cohorts of genes distinguishing normal prostate, BPH, localized prostate cancer and metastatic hormone-refractory prostate cancer were identified. Examples of genes differentially-expressed between benign and neoplastic prostate tissue include *Hepsin*, *PIMI*, *IGFBP-5*, *DAN1*, *FAT*, *RAB5A* and *HEVIN*. This study further explored the correlation of *Hepsin* and *PIMI* protein expression using tissue microarrays comprised of 700 prostate-cancer specimens with known clinical attributes. The expression levels of *Hepsin* and *PIMI* were shown to independently correlate with cancer progression. Overall, these microarray-based studies of prostate gene expression demonstrate consistent alterations in a subset of genes that can be grouped into a cancer-associated profile. These results provide important substrates for mechanistic studies designed to evaluate their utility as diagnostic, prognostic and therapeutic targets.

Transcript Profiling and Predicting Cancer Outcomes

Despite a variety of clinical and histological parameters used to classify prostate and other cancers, patients receiving the same diagnosis can have strikingly different disease progression rates and responses to treatment.

Table 1. Studies Using Microarray Analysis to Identify Differentially Expressed Genes in Prostate Carcinoma

Tissues		# Genes Studied	# Differential Genes	Differentially-Expressed Genes: Examples	Reference
Benign	Cancer*				
1	1	588	13 ^a	<i>GSTM1</i>	Chetcuti <i>et al.</i> (2001) ⁵³
1	1	588	15 ^b	<i>VEGF</i>	Chaib <i>et al.</i> (2001) ⁵⁴
9	16	~6112	210 ^c	<i>Hepsin</i>	Luo <i>et al.</i> (2001) ¹⁸
Pool	36 ²⁰	~9984	> 200 ^d	<i>Hepsin, PIMI</i>	Dhanasekaran (2001) ¹⁹
4	11 ³	~4712	4 ^e	<i>Hepsin</i>	Magee <i>et al.</i> (2001) ¹⁷
9	24 ¹	~8920	> 400 ^f	<i>Hepsin, MIC1, FAS</i>	Welsh <i>et al.</i> (2001) ⁵⁵
8	9	~6800	86 ^g	<i>Hepsin, PSMA</i>	Stamey <i>et al.</i> (2001) ⁵⁶
15	15	~35,000 [#]	84 ^h	<i>Hepsin, AMACR</i>	Luo <i>et al.</i> (2002) ⁵⁷
9	17	~12,600	216 ⁱ	<i>Hepsin, AMACR</i>	Ernst <i>et al.</i> (2002) ⁵⁸
3	32 ⁹	~12,000	> 3400 ^j	<i>USP13, STK11</i>	LaTulippe <i>et al.</i> (2002) ⁵⁹
50	52	~12,600	456 ^k	<i>Hepsin, Tetraspan1</i>	Singh <i>et al.</i> (2002) ²⁹
8	28 ⁵	~12,600	50 ^l	<i>Hepsin, AMACR</i>	Vanaja <i>et al.</i> (2003) ⁶⁰
Pool	13	6400	136 ^m	<i>Hepsin, Nectin3, CALLA</i>	Best <i>et al.</i> (2003) ²⁸
NA	72	~46,000	266 ⁿ	<i>trp-p8, seladin-1, SOCS2</i>	Henshall <i>et al.</i> (2003) ³¹

[#]Estimated by the authors.

*Superscript indicates the number of metastatic prostate cancer samples analyzed.

^aDifferential gene expression was determined by calculating the ratios of hybridization intensities between normal and neoplastic samples following array normalization. Genes with ratios ≥ 3 -fold were called differentially expressed.

^bDifferential gene expression was determined by calculating the ratios of hybridization intensities between normal and neoplastic samples following array normalization. Genes with ratios ≥ 2 -fold were called differentially expressed.

^cDifferential gene expression was determined by computing the discriminative weight (w) of each gene to separate cancer from benign tissue. P values were assigned based on the (w) values of randomized data.

^dSeveral approaches used: *t*-statistics (of prostate cancer versus benign tissue) ranked based on effect size: 200 genes reported. An analysis using fold-change identified 1520 genes with 2-fold changes between 50% of cancer samples and normal adjacent tissue and 1006 genes with 3-fold change in 75% of cancer samples compared with a normal commercial pool.

^eDifferential expression between benign and neoplastic prostate tissue; ≥ 3 -fold change in all 11 tumors compared with four benign samples. *P* values were calculated by a two-tailed *t*-test for independent data sets of unequal size and variance.

^fDifferential expression between benign and neoplastic prostate tissue was determined by equally weighing contributions from differences in hybridization intensities, the quotient of hybridization intensities and the result of an unpaired *t*-test between expression levels in tumor and normal tissues.

^gDifferential expression between BPH and neoplastic prostate tissue; $p < 0.0005$ and expressed in all samples examined.

^hDifferential expression between benign and neoplastic prostate tissue; $p < 0.05$ by Student's *t*-test; signal intensity > 500 units.

ⁱDifferential expression between benign and neoplastic tissue; $p < 0.05$ by Student's *t*-test and fold change > 2.5 .

^jDifferential expression between non-recurrent primary prostate cancers and metastasis; fold-difference ≥ 3 -fold.

^kDifferential expression between normal versus tumor samples was determined using a variation of a signal-to-noise metric. The statistical significance of the gene expression correlations was determined by comparing the observed correlations to the results mined by comparing the observed correlations to the results derived from permutations of the class labels (normal and tumor).

^lDifferential expression between benign and cancer samples was determined by combining equally weighted contributions from differences in hybridization intensities, the quotient of the hybridization intensities, and the results of an unpaired *t*-test between expression levels. Selection criteria were further narrowed to a fold-change > 2.35 and a $p < 0.001$ by Student's *t*-test.

^mDifferential expression between normal and cancer tissue was determined at the $p < 0.001$ level by a one sample *t*-test and Wilcoxon ranking.

ⁿThis report was designed to identify gene expression changes associated with disease progression. Cox proportional hazard survival analysis predicting relapse was used to evaluate each gene (probe set). Gene expression predictors of relapse at the $p < 0.01$ level were reported. A False-Discovery Rate (FDR) was calculated to be 23% (61 of the 266 findings are false positives).

A major objective in the field of oncology centers on accurate prediction of outcome and patient stratification for planned interventions such as surgery, radiation treatment and chemotherapy. Clues to tumor behavior may be found in the molecular heterogeneity within individual cancer diagnostic categories such as chromosomal translocations, deletions of tumor suppressor genes, amplifications or mutations of oncogenes, and numerous chromosomal abnormalities. Ultimately, the majority of these molecular alterations will produce changes in gene expression that manifest in phenotypic behaviors of invasion, metastasis and drug resistance.

A landmark study involving the molecular analysis of Non-Hodgkins Lymphoma (NHL) proved the hypothesis that gene expression profiles could be used to stratify cancers into clinically relevant categories. This study compared the variability in the natural history of a NHL subtype with analyses of molecular heterogeneity in the tumors as assessed by cDNA microarray fingerprints.²⁰ Although histologically-indistinguishable, two distinct forms of this NHL subtype were identified by expression patterns that reflected different stages of B-cell differentiation. The outcome of patients with different expression profiles was demonstrated by divergent survival curves following the best available therapy. The study concluded that a molecular classification of tumors on the basis of gene expression can identify previously undetected and clinically significant subtypes of cancer.²⁰ Similar studies have now been performed in other tumor types. van't Veer *et al.* used microarrays to profile genes expressed in breast cancers.²¹ A classifier comprised of 70 "prognosis reporter genes" was developed that distinguished which primary tumors were destined to progress to distant metastases versus those that did not relapse. These and other studies point to an emerging use for genomics in the management of patients with neoplastic diseases.

The strongest predictive factors for prostate cancer disease progression are the Gleason score, serum PSA, and clinical stage.²² The Gleason grading system in biopsy or prostatectomy specimens is a measure of biological aggressiveness.²²⁻²⁴ Several groups have recently combined clinical stage, serum PSA level, and Gleason score to generate "nomograms" that predict pathological stage or outcomes^{23,25-27} and allow prognostic estimates to be made with readily available clinical data. To identify biological correlates of the Gleason histology, Best *et al.* used microarray analysis to

profile transcripts expressed in 13 high and moderate-grade prostate cancers.²⁸ Using permutation *t*-tests, 21 genes were found to segregate based on tumor grade ($p < 0.001$). Genes with this discriminatory power included tumor protein D52, BRCA1 binding protein 1 and CD69. Future studies will demonstrate the utility of these genes in delineating patient outcomes prospectively.

To date, three studies have been published with designs aimed toward independently identifying molecular signatures correlating with outcomes of relapsed disease following radical prostatectomy. Singh *et al.* used oligonucleotide microarrays to characterize the expression levels of ~12,600 transcripts in primary prostate carcinomas.²⁹ The study identified 456 differentially expressed genes between benign and malignant prostate tissues. A molecular classifier comprised of five genes (*ITPR3*, *sialyl-transferase 1*, *PDGFR- β* , *chromogranin A* and *HoxC6*) was capable of segregating primary cancers into two groups, those cancers that relapsed within four years of radical prostatectomy and those that did not recur within four years. In a second study, Ramaswamy *et al.* used microarrays to define a gene expression signature of metastasis present in multiple tumor types, including prostate cancer.³⁰ Comparing metastases with primary cancers identified a group of 17 genes capable of discriminating metastases from primary tumors. A subset of primary tumors was also found to contain the metastasis signature and these tumors exhibited a high likelihood of disease relapse and poor clinical outcome following primary therapy. Henshall *et al.* used microarrays comprised of 46,000 unique sequences to profile 72 cases of primary prostate carcinoma.³¹ At the time of analysis, 17 patients were known to have relapsed as determined by rising PSA measurements and the expression levels of 266 genes were shown to correlate with disease relapse. This cohort included the putative calcium channel protein *trp-p8*, a transcript whose loss of expression in neoplastic tissue was strongly associated with disease relapse ($p \leq 0.001$) independently of pre-treatment PSA levels.

Although both the five-gene molecular classifier defined by Singh *et al.*²¹ and the 17-gene metastasis signature defined by Ramaswamy *et al.*³⁰ independently predicted disease relapse, no genes are shared between the two predictive cohorts. Similarly, none of the progression-associated genes identified by Henshall *et al.*³¹ are included in the five-gene outcome

classifier, though there may be overlaps in the biochemical pathways in which these genes participate. Of the 70 genes used to predict breast cancer progression in the van't Veer study,²¹ none are shared with the five or 17-gene prostate cancer outcome predictors. Therefore, while quite provocative and statistically valid, the utility of these predictive signatures will require validation in additional independent data sets and in prospective studies.

Proteomic Approaches for Assessing Gene Expression in Prostate Carcinoma

While important information has been gained through the profiling of transcripts, it is important to keep in mind that the end-point for gene expression is the protein, as proteins represent the actual scaffolds, molecular engines and communication mechanisms utilized by cells. In addition, the development of most biomedical interventions center on protein endpoints. Large-scale efforts are underway to analyze the proteome: the total protein complement of the genome.³² However, the proteome represents a complex dynamic entity due to the many forms of a given protein that result from alternative transcript splicing and the numerous post-translational modifications that often define functional protein states.^{33,34}

A core technology developed for the global analysis of proteins involves the electrophoretic separation of proteins along two dimensions: size and charge, using polyacrylamide gels (2D-PAGE).³⁵ Comparisons of gel profiles from two different cell states (e.g. normal versus cancer) can identify differential protein expression or protein modifications. The identity of individual protein spots can be determined by immunodetection or by calculating the theoretical locations of known proteins based upon charge and mass. More recently, microsequencing and mass spectrometry have gained widespread use for characterizing protein spots of interest.^{36,37} The 2D-PAGE technique is theoretically capable of resolving more than 10,000 proteins and peptides from complex mixtures. However, practical applications of 2D-PAGE have rarely identified more than a small percentage of proteins comprising the cellular proteome.³⁸ Despite this drawback, 2D-PAGE has been used to identify androgen-regulated genes in prostate epithelium³⁹ and prostate cancer-associated protein alterations such as NEDD8 and calponin.⁴⁰

Mass spectrometry (MS) has evolved to become a key technology in proteomics research. The basic mass spectrometer is comprised of an ion source, a mass analyzer, and a detector to record the spectra of ion intensity versus the mass to charge ratio of proteins or peptides under analysis.³⁶ Currently, surface enhanced laser assisted desorption (SELDI), matrix assisted laser desorption (MALDI) and electrospray ionization (ESI) represent the preferred methods for ionizing peptides and proteins. Comparing the mass spectra of complex protein mixtures such as human serum offers the potential for identifying proteins or protein fragments that are present in different abundances and that associate with disease states.^{33,41} Several reports have used SELDI mass spectrometry as a biomarker discovery method to distinguish serum profiles or fingerprints that reflect the presence of prostate carcinoma in comparison to serum protein profiles derived from normal controls.^{42,43} A major drawback to these profiling approaches involves a lack of reproducible quantitative accuracy and a difficulty in positively identifying the peptides and parent proteins that account for the fingerprint differences.

Several strategies have been devised to provide MS with the ability to accurately quantitate differences in protein levels between two cellular states.⁴⁴⁻⁴⁷ One technique employs Isotope Coded Affinity Tags (ICAT) comprised of three components: a biotin affinity tag, a linker with either eight hydrogen or eight deuterium atoms (generating a light and a heavy form of the molecule), and a SH-reactive group capable of covalently linking to cysteine residues (Fig. 2).⁴⁸ The proteins of one cell state (e.g. normal epithelium) are labeled with the light reagent and those of a second cell state (e.g. neoplastic epithelium) with the heavy reagent. Equal quantities of labeled cells are mixed and the proteins are separated, digested with a proteolytic enzyme and the resulting mixture of peptides are passed over an avidin column to isolate the cysteine labeled peptides (about 90% of proteins have cysteine residues). These can be fractionated and analyzed by tandem mass spectrometry (MS/MS). The first MS analysis gives the areas under the curves of the paired isotopic peptides (hence, their relative abundances); the second MS analysis provides a peptide fingerprint that can be used to identify the parent protein. Thus, the ICAT method dramatically increases throughput by reducing sample redundancy (only cysteine-containing peptides are assessed) and

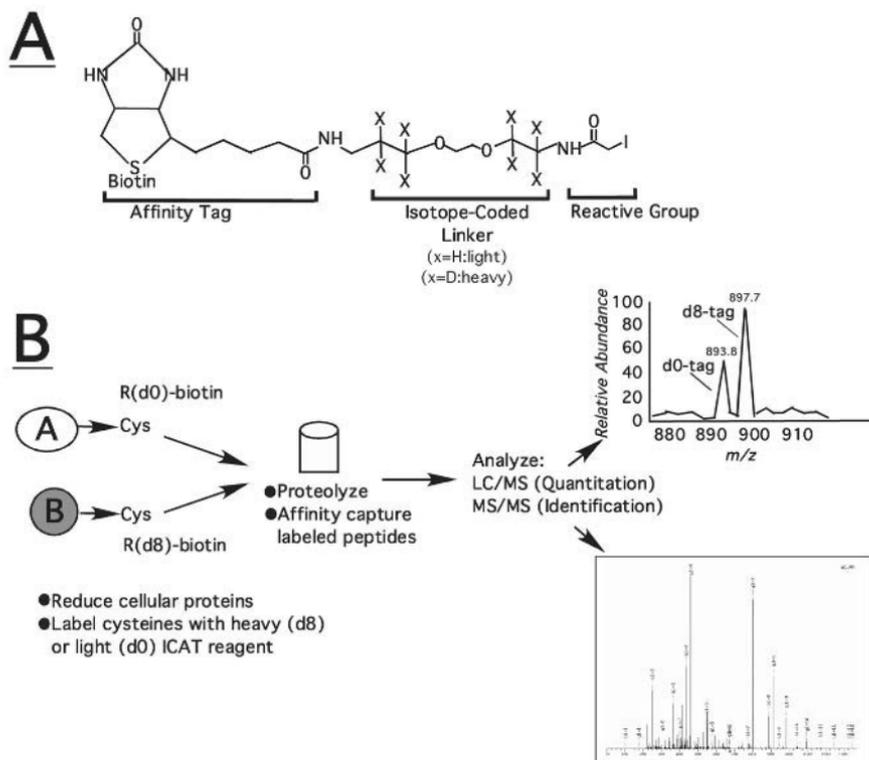


Fig. 2. Relative Quantitation of Cellular Proteins using Isotope Coded Affinity Tags (ICAT) and Mass Spectrometry. (A) To facilitate the quantitative analysis of proteins in complex mixtures, peptides are labeled with an isotope-coded affinity tag (ICAT) reagent that consists of three parts: an affinity tag (biotin) that is used to isolate ICAT-labeled peptides, a linker that can incorporate stable isotopes, and a reactive group with specificity toward thiol groups (cysteines [Cys]). Two forms of the reagent are made: heavy (contains eight deuteriums [d8]) and light (no deuteriums [d0]). (B) The strategy for ICAT-based differential protein quantitation involves separately extracting proteins from two different cell states (e.g. normal and cancer) followed by labeling each with a different (d0 or d8) ICAT reagent. The ICAT reagent covalently bonds to each cysteinyl residue in every protein. The protein mixtures are combined and proteolyzed to peptides and only ICAT-labeled peptides are isolated using the biotin tag. These peptides are separated by microcapillary high-performance liquid chromatography (LC). ICAT-labeled peptide pairs are chemically identical and easily visualized. Peptide fragments faithfully maintain the ratios of the original amounts of proteins from the two cell states. Relative peptide/protein quantification is calculated by the ratios of the d0- and d8-tagged peptide pairs. Periodic scans are devoted to fragmenting peptides and recording sequence information (tandem mass spectrum [MS/MS]). Protein identifications are made by database searches of the tandem mass spectrum.

retains sample complexity while allowing for accurate relative protein quantification.^{48,49}

The ICAT approach has been used to identify secreted⁵⁰ and androgen-regulated proteins⁵¹ expressed by prostate cancer cells. In addition to identifying genes previously not known to be regulated by androgens, comparisons of global transcript and protein abundance levels showed that for most genes (>90%), protein levels were concordant with transcript abundance. However, there were distinct outliers that indicate multiple levels of gene expression regulation (e.g. post-transcriptional, post-translational). These results suggest immediately testable hypotheses for characterizing mechanism(s) of gene expression regulation. The results also demonstrate that to *fully* delineate a gene expression profile, measurements of protein levels are necessary, since, for some genes, protein alterations would not have been predicted by transcript abundance measurements.

Conclusion and Future Directions

Gene expression profiles offer an extraordinary assessment of both the diversity and consistency of genetic alterations in prostate carcinomas. Published studies have provided the first glimpse of the potential of profiling technologies to identify individual genes that participate in neoplastic growth and to define molecular predictors of cancer behavior. However, there clearly are limitations to the approach and significant hurdles to be overcome. It is somewhat disconcerting that cohorts of genes statistically associated with disease outcome in different studies do not correlate with each other. This may be explained by the extreme heterogeneity of neoplastic prostate lesions. In addition, tissue samples used in most studies are comprised of various amounts of normal epithelium, stromal cells, inflammatory cells, blood vessels, and other constituents. While gene expression changes in these cell types may influence neoplastic transformation, growth, and invasion, the variable amounts of these cell types between different studies may make inter-study comparisons difficult. To address this problem, microdissection techniques that compare only specific cell types can be employed.⁵² The ultimate endpoint of such an approach is the characterization of individual cell expression profiles within the tumor environment. Although such an approach represents a daunting task, the pace of biotechnological advances

do not rule out single-cell analysis using high-speed flow cytometers and nanotechnology in the near future. Finally, a major factor that could influence the outcomes of tumors with identical gene expression profiles centers on variables within the host. Most expression profiling studies have focused on defining molecular determinants within tumor tissue. However, host characteristics involving immune response, dietary factors and the hormone milieu may influence tumor cell proliferation, invasion and metastasis. In the future, we will likely gain important additional knowledge of tumor behavior and response to therapy through the integration of profiles reflecting both tumor and host gene expression.

Acknowledgments

Peter Nelson is supported by a Scholar Award from the Damon-Runyon Cancer Research Foundation.

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STUDY OF ANDROGEN-ANDROGEN RECEPTOR ROLES IN PROSTATE CANCER USING MICE LACKING FUNCTIONAL PROSTATE ANDROGEN RECEPTOR

Chun-Te Wu, Shuyuan Yeh, Qingquan Xu, Zhiming Yang,
Philip Chang, Yueh-Chiang Hu and Chawnshang Chang

*George H. Whipple Lab for Cancer Research
Departments of Urology, Pathology and Radiation Oncology, and
the Cancer Center, University of Rochester
Rochester, New York 14642, USA*

Introduction

Prostate cancer is an androgen-dependent disease and a leading cause of cancer mortality in men. The vast majority of prostate cancers express the androgen receptor (AR), have androgen-dependent growth, initially respond to androgen ablation therapy, but eventually become androgen-independent (hormone refractory stage).¹ The mechanisms for transition of tumors from androgen-dependent to androgen-independent are generally explained by clonal selection, adaption, alternative pathways of signal transduction and aberrant AR signaling.²⁻⁴ The AR is now known to participate in tumor progression through three mechanisms: expression, development of point mutations, and ligand-independent activation via coregulators.⁵ Histological and epidemiological data have indicated the role of AR mutations and poly-Q polymorphisms in the anti-androgen withdrawal syndrome and prostate cancer susceptibility.⁶ The AR point mutation T877A is common in the hormone-refractory prostate cancer.⁷ *In vitro* studies have indicated the transactivation of this mutated AR can be induced by the anti-androgen, flutamide, in human prostate cancer LNCaP cells, which contain the mutant T877A (tyrptophen to alanine) AR.⁸

The involvement of this mutation in the transition to hormone-refractory stage and the anti-androgen withdrawal syndrome, remains controversial. Alleles of the CAG repeat in the first exon of the human AR gene have been shown to be associated with the risk of prostate cancer. Even in low-risk Asian populations that are favored by higher median CAG repeat lengths, a shorter CAG repeat length influences prostate cancer risk.⁹ Animal models without functional AR in the prostate provide excellent tools to investigate the AR role in normal prostate development, prostate cancer carcinogenesis, progression, metastasis and transition to hormone-refractory. It is hoped that continuing research on AR expression and function in prostate cancer will provide new therapeutic strategies.

Generation of Androgen Receptor Knockout (ARKO) Mice

The AR, a member of the nuclear receptor superfamily, was first cloned in 1988.¹⁰⁻¹¹ It contains an N-terminal transactivation domain (NTD), a central DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD). To generate ARKO mice, a cre-lox strategy for conditional KO is necessary. The cre-lox system utilizes the expression of P1 phage cre recombinase (Cre) to catalyze the excision of DNA located between flanking lox sites in exon 2 of the DBD.¹² We generated two separate lines of transgenic mice to carry the Cre and the homozygous floxed AR genes in both X chromosomes, as illustrated in Fig. 1. The generation of ar/Y-ACTB/Cre male mice in F3 resulted in total ARKO in all of the cells. Phenotype analyses showed that ARKO male mice have a low body weight and the external genitalia was ambiguous or appeared feminized, had a microphallic penis, and the urethra showed hypospadias. The scrotum is poorly developed and all vas deferens, epididymis, seminal vesicle and prostate are agenetic. Testes are small in size and cryptorchid in the low abdominal area close to the internal inguinal ring. This study indicated that disruption of AR gene in early embryonic stage had great impact in the development of normal prostate.¹² However, the systemic effects of lower androgen level in the total ARKO mice should be further explored.

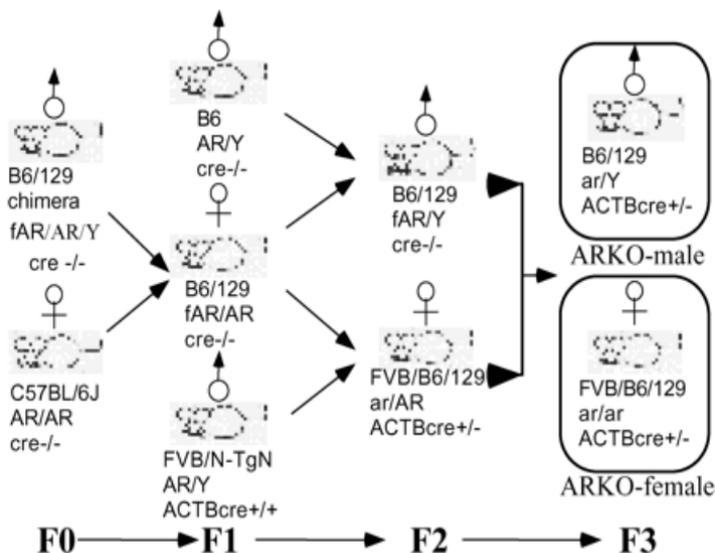


Fig. 1. Generation of ARKO female mice by mating among floxed AR (fAR) founders and cre founders. The chimera founder is B6/129 mosaic strain. The mating of the founder with the B6 female created female mice heterozygous with fAR. The following F2 generation resulted in fAR male mice. The mating between the heterozygous fAR female and the homozygous FVB/N-TgN ACTB cre male that carry Cre under β -actin promoter created a heterozygous KO of AR female carrying the Cre. The mating between the fAR male and the heterozygous ARKO female carrying the Cre generated the homozygous ARKO Cre⁺ female mice with a 1:8 ratio.

Prostate Development and Carcinogenesis in Prostate-Specific ARKO Mice

The cre-lox ARKO mouse model provides a much-needed *in vivo* animal model system to study androgen functions in the selective androgen target tissues in male mice. In this regard, it is of paramount importance to derive a cre recombinant transgenic animal system with robust expression of a biologically active Cre protein in a prostate epithelial cell-specific manner. We selected a rat probasin (PB) promoter to drive expression of the Cre gene.¹³ The AR gene was gradually deleted during the adolescence stage when increased androgen levels activated the probasin promoter expression. Specific ARKO in prostate epithelium was evidenced by

genotyping, RT-PCR and immunohistochemistry (IHC). Hormonal studies revealed normal serum testosterone levels in prostate-specific ARKO as compared to wild-type (WT) mice. The growth of urogenital organs other than prostate remained unchanged after the prostate epithelium no longer has a functional AR, however the size and weight of prostate glands were slightly decreased. The secretion proteins, probasin and prostatic secretory protein 94 (PSP94), were dramatically decreased in prostate-specific ARKO mice. When androgen ablation by castration was induced at 12 weeks old, both WT and prostate-specific ARKO mice showed dramatic regression of prostate and seminal vesicle growth. This model implies that the AR plays specific roles in epithelium and in stromal development. By crossing male probasin-cre mice to flox AR TRAMP female mice, the prostate-specific ARKO TRAMP mice were generated. This model will add more clues about the role of AR in the prostate cancer carcinogenesis and progression.

TRAMP Mice Lacking the Endogenous AR but Carrying the T877A Mutated Transgene

In a subgroup of patients with endocrine therapy-resistant prostate cancers, amino acid substitutions in the AR LBD have been found, which result in a broadened ligand response spectrum. The most common substitution in these cancers was T877A, which was first also described in the LNCaP prostate cancer cell line. The T877A substitution renders the AR responsive to natural low-affinity ligands and anti-androgens, such as flutamide. This mutation is believed to contribute to the prostate cancer progression and its transition to the hormone-refractory stage as well as the anti-androgen withdrawal syndrome.

By crossing the T877A transgene knock-in male mice with our ARKO female mice, we are in the process of generating ARKO male mice expressing prostate T877A AR transgene only. This will enable us to compare the effects of mutated AR and WT AR on the development of prostate gland. Further crossing female ARKO mice with T877A transgene male TRAMP mice will generate TRAMP mice which only express T877A mutated AR in the prostate. By these strategies, we can continue to explore the AR mutation on prostate cancer carcinogenesis and compare

the tumor initiation and progression in the TRAMP mice with two different AR expressions.

ARKO Mice with AR-97Q and AR-24Q Transgene Expression

Despite the substantial public health impact of prostate cancer, little is known about its etiology. The generally accepted risk factors for the development of prostate cancer are advanced age, familial predisposition and perhaps ethnicity. The exon 1 of the AR contains several polymorphic repeats; the most variable is a polymorphic CAG repeat, which encodes a polyglutamine (poly-Q) chain. The range of CAG repeat lengths is from 14 to 35 repeats in man and may vary somewhat with ethnicity and race. Because the length of the polymorphic CAG trinucleotide repeat is inversely correlated with the transactivation function of the AR *in vitro*, it has been proposed that men with shorter repeats will be at higher risk for prostate cancer.¹⁴ Several previous studies have shown that shorter CAG repeat length in AR NTD is associated with the occurrence of more aggressive prostate cancer, earlier age of onset and likelihood of recurrence.¹⁵ However, one small study in a European population failed to support the association between CAG repeat length and prostate cancer risk.¹⁶ The role of CAG repeats in prostate needs to be determined more clearly. By crossing AR-97Q and AR-24Q transgene male mice with our ARKO female mice, we are generating the ARKO mice carrying only 97-Q and 24-Q transgenic AR to compare the effects of different poly-Q lengths on the development of the prostate gland. Further crossing to TRAMP mice will create ARKO TRAMP mice with different poly-Q AR transgenes. By studying the differences in carcinogenesis and tumor progression in these mice, we will determine the effect of poly-Q lengths on the prostate cancer *in vivo*.

Inducible ARKO and ARKO TRAMP Mice

The normal prostate development depends on the androgen-AR signaling. In the total ARKO model, agenesis of prostate revealed that AR plays a critical role in the embryonic development of prostate. The prostate-specific ARKO model using probasin-cre lox strategy had nearly normal

prostate growth after adolescence. These two models suggested the time-specific role of AR in the development of prostate. What is the role of AR between embryonic and adolescence? The time-specific role of AR in the normal prostate development can also be elucidated by the study of the inducible ARKO mice. The method uses an interferon-responsive promoter to control the expression of Cre recombinase. Here, Cre was used to delete a segment of the AR gene flanked by loxP recombinase recognition sites. Deletion was complete in liver and nearly complete in lymphocytes within a few days, whereas partial deletion was obtained in other tissues. Crossing the flox-AR female mice with Mx-1-inducible Cre male mice¹⁷ will generate interferon-inducible ARKO mice. Our preliminary data (Xu and Chang, unpublished data), showed the efficiency of deletion of AR gene in prostate tissue was about 20% using the intraperitoneal injection of pI-pC. Some modifications can be made to improve the percentage of AR deletion, such as multiple injections and intra-prostate injection. Yet the partial deletion of AR in prostate may be sufficient for gene inactivation and result in easily detectable phenotypes. By crossing TRAMP male mice with flox-AR-cre-MX-1 female mice, the inducible ARKO TRAMP mice can be generated. As in Fig. 2, induction of IFN in 5-week-old inducible ARKO TRAMP mice resulted in a prostate tumor in one out of three mice, while all three mice without the induction of IFN had prominent prostate tumors at 20-week-old. If the AR gene was inducibly knocked out in 20-week-old mice, all the mice in WT AR and inducible ARKO groups grew prostate cancer, but the tumor size was smaller in the ARKO group. This model indicated that AR deletion in TRAMP mice might inhibit carcinogenesis at an earlier stage and delay cancer progression at a late stage. Further study is necessary to confirm the results of the preliminary observations and to explore the actual role of AR in prostate cancer carcinogenesis and progression.

ARKO Human Prostate Cancer CWR22R Cells

The advantage of using gene targeting in human somatic cells is that it provides a tool to study the roles of the signaling of interest in human cells instead of ARKO animal model. For this purpose, we attempted to disrupt AR signaling in human androgen-refractory prostate cancer cells in order

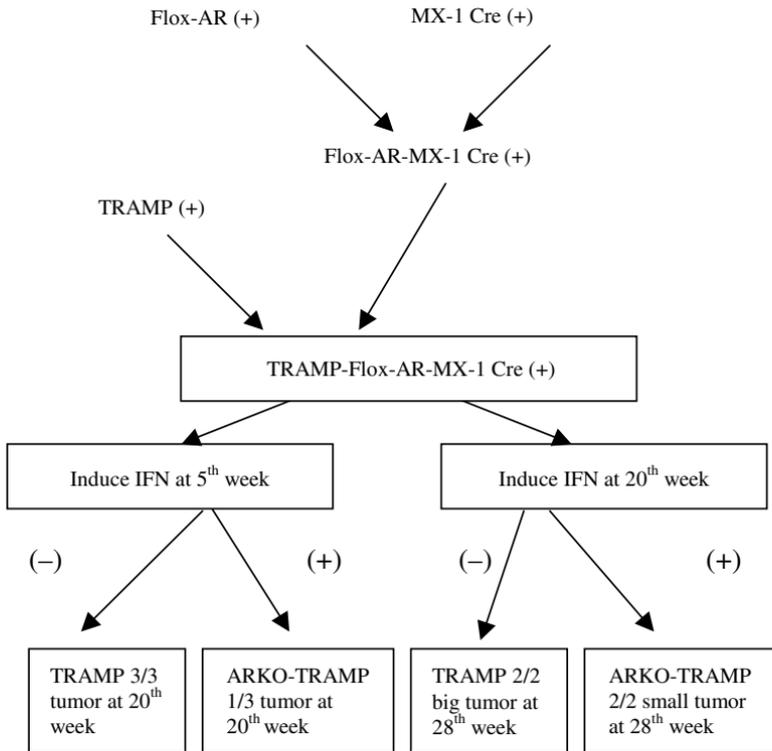


Fig. 2. Model for development of inducible ARKO-TRAMP mice.

to investigate the biological importance of AR signaling in those prostate cancers. Human prostate cancer CWR22R cells were transfected with the AR targeting vector using Superfect transfection kit (Qiagen) and selected with 400 $\mu\text{g}/\text{ml}$ neomycin reagent.¹⁸ The genotypes of the surviving clones were detected by Southern blotting using a DNA probe containing the 5'-UTR sequences of the AR gene. The untargeted and targeted loci produced approximately 9.0-kb and 3.46-kb bands, respectively. AR expression was reduced in those heterozygous clones compared to that in the parental CWR22R cells¹⁹ confirming that one of the AR genes is targeted by homologous recombination. Therefore, this method provides a promising result and indicates that it is possible that we could target AR genes in any other cell lines of interest. Efforts to obtain homologous AR-null CWR22R cells failed after extensive screening, although homologous

AR-null MCF cells were successfully established. This suggests that AR is an essential survival factor in prostate cancer cells. The essential role of AR in androgen-refractory prostate cancer has been depicted in many other studies.^{20,21} In order to knockout both AR genes, we are trying to stably transfect the tetracycline-inducible AR construct into heterozygous CWR22R cells and then target the second allele of AR in the cells. We believe this will provide a powerful tool for us to manipulate AR expression in the prostate cancer studies.

Conclusion

Although AR plays the pivot role in prostate development and prostate cancer carcinogenesis and progression, several aspects of AR function in the prostate are still not clear due to lack of appropriate animal models. One advantage of creating the floxed AR mice is to provide a base to generate tissue-specific ARKO in the prostate. Combined with different Cre-recombinases, a time-specific role of AR can also be studied by using the inducible Mx-1 mice. Based on the ARKO mice, other mutated genes can be tested as a combination of transgenic and knockout mice, such as T877A and poly-Q ARKO mice. Continued expansion of our knowledge of AR action in prostate cancer will contribute to more effective therapies. It is likely that in the future, AR ablation with a target to degenerate and/or eliminate AR protein, rather than androgen ablation to block androgen binding to the AR, will become the treatment of choice for advanced prostate cancer and hormone-refractory prostate cancer.

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CAPTURING SIGNAL ANOMALIES OF HUMAN PROSTATE CANCER INTO MOUSE MODELS

Hong Wu

*Department of Molecular and Medical Pharmacology
University of California Los Angeles School of Medicine
Los Angeles, California 90095, USA*

Ani Khodavirdi* and Pradip Roy-Burman*,†

**Department of Pathology and
†Department of Biochemistry and Molecular Biology
Keck School of Medicine
University of Southern California, Los Angeles
California 90033, USA*

Introduction

Prostatic adenocarcinoma, the most common malignant visceral neoplasm of men, displays extensive phenotypic heterogeneity, both morphologically and genetically, and is typically also admixed with non-cancerous cells within the glands.^{1,2} The disease is further confounded by the presence of multiple malignant foci in a majority of cancerous glands.³ Such complexities have hindered progress in elucidating the clinical course and the molecular parameters for the development of prostate cancer. Some evidence for molecular anomalies has been documented, albeit in a fragmentary manner. In terms of the genesis of the disease, it is recognized that the most likely primary precursor of human prostate cancer is the histopathological lesion known as intraepithelial neoplasia (PIN). PIN lesions could be described as a low grade (LG) or high grade (HG), and it is widely perceived that HGPIN is a precursor of prostatic adenocarcinoma.⁴⁻⁷ Since

the initial growth of prostate tumor or cancer is dependent on androgens, hormone therapy in the form of medical or surgical castration constitutes a common approach for systemic treatment. However, over a period of time, most cancers will develop androgen-independence, thereby making the continued androgen deprivation therapy ineffective.⁸⁻¹⁰ While the mechanisms that drive the genesis of subclinical, microscopic PIN lesions, their progression to invasive cancer and androgen independence remain largely unknown and evidence collected in recent years point to certain molecular aberrations that pave the path of disease progression. For example, anomalies in specific signaling molecules, including extracellular growth factors, protein tyrosine kinase cell surface receptors, intracellular transcription factors, nuclear receptors and their ligands, growth suppressors, cell cycle regulators, and others, have been indicated in some prostate carcinomas.

Some of these issues have been well described in a number of recent reviews.^{2,11-16} There is currently a strong focus on the genetic alterations or aberrations in gene expression that are frequently encountered in human prostate cancer in the design of mouse models. The goal is to recapture the pathophysiologic characteristics of the human disease in a "natural" manner in immuno-competent mice to facilitate exploration of the molecular mechanism underlying prostate cancer as well as for development or testing of new targeted therapies. The strategies, however, are logical extensions of the earlier successful efforts to derive transgenic mouse models with prostate-specific expression of viral oncogenes.¹⁷⁻²¹ The present report only considers the mouse models that have been engineered to depict one or more genetic defects seen in human prostate cancer.

Another important issue to note at the onset is that, although both men and mice harbor functionally equivalent prostate glands, there are similarities as well as differences in the anatomy and histology of the prostate in the two species. Similar epithelial cell types, namely, secretory, basal, and neuroendocrine are found in both mouse and human prostate, although their proportions vary. While human prostate has a robust fibromuscular stroma, the mouse contains a modest stromal component. Anatomically, the human prostate gland is a single alobular structure with central, peripheral, and transitional zones. In contrast, the mouse prostate is

composed of four paired lobes, namely, anterior (AP), dorsal (DP), lateral (LP), and ventral (VP) prostate. Since DP and LP share a ductal system, they are often dissected together and referred to as the dorsolateral prostate (DLP). The mouse DLP is perceived to be most similar to the human peripheral zone in which the majority of clinically diagnosed prostate cancers are found. The mouse VP does not appear to have a human homologue and the human transitional zone does not have a murine homologue. The transitional zone constitutes a site where human nodular hyperplasia (BPH) is commonly seen. The mouse AP is analogous to the human central zone, which only infrequently represents a site of neoplasia in humans. A summary of the comparative aspects of anatomy, histology, and pathology of the human and mouse prostates can be found in a recent review article.¹⁶

Cell Surface Signaling Molecules

Signaling interactions between various extracellular growth factors and the corresponding cell surface receptors converge to determine the fate of the cell with respect to proliferation, survival, or death. In this context, dysregulation of several growth factors or their receptors has been implicated in prostate tumorigenesis. A number of transgenic mouse lines have been produced in which genes that are known to be overexpressed in human prostate cancer are targets. All of the models reviewed here, however, concern usage of a robust prostate-specific promoter to drive the expression of the transgene, and thus exclude those which were based on promoters that are not sufficiently prostate specific. For example, the survival factor, insulin-like growth factor 1 (IGF1), which is generally overexpressed in human prostate and which may potentially be a good tumor marker in prostate cancer,²² was a target in a transgenic line. Its expression in the mouse tissues was designed by using the bovine keratin 5 promoter.²³ These mice develop squamous papillomas, some of which progress to carcinomas of the skin. The increased IGF1 levels also lead to pathologic changes in the prostate and in other male accessory glands of these animals.²⁴ The severity of the lesions in the prostate ranges from PIN to carcinoma *in situ* as well as tumors with neuroendocrine differentiation.

Fibroblast Growth Factors (FGFs)

The FGF family of heparin-binding proteins is intercellular signaling molecules of which at least 23 different members (FGF1–FGF23) have been identified to date. FGF proteins are generally secreted and their effects are mediated by a complex system of FGF receptor (FGFR) tyrosine kinases, either through autocrine or paracrine mechanisms, or both.^{25,26} While dysregulation of several FGFs has been described in prostate development and tumorigenesis,^{27,28} two members, FGF7 and FGF8, have been further pursued through mouse modeling experiments.

Alternative splicing of the mouse *fgf8* mRNA gives rise to eight potential protein isoforms that vary in their amino termini.^{29,30} In humans, however, only four isoforms (FGF8a, FGF8b, FGF8e and FGF8f) are predicted due to a blocked reading frame in an exon of the human gene. Of the four possible isoforms, FGF8b has been demonstrated to possess the most transforming and tumorigenic potential.^{31–33} While expression of FGF8b appears to represent the primary species in prostatic epithelial cell lines or malignant epithelium, its expression is practically undetected in the stromal component of prostate cancer.^{29,31,34,35} Increased expression of FGF8b in prostatic lesions beginning from PIN to adenocarcinoma and its persistence in androgen-independent disease has been described.^{34,35} The overexpression of FGF8b in prostate cancer cells has been shown to increase proliferative and invasive properties of the affected cells directly, and proliferation of prostatic stromal cells indirectly.³⁶ Consistent with these results, antisense down-regulation of *FGF8* mRNA reduces the growth rate, inhibits clonogenic activity, and decreases *in vivo* tumorigenicity of prostate tumor cells.³⁷ Recent demonstrations that FGF8 expression in prostate cancer is, at least in part, regulated by the androgen receptor at the transcriptional level,³⁸ and that FGF8b is angiogenic³⁹ further enhance the biological relevance of this factor in prostate cancer.

To assess the effects of FGF8b overexpression in the normal prostate secretory epithelium, transgenic mice were produced by using an improved rat probasin gene promoter.⁴⁰ This promoter, ARR2PB, has been found to confer a high level of reporter transgene expression specifically in the prostatic luminal epithelium and is strongly regulated by androgens.^{41,42} Prostatic hyperplasia appears in the LP and VP in some FGF8b

transgenic animals as early as 2 to 3 months, and in DP and AP between 6 to 16 months. LGPIN lesions manifest from 5 to 7 months. One hundred percent of the mice display multi-focal prostatic epithelial hyperplasia during the first 14 months, with 35% also having areas of LGPIN. In subsequent months (15 to 24 months) the profile changes to a higher incidence of LGPIN (66%) along with HGPIN (51%). Occasionally, HGPIN lesions resemble the histopathology of human prostatic carcinoma *in situ*. Figure 1 represents the progression pattern in the FGF8b transgenics. Like HGPIN, stromal proliferation and appearance of papillary hyperplasia with atypia, display a delayed pattern. Together, these findings suggest that FGF8b is an etiological factor in prostate tumorigenesis. The stochastic pattern of disease progression from hyperplasia to carcinoma *in situ* also implies that hyperplasia initiated by the FGF8b mitogen is conducive to the manifestation of other genetic lesions, which may represent the rate-limiting factors responsible for a temporal progression and the severity of the lesions. Additionally, the delayed development of stromal hypercellularity in the prostate of these mice mimics the results of the epithelial-stromal cocultures *in vitro*,³⁶ invoking an indirect effect of FGF8b signaling in the epithelial cells on the stromal cells. Thus, besides being

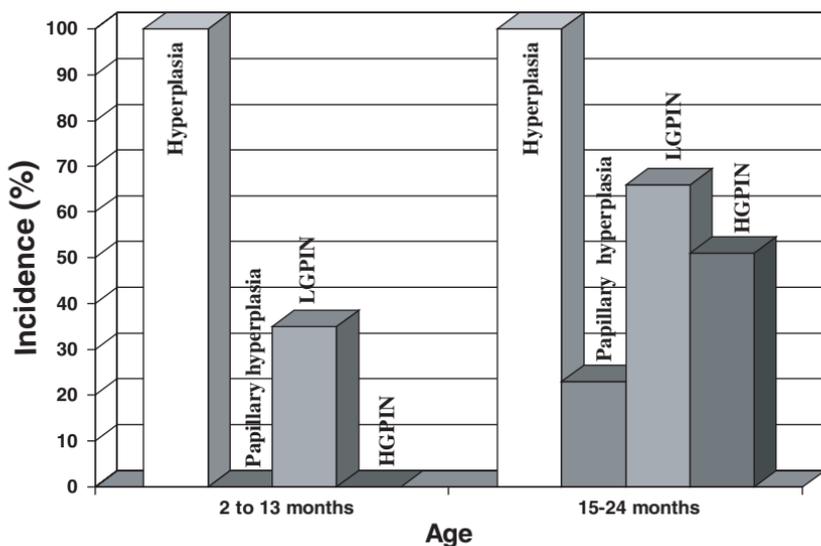


Fig. 1. Temporal incidence of appearance of prostatic lesions in FGF8b transgenic mice.⁴⁰

an initiation factor, FGF8b is likely to act as a progression factor in the prostate disease. The reported properties of FGF8b, such as induction of tumorigenic and invasive activity to weakly tumorigenic prostate carcinoma cells or the ability to induce angiogenesis,^{36,39} are noteworthy in this regard.

While FGF8 is produced in the prostate epithelium, FGF7 (keratinocyte growth factor, KGF) is made by the stroma and plays a role as a mediator of stromal-epithelial interactions in the prostate development.⁴³⁻⁴⁵ It is also reported that a switch in the spatial pattern of FGF7 expression may occur in clinical prostate cancer such that cancer cells, rather than stromal cells, display stronger expression.⁴⁶ The cognate receptor for FGF7 is FGFR2 isoform b, which is normally found on the epithelium. It is also interesting to note that during prostate cancer progression, FGFR2 may switch from isoform b to isoform c in the rat Dunning tumor system.^{26,47} Thus, it is possible that changes in FGF7 and its receptors may be linked to malignancy or gain of independence from stromal control. To convert FGF7 from a paracrine to autocrine factor, transgenic mice were produced in which the minimal rat probasin promoter was used to target FGF7 expression directly to the prostatic epithelium.⁴⁸ After one year of age, most of these mice develop prostatic epithelial hyperplasia, which, however, does not progress to dysplasia or tumors. These results imply that while FGF7 misexpression in the prostate could be a contributing factor, at least, in the induction of proliferation of the epithelial cells, the primary role of FGF7 may actually rest on its paracrine signaling in prostate biology and disease.

Fibroblast Growth Factor Receptors (FGFRs)

The role of FGFR signaling axis, focusing on FGFR1 and FGFR2, has been evaluated in the prostate of several transgenic mouse models. Considering that growth factor receptors like FGFRs⁴⁹ are naturally activated by oligomerization, a chemically induced dimerization technology was cleverly employed to achieve temporal control of FGFR expression.⁵⁰ Transgenic mice generated for this study had ARR2PB driven expression of either ligand-inducible FGFR1 or FGFR2. Examination of the prostates of these animals indicates that while activation of FGFR1 triggers hyperplasia

followed by PIN lesions, such activation of FGFR2 does not elicit any observable changes even after 12 weeks of treatment with the inducer. All mice with the FGFR1 transgene show PIN in virtually every acinus when treated for 12 weeks with the inducer, and by timed removal of this extra FGFR1 signaling, the investigators show that induced hyperplasia is reversible until intraductal vascularization occurs. After that stage, however, it appears that hyperplastic cells become independent of FGFR1 signaling.

Depression of the FGFR2 signaling in the prostate was approached by targeting expression of a dominant negative, truncated FGFR2 form by using the minimal rat probasin promoter.⁴⁸ As with the overexpression of FGFR2 in the prostate, interference of signaling by the dominant negative mutant also does not yield gross changes in the prostate except that the size is smaller than the littermate controls. However, blocking of FGFR2 appears to lead to some histological changes, such as disorganization of the prostatic ducts and areas of hyperplastic stroma. Additionally, a trend in the emergence of epithelial-neuroendocrine transition is noted in the prostate gland of these mice.

In other studies, a potential cooperation between ectopic FGFR1 and down-regulation of FGFR2 in the induction of PIN lesions in the mouse prostate was examined.^{51,52} Both probasin minimal promoter and the stronger ARR2PB promoters were used in developing separate lines of mice with either low or high expression of a constitutively active FGFR1 mutant in the prostate. While low levels of expression lead to LGPIN lesions only when the mice become older than 18 months, increased expressions dramatically accelerate PIN development. HGPIN is detected in the ARR2PB driven transgenic mice within 8 months. The results indicate a correlation between the FGFR1 expression level and the development or progression of PIN lesions. Studies of bigenic mice with repressed FGFR2 via dominant negative mutant and either low or higher expression of the constitutively active FGFR1 also led to interesting results. While a combination of depressed FGFR2 and low level of FGFR1 appears to contribute to preneoplastic lesions in the prostate, expression of the dominant negative form of FGFR2 does not display any measurable synergy with high expression of FGFR1. The combination of low FGFR1 and dominant negative FGFR2 also synergistically increase the population of neuroendocrine cells in the prostate.

Together, these different models highlight the physiological differences between FGFR1 and FGFR2 receptor functions in the prostate epithelium. Similar to what is described for FGF8 and FGF7 transgenic mice, these studies underscore the specificity and potency of the individual players as well as their dose-dependent effects in FGF-FGFR signaling, which could potentially be exploited for targeted intervention of prostate tumorigenesis.

Intracellular Signaling Molecules

A number of transgenic mouse lines have been produced in which a single gene encoding an intracellular signaling molecule is overexpressed or disrupted in the prostate epithelium. The design to increase expression concerns genes that are known to be overexpressed in human prostate cancer. Similarly, the targets of inactivation are those genes each of whose function is diminished or abolished in human prostate cancer. While the first approach is straightforward transgenic development, the latter invokes bigenics, particularly when conventional knockout is unproductive because of embryonic lethality or premature death. The powerful approach of conditional, cell-type, and tissue-specific deletion of genes, through the commonly referred Cre-loxP recombination, has been productive. Although three reports have recently appeared describing alternative prostate-specific Cre systems,^{42,53,54} the power and utility of the PB-Cre4 line⁴² has been fully documented.

Androgen Receptor (AR)

AR is expressed in normal prostate in the secretory epithelial cells at high levels and in a subset of smooth muscle cells.^{55,56} All phases of prostate tumorigenesis ranging from preneoplastic lesions to androgen-dependent adenocarcinoma to androgen-independent disease require the activity of this nuclear receptor.^{9,10,15} The failure of androgen ablation therapy and development of “androgen-resistant” prostate cancer is thought to be, at least partly, due to hypersensitized AR signaling, either by gain-of-function mutations or through increased AR levels, that operates in the absence of optimal levels of the ligand.^{10,15,57} To assess the effects of AR

overexpression in the normal prostate secretory epithelium, a transgenic mouse line was produced by using the minimal PB promoter to drive a murine AR transgene expression.⁵⁸

Although the effect of AR overexpression in the model is not significant up to one year of age, with further aging focal areas of VP and DLP begin to display PIN lesions. Interestingly, hyperplasia is not prominent in the prostate of these animals, which may be a consequence of simultaneous increase in the rate of apoptosis. Since PIN lesions are focal and increased with age, it is suggested that a balanced increase in proliferation and apoptosis may be a conducive factor for secondary genetic or epigenetic events that lead to dysplasia.

Homeobox Transcription Factor, NKX3.1

NKX3.1, which displays prostate-specific expression, is a candidate tumor suppressor in its properties. *NKX3.1* gene is mapped in a chromosomal region (8p21), which is found to undergo allelic deletion in about 80% of prostate cancers.⁵⁹ Unlike the conventional inactivation of the remaining allele of a tumor suppressor gene by mutation, epigenetic modifications, yet to be clearly understood, may be involved in loss of NKX3.1 protein expression in prostate cancer.^{60,61} Both conventional and conditional knockouts of the *Nkx3.1* gene in mice were attempted to define the role of NKX3.1 in prostate tumorigenesis.^{53,62–64} All models point to this homeobox transcription factor as a regulator of ductal branching and secretory activity of the glandular epithelium, and when lost, as a factor in the development of LGPIN by one year of age, largely in AP. Mice with further aging up to two years reveal progression of lesions but not beyond HGPIN.⁶² To extend the “life” of these lesions, when tissue recombination/transplantation approach⁶⁵ was used, the lesions did progress to a more advanced stage of PIN, but did not convert to invasive cancer.⁶⁶ The results implied that other secondary events for progression might not manifest in the *Nkx3.1* knockout model. However, studies of both homozygous and heterozygous mutants clearly indicated that not only null status, but also reduced NKX3.1 expression, may be directly related to initiation of preneoplastic lesions in the prostate, a point which is clinically very relevant.^{62,67}

Retinoid Receptor, RXR α

There has been long term interest in retinoids and prostate biology.⁶⁸ Actions of retinoids are largely mediated by their nuclear receptors comprising retinoic acid receptor (RAR) and retinoid X receptor (RXR) families. The physiological consequences of RARs and RXRs inactivation have been investigated via conventional knockout technology.⁶⁹ Considering that the active retinoid receptor is mostly a heterodimer of one RAR and one RXR,⁷⁰ it was noteworthy that RAR γ null mutant mice develop squamous metaplasia of the prostate.⁷¹ Since mice lacking both RXR β and RXR γ are normal in terms of prostate morphology and function,⁷² the critical RXR in prostate biology appears to be RXR α . The human chromosomal region, 9q34.3, in which *RXR α* gene is mapped, is characterized by a high rate of recombination,⁷³ and, the incidence of loss of heterozygosity at this locus has been reported to be 20% in prostate cancer.⁷⁴ In a recent study, it was demonstrated that the nuclear expression of RXR α is generally down-regulated in human prostate cancer cell lines and specimens, and that manipulated overexpression of just RXR α subtype in prostate cancer cells can significantly induce cell death by apoptosis.^{75,76}

Since conventional disruption of the *RXR α* gene is embryonic lethal, the PB-Cre4/loxP system was used to inactivate *RXR α* alleles in the mouse prostate.⁷⁷ Developmentally, prostatic branching is increased from the loss of RXR α function. Histopathologically, homozygous *RXR α* -deficient prostates show multifocal hyperplasia as early as 4 months of age. Lesions, which are like LGPINs, are detected after 5 months. Subsequently, beginning at about 10 months of age, HGPINs develop in some animals, and could be present in any of the lobes. Similar to homozygous mice, the monoallelic mice appear to develop hyperplasia, LGPIN, and HGPIN in a temporal fashion, except that incidence is substantially delayed by several months (Fig. 2). Thus, it appears that, like NKX3.1, haploinsufficiency of RXR α could be a factor in prostate disease, the reduced production or delayed accumulation of which might promote a positive environment for proliferation and, perhaps following other secondary events, transformation to preneoplastic lesions. All in all, results from the model underscore that a major component of retinoid

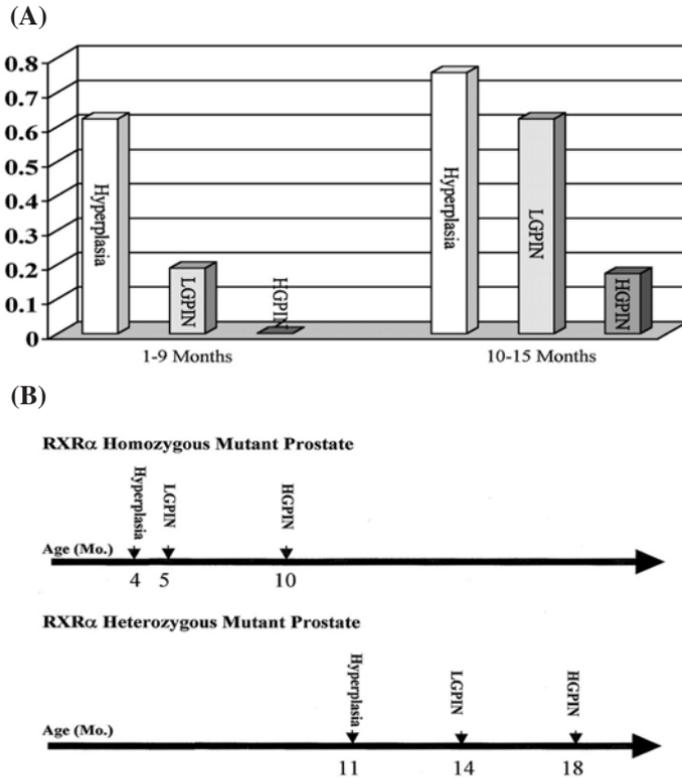


Fig. 2. Incidence and onset of prostate lesions in mice with monoallelic or biallelic inactivation of $RXR\alpha$ in the prostate epithelium. **(A)** Homozygous mutant prostate displays LGPIN after 5 months, while HGPN begin to appear after 10 months. **(B)** The heterozygous mutant mice also develop similar phenotypes, but in a delayed manner, implying a dose effect.⁷⁷

action in the prostate is mediated by a retinoid receptor, $RXR\alpha$, the inactivation of which in the prostatic epithelium leads to early stage neoplastic disease.

Cell Cycle Inhibitor p27Kip1 and Its Regulator SKP2

Protein ubiquitinylation for proteolysis of regulatory proteins in the cell is a complex process involving a series of components, both invariable and variable, which is targeted towards a specific protein. A ubiquitin E3

ligase, the SCF SKP2 complex, consisting of SKP1, Cull, Rbx1, and the F-box protein SKP2, and mediates the polyubiquitination of the CDK inhibitor p27Kip1.^{78–80} In this complex SKP2 serves as a substrate-targeting subunit that binds to the phosphorylated p27. Based on the knowledge of inverse relationship between the levels of SKP2 and p27, and by the general observation that low or lack of p27 expression may be associated with human cancers including prostate cancer,^{81–83} transgenic mouse lines were generated that express SKP2 under the control of the ARR2PB promoter in the prostate epithelium.⁸⁴ Overexpression of SKP2 down-regulates p27 and is associated with incidences of epithelial hyperplasia and dysplasia up to HGPIN in both VP and DLP glands. It is likely that SKP2 acts, at least partly, as an oncoprotein in the mouse prostate through induction of ubiquitin-dependent degradation of p27. There appear to be other mechanisms in play which, however, remain to be defined. For instance, although hyperplasia and dysplasia are prominent by 3 to 7 months of age in SKP2 transgenic mice, only hyperplasia, but not dysplasia, develops in p27-deficient mice at the advanced age of 14 months.^{83,85} One possibility could be a consequence of differences in the mouse strains used, but potentially more intriguing would be a relationship to differential levels of p27. The p27 protein, besides being a major CDK inhibitor in the cell cycle, may also function in the assembly of the cyclin D/CDK4 or CDK6 complex.⁸⁶ It is thought that if SKP2 expression fails to completely abolish p27 levels, as in p27 null mice, the residual p27 may be critical in sustaining the assembly role.⁸⁴ Still another possibility could be that SKP2 might promote earlier onset of hyperplasia and subsequent progression to dysplastic lesions by not only targeting p27, but perhaps other cell cycle regulators for proteolysis.

c-MYC Transcription Factor

c-MYC has been widely implicated in various human cancers.⁸⁷ Involvement of c-MYC in the multi-step tumorigenesis was first demonstrated in Burkitt's lymphoma in which the translocation from chromosome 8 to chromosome 14 results in deregulated c-MYC expression. Increased c-MYC gene copy number has been described in a significant portion of human prostate cancers^{88,89} and overexpression of c-MYC mRNA is detected in several prostate cancers.⁹⁰ To address the role of c-MYC in the

prostate, transgenic mice were generated in which the transgene is expressed from two different strength prostate epithelium-specific promoters, namely minimal PB and ARR2PB.⁹¹ Several interesting observations were made from the examination of these two model systems, designated Lo-Myc and Hi-Myc, respectively.

Multifocal proliferative lesions and dysplasia develop in both Lo-Myc and Hi-Myc mice mostly in VP and DLP. The PIN lesions appear to progress to invasive adenocarcinomas by 3 to 6 months in the Hi-Myc mice and by 10 to 12 months in the Lo-Myc mice. These results imply a dose effect of c-MYC in the rate of disease progression. Invasion noted to date is mostly local as determined from the penetration through the fibromuscular layer, and in some cancers, with detection of foci indicative of lymphovascular invasion. On the molecular level, c-MYC is known to normally regulate early cell growth and proliferation. In certain contexts, c-MYC also induces apoptosis, and, in fact, in *c-myc* transgenic models, both proliferation and apoptosis are evident, although it appears that proliferation may outpace apoptosis to some extent.⁹¹ It is also noteworthy that c-MYC levels are increased in the prostate after castration.⁹² Furthermore, a recent study describes that overexpression of c-MYC may lead to defects in double-strand DNA break repair and induction of chromosome translocations, suggesting that c-MYC may serve as a “dominant mutator” to accelerate tumor development.⁹³

Microarray-based expression profiling of the *c-myc* transgenics has led to identification of some signature gene activities, which are shared between human and mouse prostate cancers. For example, while NKX3.1 protein is detected in PINs at variable levels, it is not detected in the prostate tumor in the transgenics. Another gene, *pim1*, whose serine/threonine kinase product is known to cooperate with c-MYC in murine lymphomagenesis, is also overexpressed in tumors in the *c-myc* transgenic mice. Interestingly, PIM1 expression is increased in a subset of human prostate cancers that also display poor clinical outcome.⁹⁴

Protein Kinase AKT and Master Tumor Suppressor PTEN

The cellular proto-oncogene *c-Akt* was originally identified as a homologue of the viral oncogene *v-Akt*.⁹⁵ AKT is a protein kinase whose activity is positively regulated by PI3-kinase.⁹⁶ Activated AKT, in turn, controls

multiple signaling pathways, including cell survival and cell proliferation. Phosphatase and tensin homologue detected on chromosome 10 (*PTEN*) is a tumor suppressor gene, which is most frequently mutated or deleted in various human cancers. Germ line mutations in the *PTEN* gene are associated with Cowden Syndrome and related diseases in which patients develop hyperplastic lesions (hamartomas) in multiple organs with increased risks of malignant transformation.^{97,98} *PTEN* alteration is strongly implicated in prostate cancer development. Its deletions and/or mutations are found in 30% of primary prostate cancers^{97,99} and 63% of metastatic prostate cancers,¹⁰⁰ placing *PTEN* aberrations among the most common genetic alterations reported in human prostate cancers. The major function of PTEN relies on its lipid phosphatase activity that antagonizes the PI3K/AKT pathway.^{101–103} Loss of PTEN function results in accumulation of PIP3 and activation of its downstream effectors including AKT/PKB.^{42,104,105} As a serine/threonine protein kinase, AKT functions by phosphorylating key intermediate signaling molecules, such as glycogen synthase kinase 3, BAD, caspase 9, I κ B, and others leading to increased cell metabolism, cell growth, and cell survival.^{102,106,107}

To determine whether AKT activation is sufficient for the transformation of normal prostatic epithelial cells, a transgenic mouse line, with the use of a probasin promoter driving the expression of a constitutively activated form of *Akt*, was generated.¹⁰⁸ These animals develop hyperplastic/dysplastic lesions, which do not progress further, and the longevity of the animals is compromised apparently related to a bladder outlet obstruction. Analyses of gene expression profiles in the VP of this transgenic line led to some interesting findings that have relevance to human prostate tumorigenesis. For example, the up-regulated genes include prostate stem cell antigen (PSCA), which is expressed in prostate ductal tips during prostate development,¹⁰⁹ osteocalcin (or gla protein), which is overexpressed in prostate cancer,^{110,111} and angiogenin-3 and other family members which induce angiogenesis.

To directly assess the role of prostate-specific *Pten* deletion, mice harboring floxed alleles of *Pten* were crossed with the PB-Cre4 line.^{42,112,113} In another study, Cre/loxP strategy involving PB-Cre4⁴² or PB-Cre,⁵⁴ was used to define the effect of *Pten* deletion in the prostate.¹¹⁴ A “hypomorphic” strain of mice was also developed which exhibits only 25%–35% of PTEN compared to heterozygous *Pten* alleles (50%) and

wild-type (100%).¹¹⁴ Several exciting sets of information have been derived from these new models. In homozygous *Pten* null prostates,¹¹³ multifocal hyperplasia is evident starting from DLP and VP from 4 weeks and later reaching AP. These mice develop PIN at 6 weeks and prostatic adenocarcinoma at 9 weeks. The latency of PIN formation is shorter than heterozygous animals that display these lesions from 8 to 10 months. While heterozygous mice of this strain progress only up to HGPIN in their late life, mice with homozygous *Pten* deletion display invasive adenocarcinoma as early as 9 weeks. Practically all lobes are involved, and from 15 weeks, there is evidence for the cancer cell invasion into lymph and blood vessel systems. Some of the cancer cells appear to survive in the circulation, as seeding into distant sites such as subcapsular sinus of lymph nodes and the lung is evident. The metastatic tumor cells remain AR positive and null for PTEN immunostaining. The progression of the disease in this conditional *Pten* null mouse strain is depicted in Fig. 3.

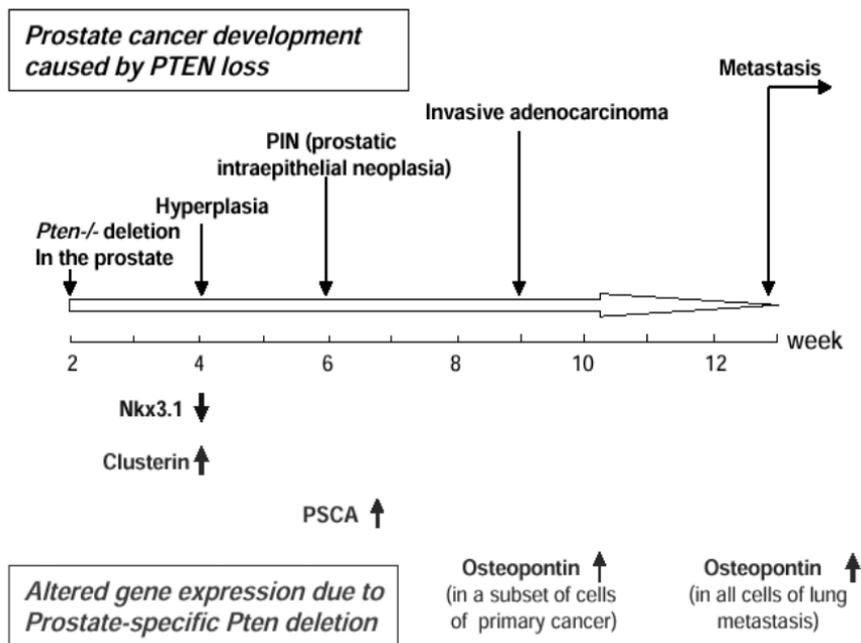


Fig. 3. A summary of onset and progression of prostate cancer development in mice with homozygous *Pten* deletion in the prostate epithelium.¹¹³

Androgen ablation in these mice reveals that the cancer cells do respond to the treatment, as there is a significant reduction of prostate volume. However, even though the survival of *Pten* null prostate cancer cells appears to be androgen-dependent, their proliferation is not sensitive to androgen withdrawal. This property of androgen-independent growth observed in this model is likely to contribute to formation of hormone-resistant cancer.

Although the mixed genetic backgrounds of the mouse strains used in different laboratories do limit direct correlation of the results, it is clear that the activity of PTEN is critical in suppression of prostate tumorigenesis. Inclusion of the hypomorphic *Pten* allele in the work of Trotman *et al.*¹¹⁴ underscores the point that PTEN dose dictates cancer progression in the prostate. Furthermore, this study also illustrates that qualitative morphological changes are a function of the number of cells suffering complete *Pten* inactivation as well as the strength of Cre expression.

As expected, in all *Pten* null prostate mouse lines, the AKT is activated. AKT-activated prostate epithelial cells are also larger than the wild type or heterozygous control cells, consistent with the role of PTEN in controlling cell size.^{115,116} While AKT activation is likely to be an important mechanism in this model, there must be other PTEN-directed signaling pathways that allow progression to invasive prostate cancer, since disease progression stops at dysplasia with AKT activation alone. In this regard, interesting clues are emerging from the global assessment of molecular changes caused by homozygous *Pten* deletion in the prostate, including changes in levels of factors like NKX3.1, PSCA, clusterin and osteopontin.¹¹³

Collaboration Between Signaling Molecules

Since carcinogenesis is a multi-step process involving a number of genetic changes or aberrations, successive development of increasingly complex mouse models with respect to these aberrations should be important in critical analysis of the disease progression. To date, only a few models have been developed incorporating up to two gene functions in a single system for prostate tumorigenesis, although most of them may lack the desired degree of tissue specificity. As the field is still at an early stage, only two examples are described below.

NKX3.1 and PTEN

The conventional knockout mice for *Nkx3.1* and *Pten* alleles were cross-bred to examine progression of prostatic lesions. The latency of PIN development in the NKX3.1 insufficiency background is reduced by half in the *Nkx3.1* +/-; *Pten* +/- mice.⁶⁷ Furthermore, evidence is obtained for the loss of NKX3.1 protein as well as the loss of the second allele of *Pten* in focal areas of tumors formed in the prostate of these compound mutants carrying each of the two genes as heterozygous alleles. The malignant potential of the pre-invasive lesions from the compound mice have further been investigated using a serial tissue recombination/transplantation assay or as a consequence of aging beyond one year of life.¹¹⁷ Such prolonged observation or propagation appears conducive to the development of invasive adenocarcinoma, which is also frequently accompanied by metastases to lymph nodes. These results are quite consistent with the observations made with the conditional inactivation of *Pten* alleles in the prostate, in which there is also concomitant disease in NKX3.1 protein levels.¹¹³

p27Kip1 and PTEN

Since loss of p27 and PTEN is a frequent observation in human prostate cancer, one approach was to attempt concomitant inactivation of one *Pten* allele and one or both p27 alleles.⁸⁵ p27 is a downstream target of the PTEN controlled signaling pathway. Activated AKT phosphorylates the forkhead transcription factors, such as AFX, FKHR, and FKHR-L1, which, in turn, act to reduce p27 mRNA and protein levels, leading to accelerated G1-S cell cycle transition.^{105,118,119} PTEN and PI3K pathways also regulate p27 protein stability. For example, PTEN deficiency in embryonic stem (ES) cells causes a decrease of p27 levels and concomitant increase of SKP2, a key component of the SCF SKP2 ubiquitin E3 ligase complex as described above.

While most of the *Pten* +/- heterozygous mice die within eight months from massive lympho-splenomegaly, long-term survivors develop a spectrum of epithelial tumors with propensities for those of adrenal, endometrial, and thyroid tissues. They also manifest a high incidence of

PIN after nine months.^{85,120} Mice null for *p27* alleles do not display enhanced spontaneous tumors.^{121,122} In this regard, it is noteworthy that when *Pten* +/- mice are produced in the background of *p27* null genotype, the compound mutants develop prostate carcinoma within 3 months postnatally with complete penetrance along with tumors of various other histological origins.⁸⁵ The prostate cancer is formed in DLP and AP, but not in VP, and is described to be locally invasive as rupture of the basal membrane of the epithelium is frequently seen. These results testify to a cooperative role of PTEN and *p27* in tumor progression in epithelial tissues including the prostate.

Summary and Conclusions

Evidence has been collected to demonstrate that dysregulated expression of endogenous genes in the mouse prostate can lead to stages of prostate tumorigenesis. Transgenic mouse models that represent robust and tissue-specific activation of FGF8b, FGFR1, or SKP2 in the prostatic secretory epithelium, and those that address genome-wide knockout of a target gene, such as *Nkx3.1* or conditional prostate-specific gene inactivation, such as RXRalpha, display a stochastic pattern of increasing degree of phenotypic abnormalities of lesions, beginning with epithelial hyperplasia followed by presentations of preneoplastic lesions. Other types of modeling, like overexpression of a strong proto-oncogene *c-myc* or disruption of the master tumor suppressor gene *Pten*, in the prostate epithelium, lead to the rapid development of invasive adenocarcinoma of the prostate with 100% penetrance. Evidence is accumulating to suggest that some of prostate cancer models, such as *Pten* null prostate, further progress to distant metastases in lung and lymph nodes, and possibly also to the bones for which more compelling data remain to be derived. While the adenocarcinomas in the advanced disease models respond to androgen ablation through increased apoptosis, the *Pten* null prostate cancer cells do appear to survive and eventually proliferate. With time, it is expected that the ability to proliferate under the condition of androgen deprivation may actually lead to development of hormone refractory prostate cancer as it happens in humans after prolonged hormone therapy.

The models generated to date are beginning to shed light into the molecular features of the disease. Global assessment of molecular changes in the advanced disease models has already identified key genes known to be relevant to prostate cancer. The preneoplastic disease models are being similarly scrutinized to identify molecular expression changes that are associated with progression from one early step to the next in time. Revelations of new molecular targets will likely contribute to the current theme of compound mutants to recapture the pathologic characteristics of the human disease to a fuller extent. In this regard, it would be important to increase focus on the models that favor the genesis and progression of the tumor in the DLP so as to increase relevance to the pathology that is frequently seen in the peripheral zone of the human prostate.

It is predicted that the next generation mouse models of prostate cancer, which are founded on prostate-specific, regulatable expression of appropriate proto-oncogenes in combination with conditional as well as temporal inactivation of relevant tumor suppressor or metastasis suppressor genes, will be forthcoming not too far in the future. These models will be of further value if simultaneously engineered with the power for non-invasive quantitation of tumor burden, its spread or colonization to distant sites. This capability is reflected in the recent successful generation of a reporter mouse that facilitates visualization of spontaneous tumor development via *in vivo* bioluminescence imaging.¹²³ Potentially, any Cre/loxP models for prostate cancer can be combined with this reporter line to make them amenable to longitudinal monitoring of tumor growth. Thus, the future complex models mimicking the sporadic prostate cancer and equipped with new approaches to follow the disease progression are anticipated to compliment the current models to a great extent. All of these will contribute to new insights into the pathogenesis of this common disease as well as for initiating the essential preclinical tests for therapeutic or chemopreventive regimens.

Acknowledgments

The research concerning modeling human prostate cancer in mice is primarily supported by NIH grant R01CA59705 (to P.R-B), and grants

U01CA98013 and DOD PC991538 (to H.W.). The authors thank Leslie Wauke for secretarial assistance in the preparation of this manuscript.

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