Dysregulation of p53 Gene Expression in Human Prostate Carcinogenesis and Its Relationship to Angiogenesis

A Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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*****

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Abstract

Prostate cancer is a common malignancy in affluent nations. Indeed, it is the most common visceral malignancy in American men impacting over 200,000 annually. The disease is heterogeneous in biology and the molecular biomarkers that define the various subtypes of prostate cancer have not been clearly defined. The host genetic factors and the acquired mutations that drive prostate cancer progression are only beginning to be elucidated. TP53 is a tumor suppressor gene that is known as the "guardian of the genome" due to its central role in the inhibition of the cell cycle and promotion of DNA repair, among many other anticancer activities. Recently our laboratory studies have implicated p53 dysfunction in the early stages of rodent carcinogenesis. Coincidentally, a patient with Li-Fraumeni Syndrome (LFS) presented to The Ohio State University Clinic with prostate cancer at a young age. LFS is an inherited cancer syndrome, with an autosomal dominant Germline mutation in the TP53 gene. Our extensive review of the literature revealed that guidelines for prostate cancer screening and treatment for men with LFS have not been established. We have provided a review focusing on the relationship of LFS and p53 to prostate carcinogenesis. Indeed, men with LFS warrant earlier screening in hope of diagnosing disease at an early stage where therapy can be provided with curative intent. We recommend that LFS men avoid, if at all possible, external beam irradiation and chemotherapy that is associated with DNA damage and risk of secondary cancers. We then pursued the frequency of p53 dysfunction in a set of prostatectomy cases using immunohistochemical techniques. We procured and examined samples from the Health Professionals Follow-up Study (HPFS) initiated in 1986 at the Harvard School of Public Health.
with recruitment of over 50,000 men volunteering to participate in this prospective cohort. We created a prostatectomy tissue bank for tissue samples and over 500 cases were available for our studies. The rate of intense nuclear p53 staining indicating a dysfunctional protein that cannot be degraded in noncancerous tissue is 0%, but is 20% in cancer. We further showed that p53 staining is associated with more aggressive disease in relation to cancer grade. Our rodent studies also suggest that the early overexpression of p53 occurs in parallel with establishment of tumor vascularity. Thus, we hypothesized that prostate tumor vascularity may be related to p53 expression. In order to address this relationship, we first needed to fully characterize objective biomarkers of tumor angiogenesis. Thus, we have focused upon digital image analysis to provide quantifiable endpoints related to angiogenesis. We first used antibodies to clotting factor VIII, known as von Willebrand’s factor, to outline vascular endothelial cells in human prostate tissue by immunohistochemistry in both normal and cancer. We report a dramatic increase in microvascular density in cancer samples (p<.0001). In addition, irregularity of the vessels is much greater in cancer (p<.0001). Finally, vessels of smaller diameter and perimeter are found in cancer compared to noncancer (p<.0001). Based upon preliminary data indicating that CD34 antigen on vascular endothelial cells is a marker of even more immature vessels than FVIII, we chose immunohistochemical staining with anti-CD34 to evaluate vascularity in prostate cancer relative to tumor grade and stage. Our analysis shows a significant correlation with grade (p<.0001). Finally, we report that p53 staining is associated with angiogenesis. Future studies will assess the mechanisms of their interaction and how these two biomarkers predict prognosis.
Dedication

To Stevyn, Bryce, and Bailey – thanks for doing your homework with mommy!

To my husband, Brad, who sacrificed much, always without complaint.

I love you all.

To all those families, including my own, affected by the horrific disease known as Li-Fraumeni Syndrome.

This is for you...
Acknowledgements

It takes a village...

This labor of love has been an incredible journey, both personally and professionally. Along the way, I have relied on MANY incredible individuals to serve as mentors, teachers, advisors, counselors, and constructive critics. As such, it is with tears that I write these acknowledgments. Although I could never pay appropriate homage to these individuals with mere words; I will forever hold them in my heart.

"Never doubt that a small group of thoughtful, concerned citizens can change world. Indeed it is the only thing that ever has." - Margaret Mead

Dr. Kay Wolf is the living definition of a servant leader. Her quiet, self-sacrificing (and sometimes misunderstood) demeanor has given me great strength in times of doubt and uncertainty. Kay knows me and still likes me. I've had lots of bosses; Kay is simply the best.

“Some men see things as they are and ask why.
Others dream things that never were and ask why not.” - George Bernard Shaw

Dr. Steven Clinton is one of the most unique and eclectic forces of nature that I have ever had the pleasure of meeting. Although I have spent much time under his direct guidance, he remains a mystery. Dr. Clinton demands excellence, yet somehow remains gentle, unbiased, and noncritical in terms of delivery and mannerism. He is an incredibly talented and gifted
teacher that continually challenges me to “think like a scientist” while pushing me to step outside my comfort zone (even when I REALLY don’t want to). As a research mentor and investigator, he is unparalleled.

I would also like to acknowledge Dr. Diane Habash for her enthusiasm, energy, and friendship. Diane has spent countless hours listening to my grandiose ideas on how to save the world. Then, with a sweet smile on her face, she gently pulls me back to reality and sets me on my way, more focused and determined than ever. Diane is a trusted friend and respected colleague. She is the best kept secret at The Ohio State University.

I would like to express my gratitude to all members of the Medical Dietetics and Health Sciences hallway for their resolute support. Dr. Chris Taylor came to my rescue a million times over, and I am forever indebted. Between Chris and the others, I could always count on lots of smiles and hugs when I entered the department. Many times I needed it - thanks, I love you guys.

I would like to recognize my committee members, Drs. Anne Smith and Sally Rudmann. These two women are incredible role models for me. They are successful yet remain ‘real’ and centered. Anne and Sally give me great hope.

I would also like to thank members of the Clinton laboratory, both past and present (especially Beth, Val our “Original Lab Goddess,” Rob, Anna, Jun, Kim, Shana, Stephanie, and Jen, our “New Lab Goddess”). Each one of these individuals have made my voyage a bit easier in some way – by offering encouragement, helping me locate samples quickly in the -80 freezer, leading me to a printer that actually works, providing a smile during some tougher-than-usual presentations, working beside me into the wee hours of the night, and always lending a hand
when I was drowning in work. I must acknowledge members of my PhD cohort, especially Karen. Keep the faith.

A special thanks to Dr. Phil Binkley, Stephanie, Val, and others at the CCTS for supporting me.

Lastly, to my big brother and mentor in life, Dr. Kelly Kelleher. Where shall I start? Kelly is one of the very few people that truly appreciates and understands the suffering that has led me to this journey. Certainly, we’ve not won every battle and have many more to face, yet we’re (hopefully) headed in the right direction. Although I will always pale in comparison as a person and a researcher...mom still likes me better.

I am fortunate to remain surrounded by an abundance of mentors, friends, family, and colleagues in all aspects of my life. Without a doubt, I am one lucky girl.
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Major Field: Health and Rehabilitative Sciences
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Chapter 1: Introduction and Background

Prostate cancer (PCa) affects one in six American males, making it the most frequently diagnosed cancer in men. In the United States, it is estimated that over 240,000 new cases of PCa will be diagnosed and over 33,000 men will die of PCa in 2011 (Figure 1.1). In addition, 1 in 36 men diagnosed with PCa will die of the disease (2). Despite recent advances in both screening and treatment options, clinicians and patients are confronted with limited scientific evidence discriminating aggressive versus indolent tumors. Key molecular and environmental biomarkers related to prostate carcinogenesis remain invalidated and unable to inform treatment decisions to impact mortality.

Because TP53, a tumor suppressor gene (TSG), is central to metabolic and apoptosis pathways in the cancer cascade process, we proposed to examine p53 protein expression as one of, if not the key indicator of tumor aggression and mortality in a longitudinal cohort study. Although the TP53 gene is central to cancer research, there remains a significant gap in the literature specific to p53 protein dysfunction relative to molecular biomarkers of prostate carcinogenesis. Because the TP53 gene encodes key cell cycle and survival regulatory proteins, mutations of this gene may play a central role in PCa progression. To answer this question, we evaluated p53 status, by quantitative immunostaining technology, to define its relationship to established clinical diagnostic biomarkers.
## Estimated New Cases

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<th>Cancer Type</th>
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<th>Females</th>
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<td>Prostate</td>
<td>192,280</td>
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<td>Lung &amp; bronchus</td>
<td>116,090</td>
<td></td>
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<tr>
<td>Colon &amp; rectum</td>
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<td>Urinary bladder</td>
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<tr>
<td>Melanoma of the skin</td>
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<td>Non-Hodgkin lymphoma</td>
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<td>Kidney &amp; renal pelvis</td>
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<td>Leukemia</td>
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<tr>
<td>Oral cavity &amp; pharynx</td>
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<tr>
<td>Pancreas</td>
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<td>All Sites</td>
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## Estimated Deaths

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<td>Lung &amp; bronchus</td>
<td>88,900</td>
<td>70,490</td>
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<td>Prostate</td>
<td>27,360</td>
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<tr>
<td>Colon &amp; rectum</td>
<td>25,240</td>
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<td>Kidney &amp; renal pelvis</td>
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<td>All Sites</td>
<td>292,540</td>
<td>269,800</td>
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*Figure 1.1: Ten leading cancer types for estimated new cancer cases and deaths, by sex, in the United States (2).*
Evidence has revealed that angiogenesis contributes to tumorigenesis heterogeneity (4, 5). Formation of a hearty vascular network contributes to tumorigenesis and microenvironment alterations often noted in the cancer cascade (6). Neovascularization and continual architectural vessel changes appear to relate to a tumor's hostile and destructive behavior (1, 6). Specific to PCa, greater microvessel density (MVD) in cancerous tissue is suggestive of more poorly differentiated tumors (7-9) and disease aggressiveness and lethality (10, 11). To date, few concrete studies concentrating on PCa angiogenesis exist. In our study cohort, Mucci and Clinton (12) showed that prostatectomy patients with the most irregular vascular architecture were seventeen times more likely to advance to lethal disease. Building upon these dramatic results, we assessed immunohistochemical (IHC) staining of von Willibrand Factor VIII (FVIII) in both malignant and noncancerous post-prostatectomy tissue to determine if FVIII is a viable biomarker of PCa progression.

**Prostate Biology and Cancer**

The prostate is a gland that is anatomically located under the bladder, in front of the rectum and surrounding the urethra. The function of the prostate is to produce seminal fluid. Androgen hormones, including testosterone, are predominately produced in the testicles and can cause abnormal prostate growth that constricts the urethra. By far, aging is the primary risk factor associated with PCa. In the United States, most men diagnosed with PCa are over the age of 65. Family history, race, prostatic changes, and genetic factors also appear to contribute (13). In malignant cancers, tumor cells multiply uncontrollably and eventually break through the basement membrane to enter the lymph system. Uninhibited, cancerous cells nurture new tumor growth, leading to distant metastasis.

Prostate cancer is confirmed by pathology and graded according to tumor tissue differentiation and patterning (Figure 1.2). The principal grading system utilized in prostate
Cancer classification is the Gleason Score (3). Gleason grades (GG) are based upon the sum of the primary and secondary cancerous tissue patterns in which a score of 2 (1 + 1) represents mainly normal cells and 10 (5 + 5) represents the most poorly differentiated cell types. Tumors with higher GGs are associated with more aggressive cancers that are related to poorer prognosis and mortality (14). Cancer staging is also utilized to determine distant metastasis and is used in conjunction with GG to inform treatment decisions. For PCa, Stage I refers to the most docile tumors that may be detected during another medical procedure. In contrast, stage IV defines aggressive tumors that may have invaded nearby structures or is recognized distantly.

Histological and pathological evidence are heavily weighed when determining the best course of action. Active surveillance (“watchful waiting”), radiation, hormone therapy, chemotherapy, and surgery remain common and viable management options for PCa. If a radical prostatectomy is elected, the entire prostate gland is removed along with any suspect surrounding tissue. The clinical goal of this procedure is to completely remove the cancerous mass and prevent distant metastasis and recurrence of disease. Success rates for radical prostatectomy vary according to disease risk: low risk (76-98%); moderate risk (60-76%); and high risk (30-76%) (15-18). Indeed, PCa is an extremely heterozygous and complex disease.
Figure 1.2. The Gleason Grade (GG) is a biomarker system of PCa progression based upon microscopic appearance. Cancers with higher combined Gleason scores are typically more aggressive and less differentiated. The first score is assigned to the most prevalent tumor pattern, and the second grade corresponds to the next most prevalent tumor pattern. The sum of the two grades are added together for the total Gleason Sum. The GG ranges from 1 to 5, with 5 depicting the least differentiated cells. The Gleason sum ranges from 2 to 10, with 10 being associated with the worst prognosis. Image adapted from Gleason (1974) (3).
Cell Biology and Cancer

Cancer is a multistep process in which genetic alterations drive transformation of normal cells to malignant tumor cells. Hanahan and Weinburg originally defined six hallmarks to most, if not all, cancerous cells: growth signal autonomy, evading apoptosis, insensitivity to anti-growth signals, tissue invasion and metastasis, unlimited proliferation potential, and sustained angiogenesis (19). In their revised version (1), the authors further refined their hallmarks based upon novel discoveries that did not exist when their seminal work was published. Two new hallmarks (enabling characteristics and emerging hallmarks) have been added to the original six. Reprogramming energy metabolism and immune system evasion have yet to be permanently assimilated as more evidence is needed to define their specific roles in carcinogenesis (Figure 1.3).

Normal cell cycle division ultimately results in DNA replication and division. Inherent within are internal control mechanisms that propel cells from one stage to the next with a series of checkpoints that monitor cellular events (20, 21). Ultimately, cell cycle checkpoints scrutinize critical events and are activated in response to recognition of errors of duplication or progression. Once activated, checkpoints delay cell cycle progression until the error has been successfully averted. Unfortunately, cancerous cells evade these normal cell cycle controls and engage in exponential cellular proliferation. This vicious cycle results in checkpoint insensitivity that further eludes mechanistic controls (21).
Figure 1.3. The next generation hallmarks of cancer that include the original 6 acquired defects of cancer (growth signal autonomy, not paying attention to the stop signs, evasion of apoptosis, angiogenesis, unlimited replicative potential, and invasion and metastasis) plus enabling characteristics and emerging hallmarks (1).
Mutations in oncogenes (gain-of-function genes) and tumor suppressor (loss-of-function genes) are related to cancer development and progression (22, 23). Tumor suppressor genes (TSG) were originally recognized as inherited cancer susceptibility genes (24, 25). Unlike gain-of-function (GOF) mutations, loss-of-function (LOF) mutations are often autosomal dominant and familial patterns may be identified via pedigree analyses. Heterozygous genotypes may present as phenotypically normal until a mutational insult inactivates the remaining functional allele (26). Mounting evidence suggests that TSGs may promote a significant number of both spontaneous and hereditary forms of cancer. In terms of the cell cycle, TSGs, such as \textit{TP53}, are critical in cell cycle checkpoint activation. Loss of p53 protein function removes the ability to halt division of damaged cells. Clearly, cell cycle regulation is a vital component in carcinogenesis, and p53 is a key regulator of this process (27). The literature deviates from powerful studies confronting p53 dysregulation related to histopathologic and prognostic variables specific to PCa. Specific Aim 1 and 2 directly confront this issue.

**Biomarkers of Cancer**

A biomarker is a biological substance that can be measured and is correlated with disease risk (28). Particular cells, molecules, genes, gene products, hormones, and enzymes often serve as biomarkers. Disease-related biomarkers indicate risk of disease, prediction of disease progression, or existence of a current disease state. The U.S. Food and Drug Administration (FDA) has defined two types of clinical biomarkers: (1) a probable biomarker having well established performance characteristics for which there is a scientific framework or body of evidence that appears to elucidate its physiological, toxicological, pharmacological or clinical significance; (2) a validated biomarker having well established performance characteristics for which there is widespread agreement in the scientific and medical
community about its physiological, toxicological, pharmacological or clinical significance (29). In
terms of PCa, identification of biological stratification markers could help guide therapeutic
applications.

Prognostic biomarkers for PCa screening, detection, and prognosis have modernized
disease management. Prostate specific antigen (PSA) is a serum biomarker that is produced, in
small amounts, in normal prostate cells. In 1994, the FDA approved PSA for PCa screening and
defined 4.0ng/ml as the upper limit of “normal” (30). Since that time, widespread application of
PSA screening has resulted in stage migration as PSA levels have become a common biomarker
used to signal early PCa (31). Worldwide, PSA-based screening has been associated with overall
reductions in PCa specific mortality (32). However, much controversy exists over PSA screening
for early disease, since no randomized evidence has validated a rigorous association with a
reduction in mortality (30).

In primary care, PSA remains one of the only serum biomarkers consistently utilized and
referenced. Although the PSA-era has revolutionized PCa detection, PSA lacks specificity for
early stage disease. Approximately 15% of PCa occurs in individuals presenting with very low
PSA levels (30). Other PSA confounders include nonmalignant physiological conditions of aging
(e.g., benign prostatic hyperplasia and prostatitis, prostatic manipulation (via exercise or
physical examination), medical interventions (cystoscopy), and patient/tumor heterogeneity)
(33, 34). Indeed, PSA is a useful, yet nonspecific, biomarker of PCa. Given these limitations and
the fact that genetic testing is both cost and time prohibitive, clinicians are eager for novel
prognostic biomarkers, or combinations thereof, in which to guide treatment regimens (35).
p53: The Guardian of the Genome

p53 is a key tumor suppressor protein, encoded by the TP53 gene that is deleted or mutated in over 50% of human cancers as an acquired mutation (36-41); yet its role in prostate carcinogenesis has not been clearly defined. In addition, inherited defects in the TP53 gene are associated with the Li-Fraumeni Syndrome (LFS) inherited cancer syndrome (42, 43). p53 serves as the “guardian of the genome” (44) and is critical for normal cell senescence and programmed cell death, particularly when genetic damage and genotoxic stress has occurred (25, 45). Within the cell cycle, p53 serves as a transcriptional monitor essential in G1 phase cellular growth arrest secondary to DNA damage. Furthermore, p53 assists with spindle checkpoint regulation, homeostatic centrosome balance, and G2-M phase transition (46)(Figure 1.4). During nonstressful and normal cellular conditions, the p53 protein level remains low. DNA damage and cellular stressor signals trigger an increase in p53 proteins that result in growth arrest, DNA repair or programmed cell death. Growth arrest halts cell cycle progression, thus preventing replication of the damaged DNA. During cell cycle growth arrest, p53 may activate proteins involved in cellular DNA repair. Apoptosis is the final step to avoid uncontrolled proliferation of cells containing mutated DNA. Throughout, the concentration of p53 remains tightly regulated. While p53 can ultimately suppress tumors, high levels may accelerate the cellular aging process by acute apoptosis.

p53 and Carcinogenesis

During the carcinogenesis process, cells essentially override the function of p53 in these cell cycle checkpoints. This is due to a number of reasons, including mutation of p53, resulting in dysfunctional protein expression or complete lack of expression. This dysfunctional or
deleted TP53 gene results in significant disruptions in cell cycle regulation (47), inhibits cellular repair of injured DNA (48), stimulates proliferation rates (49), permits expression of endogenous Pro-angiogenesis factors (50), and blocks apoptotic signaling (48), all contributing to cancer progression (25).

Figure 1.4. The normal cell cycle is defined by a sequence of events that depend upon each other. Cells are equipped with cell cycle checkpoints to protect against proliferation of mutated cells. DNA damage checkpoints are classified into 3 key areas: G1/S (G1), intra-S phase, and G2/M.
**p53 as a Biomarker of Prostate Cancer**

Successful identification and classification of biomarkers correlated with a more hostile disease phenotype remains precedence in PCa and could reserve radical treatments for patients displaying highly aggressive disease (Figure 1.5). The utility of delineation of aggressive rather than indolent PCa remains a key challenge given current prognostic parameters. The TP53 gene has been recognized as a practical biomarker with significant clinical value in multiple cancers (51, 52). Specific to PCa, there is evidence from prior studies using smaller cohorts indicating that p53 immunostaining correlates with tumor progression, tumor recurrence and prognosis (53-61). To the best of our knowledge, no one has compared the differences between the areas of malignancy and the corresponding nonmalignant tissue. Hence, our current proposal is absolutely critical in correlating measures of p53 expression with PCa disease stratification in a larger, more powerful prospective cohort study that encompasses a rigorous and standardized study design.

Evidence from the literature suggests that p53 may serve as a clinically relevant biomarker for PCa. IHC enhances the morphologic differentiation assessed by hematoxylin and eosin (H&E) alone (62). Ideally, disease stratification biomarkers should be validated in large, prospective, tightly-controlled and widespread clinical studies with standardized protocols (46, 63). There are currently no studies assessing p53 expression in the normal versus cancerous prostate and the relationship with disease progression a large cohort powered to detect such differences. p53 immunostaining is not commonly utilized clinically as a standard diagnostic biomarker for PCa.
Figure 1.5: Biomarkers of prostate carcinogenesis include histological, angiogenesis, and proliferation biomarkers. By identifying biomarkers of prostate carcinogenesis, we can better predict aggressive (versus indolent) disease.
There are a number of issues to consider when using immunostaining as a standard diagnostic tool in general and specifically for p53. The first issue is related to the biological differences. It has previously been observed that there is stratification of intensity in p53 IHC. It is hypothesized that p53 protein is undetectable or is present at a very low level in resting cells and that it is found in large amounts in a wide variety of transformed cells and in many actively proliferating and nontransformed cells. The second issue relates to technical considerations. In order for any potential biomarker to be incorporated into clinical practice, standardization in biospecimen handling, processing, and assessment is required. It is well established that differences in fixation time, antigen retrieval, antibody concentration and incubation time can dramatically impact staining quality and intensity. With this stratification in staining intensity, there is a need to assess the relationship to prostate carcinogenesis where standard techniques are used to investigate the biological contribution.

Lessons Learned From Li-Fraumeni Syndrome

In addition to assessing p53 as a clinically diagnostic biomarker, we aim to evaluate the biological function of p53 in carcinogenesis and cancer prevention strategies. In 1969, American physicians Frederick Li and Joseph Fraumeni defined a cluster of familial-patterned, early onset soft tissue tumors (64). Since then, the original criteria have been expanded to include a much larger spectrum of autosomal dominant tumors linked with TP53 mutations (65, 66). The phenotype of a LFS individual presents with an array of primary tumors, making disease surveillance and risk stratification impractical, if not impossible. Early identification of LFS is particularly daunting given the heterogeneity of the symptoms, even within biologic families. Because LFS is a highly penetrant cancer predisposition syndrome (67-69), it provides an excellent human model for biomarker discovery and signature validation.
Nutritional factors also play a key role in prostate carcinogenesis (70). Although no human studies have addressed dietary intake specific to LFS, murine models have revealed that tumor development is delayed in energy restricted p53 null (-/-) mice (71) (Figure 1.6). p53 heterozygous (+/-) mice showed similar results (72). Radiation-induced secondary malignancies have been noted among LFS individuals and p53-deficient mice have confirmed cancer secondary to radiation sensitivity (73-76). These results suggest that in the LFS genotype, modifiable environmental behaviors appear to impact the phenotype and thus, improve prognosis. To date, no uniform surveillance measures or biomarkers have been identified for LFS-positive individuals (68, 77). Clinical evidence has shown an association between TP53 missense mutations and poor prognosis (78, 79).
Figure 1.6. Hursting’s murine survival curves in p53 wild type, heterozygous, and null animals. The survival curve for wild type (p53 +/+) murine models is high in comparison to heterozygous (p53 +/-) and null (p53 -/-) mice (71) indicating that survival decreases in line with mutations in p53 alleles.
Studying LFS models has great research advantages, especially within biological family studies. Early identification of at-risk individuals and families is paramount so that predictive testing may be employed for surveillance, prevention, and treatment (80). Hence, the need for research in this area is enormous as discovery of predictive and prognostic biomarkers could revolutionize our understanding of p53 and cancer biology. The interrelationship between p53 and predictors of PCa is incredibly multifaceted and impacted by numerous variables. A critical goal of Aim 1 was to thoroughly examine such relationships by utilizing a case report and extensive literature review to inform clinical recommendations for this unique population. It is critical that the results of this aim be made public. To date, clinical recommendations specific to the treatment of PCa in LFS affected patients is nonexistent.

**Angiogenesis and Carcinogenesis**

There is a deep-rooted association between carcinogenesis and angiogenesis. Indeed, angiogenesis is one of the key hallmarks of cancer (1). The establishment of a hearty vascular foundation plays a central role in the carcinogenic cascade by promoting nutrient and oxygen delivery, the elimination of waste, and providing a conduit for metastasis (6, 81)(Figure 1.7). Angiogenesis enhances cellular metastases by providing cancerous cells direct access to new vasculature that are architecturally primitive, functionally compromised, and morphologically embryonic (6).
Figure 1.7. Cartoons depicting the angiogenic switch leading to cancer progression and metastases as new vasculature provides growing cancer cells with nutrients, oxygen, elimination of waste, and a conduit for metastatic travel (82).
Microvessel density (MVD) refers to the number (whole number count) of BVs within a specified area. The gradation of neoangiogenesis, as measured by MVD, blood vessel (BV) mass, and vessel architecture, seems to be associated to a tumor’s aggressiveness of behavior (1, 6). Specific to PCa, greater MVD in cancerous tissue is symptomatic of more poorly differentiated cancers (7, 8, 83) and disease lethality (10). Surprisingly, very few prospective studies exist that focus exclusively on angiogenesis in PCa. Our laboratory has revealed (12) that irregularity of vessel shape and size correlates with lethality in PCa patients. There are no studies characterizing the differences between the cancerous tissue and the adjacent non-cancerous tissues with respect to vascular architecture utilizing Factor VIII immunostaining.

**Angiogenesis as a Biomarker of Prostate Cancer**

For MVD to be considered an effective and valuable biomarker, it must compare with survival and other previously recognized biomarkers of prostate carcinogenesis. Some generally applied biomarkers of PCa aggressiveness include seminal vesicles involvement, presence of regional lymph nodes, positive pathological margins (84), extraprostatic extension, heightened prostate specific antigen (PSA) levels (>4ng/ml) (85), and greater tumor stage (TNM), and GG (86).

Because angiogenesis encourages cancer growth and progression, greater MVD may have an undesirable impact on a patients’ prognosis. This hypothesis was confirmed in a number of studies that reported an inverse relationship between MVD and survival (8, 10, 50, 87, 88). In addition, two studies found that MVD was an independent biomarker predictive of survivorship (50, 89).

For our project, we assessed immunohistochemical staining of FVIII in both malignant and noncancerous prostate tissue. This study is unique in approach and assesses quantitative
measures of angiogenesis as a possible biomarker to predict biological subtypes of PCa that may help to inform clinical decisions in this population.

**p53 and Angiogenesis: Molecular Biomarkers of Prostate Cancer**

Uncontrollable cellular proliferation and diminished apoptosis are hallmarks of cancer (1). Tumor suppressors, such as p53, regulate anti-cancer processes under normal conditions. During cancer progression, p53 is frequently mutated (1). It has yet to be determined whether acquired mutations in key genes play a role in the process of neovascularization. Some studies do report a significant connection between mutant p53 and increased MVD (50), especially in tumors classified by a higher GG (90). These studies reveal that a higher MVD, in the presence of p53 mutations, were positively correlated with advanced disease and reduced survival (50, 57, 90, 91). These findings suggest that p53 mutational events may confer apoptotic resistance and a pro-angiogenic phenotype. Further research is necessary to fully elucidate associations between TSGs, oncogenes, and mutational events that modify PCa phenotypes and progression.

**Specific Aims and Overall Hypothesis**

We hypothesize that p53 protein expression and tumor vascularity measures correlate with the cancer phenotype (cancer versus noncancer) and established biomarkers of disease (grade and stage). Such evidence will support their establishment of potential biomarkers to segregate indolent versus aggressive disease and provide additional insights into their function during prostate carcinogenesis. We also hypothesized that cancer tissue would contain vessels of greater number (MVD), smaller diameter, smaller perimeter, and more irregular vessel architecture than noncancer prostate tissue of the same cases. We further propose that markers of angiogenesis (FVIII) will correlate with p53 overexpression in the same cases, predicting aggressiveness of disease.
We conducted a prospective cohort study of angiogenesis and PCa in 500 men that were diagnosed with localized PCa and selected radical prostatectomy for initial therapy. We quantified tissue expression levels of p53 and angiogenesis (FVIII) in relation to established PCa grading and staging (Specific Aims 2 and 3). The impact on PCa biology and progression were further defined by assessing the association between p53, angiogenesis and histopathologic biomarkers in normal and cancerous prostate (Specific Aims 2 and 3).

Li-Fraumeni Syndrome, p53, and Prostate Cancer: Implications for Screening and Therapy (SA1)

Our goal was to present a LFS-PCa case report and highlight the need for enhanced awareness of cancer genetics in those treating urologic cancers, and consideration of how inheritance may impact screening, diagnosis, therapy, and survivorship monitoring. As genetic testing allows for early detection of LFS, a greater proportion of LFS men are living to an age where PCa incidence increases. We report a case of clinically localized PCa in a young LFS patient and discuss implications for clinical assessment, ongoing surveillance and medical management.

Prostate Cancer Biomarker: p53 Dysregulation (SA2)

Our goal was to validate relevant tumor biomarkers of PCa aggressiveness as related to p53 in a large and statistically powerful prospective cohort study. Although the TP53 gene is central to cancer research, there remains a significant gap in the literature specific to p53 protein dysfunction relative to short and long term biomarkers of prostate carcinogenesis. Because the TP53 gene encodes key cell cycle and survival regulatory proteins, p53 dysfunction may play a role in PCa progression. We evaluated the relationship between p53 expression by immunostaining and cancer status and established diagnostic biomarkers.
Prostate Cancer Biomarkers: Angiogenesis (SA3)

Our goal was to authenticate pertinent biomarkers of PCa aggressiveness as related to angiogenesis (FVIII) and vascular architecture in both cancer and noncancerous human prostate in a large and statistically powerful prospective cohort. Although markers of angiogenesis are central to cancer research, there remains a significant gap in the literature specific to the relationship between the cancerous prostate and noncancerous tissue of the same case. We assessed measures of angiogenesis (i.e. MVD, BV area, roundness, perimeter, and diameter) by IHC and quantitative digital image analysis to delineate a signature of FVIII vasculature that may serve as a biomarker of critical molecular events involved in cancer progression.

Specific Aim 1. Li-Fraumeni Syndrome and prostate cancer: review of the literature and implications for screening and therapy.

After an extensive review of the literature, we determined that there remains a colossal gap in the literature focusing on PCa patients affected by LFS. We utilized a case report approach to introduce this topic and created comprehensive and evidence-based clinical recommendations for identification, surveillance, and management of patients presenting with LFS and PCa.

Specific Aim 2. We hypothesize that overexpression of p53 occurs in a significant proportion of human PCa and is associated with a higher GG and stage at the time of diagnosis. In addition, overexpression will be more commonly associated with disease aggressiveness in a longitudinal cohort.

To test this hypothesis, we utilized robotic IHC technology and digital image analysis in over 500 cases of human PCa to quantify p53 status and compare our findings with traditional
pathological grading and staging criteria for prostate carcinogenesis. We propose that p53 overexpression will correlate with more lethal grades and pathological stages of human PCa.

**Specific Aim 3.** We hypothesize that Factor VIII immunostaining will correlate with markers of angiogenesis. In addition, we proposed that FVIII expression will reveal a greater pro-angiogenic phenotype in malignant cells as compared to normal (noncancerous) prostate.

We propose that markers of angiogenesis will correlate with a more aggressive vascular architecture in cancer versus noncancer human prostate in a large prospective cohort. Markers of angiogenesis (FVIII) include newly validated and quantifiable outcomes defined by novel digital image analysis techniques and include vascular density, vessel area, vessel shape, and vessel size.

**Innovation and Value**

The proposed study directly confronts a critical and controversial scientific issue relevant to public health. This project is a direct extension of significant findings from current literature in the field and findings from our laboratory and will fill a gap in the areas of PCa biomarkers relative to human prostate carcinogenesis. Our research strategy involves a multifaceted, transdisciplinary collaboration designed to optimize the translation from research discovery to clinical application. These data will provide a basis for future clinical trials to identify the role of p53 and FVIII in human PCa and provide novel insights into biomarkers of disease progression and prognosis.
These efforts, taken together, will provide data to support future studies aimed at assessing environmental and genetic interactions that are critical to our understanding of human prostate carcinogenesis and mortality. In addition, our LFS-PCa review and clinical recommendations will serve as a landmark document to inform clinical recommendations until large-scale clinical trials can be conducted in this unique and high risk population.
Chapter 2: Li-Fraumeni Syndrome, p53, and Prostate Cancer: Implications for Screening and Therapy

Abstract

Li-Fraumeni Syndrome (LFS) is an autosomal dominant germline disorder associated with mutations in the TP53 gene and characterized by a propensity to develop a variety of malignancies typically resulting in shortened lifespan. Prostate cancer is currently not considered one of the core spectrum malignancies characteristic of Li-Fraumeni Syndrome. As the syndrome is identified at earlier ages and affected individuals are monitored and successfully treated for developing cancers, a greater proportion of LFS men are living to an age where prostate cancer incidence increases. We present a LFS patient with clinically localized prostate cancer detected by screening at age 50. Considerations of potential risks second malignancies as a result of radiation and chemotherapy in LFS men may impact management decisions for prostate cancer. Although human studies are limited, experimental studies suggest that the presence of TP53 mutations in prostate cancer is associated with therapeutic resistance, implying that LFS men with prostate cancer may have a more aggressive biology or clinical course. Most importantly, this case highlights the need for enhanced awareness of cancer genetics in those treating urologic cancers, and consideration of how inheritance may impact screening, diagnosis, therapy, and survivorship monitoring. The establishment of the global International Agency for Research on Cancer (IARC) TP53 Germline Mutation Database in Familial Cancers and the Li-Fraumeni Exploration International Consortium (LiFE) will
accumulate data on afflicted families and serve as a research resource to inform clinical care. As statistical power increases, a more precise assessment can be made regarding prostate cancer as one of the core spectrum of malignancies in LFS and the guide our efforts at early detection and cure.

Introduction

Over 4 decades ago, Drs. Frederick Li and Joseph Fraumeni described a familial cancer predisposition syndrome most consistent with a Mendelian dominant inheritance pattern (92). Later termed Li-Fraumeni Syndrome (LFS), carriers were found to display higher cancer incidence rates, beginning in children and young adults, with multiple cancers within an individual and multiple affected family members (66). In contrast to most inherited cancer syndromes, characterized by site-specific cancer, LFS exhibits various tumor types. The common primary cancers in LFS are termed “core malignancies” and include soft tissue sarcomas and osteosarcomas, breast cancer, brain tumors (astrocytomas, glioblastomas, medulloblastomas, and choroid plexus carcinomas), acute leukemia, and adrenocortical carcinoma (74, 93, 94). These core compartment cancers account for about 80% of all LFS-related tumors (94, 95). The lifetime cancer penetrance is very high, and currently estimated to be 93% by age 50 in women and 68% in men, with continued increases with aging (77, 96). Offspring of LFS carriers have a 50% risk of inheriting the mutation consistent with an autosomal dominant syndrome. It is estimated that 10-20% of LFS cases may involve de novo TP53 mutations (96).

The landmark report in 1990 showed that LFS is characterized by inherited mutations in the TP53 tumor suppressor gene (TSG) on chromosome 17p13.1 (66). LFS individuals typically carry a dominant "activating" gain-of-function (GOF) mutation at various “hot-spots” within the large TP53 gene (97). The specific type of TP53 mutation, combined with unique environmental exposures, may account for the heterogeneous patterns of cancer at different ages in those
inheriting the variant TP53 allele (98). Mutations in TP53 have not been detected in 10-30% of LFS families and searches for other missed TP53 mutations or another predisposing genes continues (99). The p53 protein, encoded by TP53, is a transcription factor that is activated by a myriad of cellular stress signals potentially impacting genome integrity and normal cell proliferation (97). Known as the “guardian of the genome” (44) p53 coordinates complex cellular responses promoting cell cycle arrest, DNA repair, apoptosis, or senescence (100).

To date, 584 families worldwide have been identified as LFS carriers (101, 102), yet LFS is very likely under-recognized due to the variety of cancers that develop at different ages, and it is expected that this number will dramatically increase as knowledge of genetic cancer syndromes is incorporated into clinical practice globally. Recent data suggests that familial TP53 mutations may be as high as 1:5000; a number much greater than originally estimated (95). Due to the fact that LFS is infrequently identified, and that core malignancies may result in death at early ages, the precise tissue-specific cancer risk estimates are thought to be grossly underestimated (103). Other putative LFS-related component tumors may include prostate, melanoma, gonadal familial carcinomas, and tumors of the lung and pancreas (65).

As recognition of LFS improves and cancer screening and surveillance of impacted individuals improves, many cancers will be detected earlier with improved rates of cure and enhanced longevity (104). Thus, those caring for urologic cancers are likely to observe a rise in the number of LFS-PCa patients secondary to longer life expectancies. If PCa risk is indeed increased by LFS, a diagnosis at a younger age with more aggressive biology may be predicted. We report a case of clinically localized PCa in a young LFS patient and discuss implications for clinical assessment, ongoing surveillance and medical management.
Case Report

A 50 year old male presented for routine primary care evaluation and underwent PCa screening with a normal DRE and PSA of 4.41ng/ml. There were no previous PSA values. The family history was significant for PCa in a paternal uncle (72y) and cousin (60y). His family history was also significant for LFS, and he was genotyped at age 49 after his family was determined to fit the criteria for LFS through a consultation with the cancer genetics program at The Ohio State University (OSU). The limited family pedigree (Figure 1) shows the individuals, their cancer diagnoses, age, survival status, and genetic test results if known. Familial TP53 genetic testing revealed a single 13220C->T transition in exon 5 of the DNA coding sequence for TP53 resulting in a missense mutation at p.Arg181Cys (R181C). Because the case’s TP53 missense mutation lies within a conserved region on the DNA-binding domain, tumor suppressor activity of p53 becomes dysfunctional (105). The patient’s history is also significant for having donated a kidney for transplant at age 44, 5 years prior to his LFS diagnosis. He has been free of malignancy with the exception of a solitary 4 cm skin lesion consistent with limited stage mycosis fungoides (T-cell lymphoma) treated with topical steroids.
Figure 2.1. Partial Li-Fraumeni Syndrome (LFS) pedigree of case. Offspring of LFS positive individuals included in pedigree; LFS negative offspring excluded. Age of death noted within geometric shape. Diagnosed cases listed under shapes: 1=prostate; 2=brain; 3=breast; 4=lymphosarcoma; 5=melanoma; 6=lymphoma; 7=renal.
He was referred to urology and transrectal ultrasound and biopsy completed with two of eight cores showing adenocarcinoma, one from the left bilateral base, Gleason grade 3+4=7 involving 40% of the specimen, and the left base with a 3+3=6 carcinoma involving 1% of the sample. After considering his options he proceeded with an uncomplicated radical robotic prostatectomy and lymph node resection. Pathologic staging showed adenocarcinoma, 3+3=6, involving 5% of the gland, bilaterally, multifocal, apex to base, within a background of diffuse high grade prostatic intraepithelial neoplasia (PIN), without nodal involvement or extracapsular or extraprostatic extension, and a staging of pT2cN0Mx.

Discussion

Knowledge of Cancer Genetic Syndromes

In spite of their rarity, caregivers involved in management of genitourinary cancers should consider heredity cancer syndromes in patients demonstrating family histories of multiple and early-onset cancers (106). Prompt diagnosis of heredity cancer syndromes and therapy may have important therapeutic and prognostic implications. In addition, evaluation of potentially impacted family members by clinical geneticists will define the diagnosis and appropriate screening can be initiated for at risk family members for cancers within or outside of the urogenital system.

With regards to LFS, cancer of the adrenal cortex is a rare urological malignancy but is one of the core cancers of LFS, and its diagnosis should trigger a careful evaluation of the family history (106). The incidence of adrenalcortical cancer is 1-2 per million annually with clustering in children and in middle aged adults (107). Additional uncommon genitourinary cancers documented in LFS affected individuals, but not currently considered as core cancers, include germ-cell tumors and nephroblastomas, but it is premature to determine if rates exceed age-adjusted non-LFS rates (106). In the past decades, the majority of LFS men would not reach the
ages where PCa risk was significant (Figure 2). However, improved awareness and identification of LFS is providing an opportunity for aggressive cancer screening and tailored surveillance in LFS families. Current studies of screening with modern imaging and laboratory testing coupled with traditional physician clinical evaluation has increasingly detected cancers at earlier and more curable stages in those afflicted with LFS (108). Indeed, a fundamental goal of LFS cancer management centers focuses upon early detection and surgical resection whenever possible (77). Emerging data suggests that this approach is allowing LFS patients to achieve greater longevity (109) and men are surviving to ages where PCa rates dramatically increase by over 100 fold between the ages of 40 and 70 (2).
Figure 2.2. Survival curve comparing non-LFS carriers, male and female LFS carriers and prostate cancer incidence by age.
At the present time, data is insufficient to determine if PCa will be more common and/or at an earlier age in LFS than non-afflicted men. International and American organizations have emerged to collect cancer incidence and survivorship data on LFS and serve the dual roles of research and as a source of information for medical caregivers and LFS families. The establishment of the global International Agency for Research on Cancer (IARC) TP53 Germline Mutation Database in Familial Cancers (102) and the Li-Fraumeni Exploration International Consortium (LiFE) will accumulate data on afflicted families and serve as a research resource and inform clinical care.

The presence of acquired TP53 somatic mutations in human prostate cancer supports the hypothesis that inherited defects of the same gene may enhance risk (102). Recent studies suggest that p53 mutations are not common in prostate cancer when reliable gene sequencing analysis is employed in contrast to studies employing immunohistochemical techniques. Keeping in mind that the p53 gene is very large, composed of 393 amino acids spanning 20 kb and sequencing studies are often limited to a few specific “hot spots,” the range of mutations is approximately 17% percent by direct sequencing for prostate cancer (41). p53 mutations can be estimated by immunohistochemical (IHC) staining based upon the finding that the p53 protein with point mutations shows a relative resistance to intracellular degradation, thus accumulating in cells. The IHC technology estimates approximately 10-30% percent of prostate cancers demonstrate overexpression and possible mutations (105). Regardless, when present, the p53 mutations appear to have very high prognostic relevance, being associated with advanced stage, higher Gleason grade and early post prostatectomy biochemical failure (26). The lack of a functional p53 protein may allow the cancer cell to accumulate additional mutations more rapidly over time, enhancing the likelihood it will emerge earlier and with more aggressive biology associated with higher grade and stage.
It is important to note that LFS men have p53 mutations in all somatic cells and this is of particular relevance to a novel hypothesis suggesting a role from stromal cell p53 mutations enhancing epithelial prostate cancer progression (110). In addition, other studies suggest that p53 mutations in the stromal fibroblasts sensitize prostate tumors against anthracycline and cisplatinum chemotherapy (111). The mechanisms may involve the induction of senescence pathways in the stromal cells which in turn stimulates the production of growth factors that may act upon the prostate cancer cells in a paracrine manor.

Prostate Cancer Screening for Li-Fraumeni Syndrome

Given the increased longevity of LFS males, decisions regarding the age at which to begin PCa screening and how often to evaluate men needs to be incorporated into a surveillance program. Public health guidelines for PCa screening remain controversial and vary dramatically around the globe, in large part due to the remarkable lack of carefully conducted prospective studies. In addition, the relatively slow and heterogeneous progression of prostate cancer, varied application of treatment options, and competing mortality make it difficult to assess the efficacy of screening on overall survival. The National Comprehensive Cancer Network (NCCN) clinical practice guidelines are a reasonable starting point (112) and provide patients and clinicians with an evidence-based approach for cancer detection, prevention, and treatment options (112). Because these guidelines are largely based upon lower level evidence and “expert” opinion, the need for high quality, evidence-based clinical recommendations remains paramount in the field of prostate oncology (113). Until such time, the authors favor treating LFS men as high risk and empirically initiating the screening program prior to age 40 in LFS males until data accumulates to guide our efforts more precisely. Obviously, in an
asymptomatic male whose life expectancy is less than 5 years (if an active lethal cancer or comorbidities), the more urgent health issues may supersede the importance of screening and detection of an early PCa.

LFS is also known to occur in African Americans, a subgroup at significantly greater risk for PCa diagnosis and mortality compared to Caucasians (114-116). At this time we have no basis upon which to propose that a significant interaction exists between LFS, African American heritage, and PCa risk or rate of progression. However, a relationship between TP53 gene alterations and excess death from breast cancer in African American compared to Caucasian women has been reported, yet similar studies in PCa have not yet been reported (115, 116).

**Prostate Cancer Therapy in Li-Fraumeni Syndrome**

**Surgery**

Surgical intervention has been successfully employed with curative and palliative intent in LFS without evidence of unexpected complications. Issues related to surgical risk, anesthesia, wound management, risk of infectious and other complications, and rehabilitation appear to be similar to non-affected individuals. Thus, otherwise healthy men with long life expectancy are candidates for a radical prostatectomy with curative intent (117).

**Radiation**

Ionizing radiation (IR) therapy, either external beam or brachytherapy, is an established single modality option for treatment with curative intent for clinically localized prostate cancer (118). Increasingly, integration of radiation therapy into multidisciplinary approaches in combination with hormonal and chemotherapy for higher risk patients or to employ radiation for post-surgical adjuvant and salvage interventions is essential (119, 120). Basic laboratory science regarding the impact of TP53 mutations on cancer cell responses to radiation raise...
concerns regarding the use of this modality in LFS patients. Ionizing radiation induces DNA damage and activates a free radical cascade that causes intracellular damage (120, 121). p53 mutations in prostate cancer are associated with relative radio-resistance.

An additional concern is that p53 dysfunction is associated with increases in the risk of developing multiple primary tumors in the treated field. This is due to the radiation induced oncogenic transformation (73, 74, 122-124). Based upon modest accumulated data, the risk to individuals with LFS of developing a radiation-related second primary cancer has been estimated at 57%, and the risk of a third primary malignancy is approximately 38% (73). In the absence of definitive clinical data for prostate cancer therapy in LFS patients, limiting exposure to radiation therapy whenever possible seems to be a sensible recommendation (125-128). Even diagnostic radiological measures or medically necessary radiotherapy procedures must be approached with caution in light of such findings (69, 129, 130). The NCCN Clinical Practice Guidelines for breast cancer screening and diagnosis now recommend consideration of breast MRI and novel surveillance imaging in lieu of annual mammography for LFS-positive woman (130, 131).

**Chemotherapy**

Chemotherapy, particularly taxotere, is an established active agent for prostate cancer in the advanced setting. Increasingly, chemotherapy is employed in neoadjuvant and adjuvant clinical trials for those with locally advanced disease at presentation with a high risk of relapse following single modality, such as surgical, therapy. There remains much to be learned regarding the risks and benefits of specific chemotherapy regimens for prostate and other cancers in LFS patients. Many agents can induce DNA damage, increasing the risk of future tumors (132). Indeed, the recent high-risk post prostatectomy adjuvant trial of mitoxantrone and hormone therapy was discontinued due to a possible increase in the risk of hematologic malignancies; particularly in those given radiotherapy, similar to findings in a breast cancer study (133). Not
surprisingly, p53 protein mutations also confer drug resistance in multiple cancers (134-136). Known chemoresistant agents in dysfunctional p53 tumors include docetaxel (137), doxorubicin (138), cisplatin (139, 140), anthracyclines (141-143), and mitoxantrone (144). Thus far the efficacy of taxane-based chemotherapeutics, do not appear to be dramatically impacted in p53 mutant disease (145). Indeed, adjuvant treatment options must be weighed cautiously in high-risk individuals, and rigorous follow-up to detect tumor recurrence or the onset of new primary tumors is critical. Thus the optimal management of LFS patients is early detection of prostate cancer at a stage where curative surgical interventions are high.

**Renal Transplantation and Cancer Genetic Syndromes**

At the time of his donating a kidney for transplant through the OSU Medical Center Renal Transplant Program Organization, the patient’s family had not yet recognized a familial cancer pattern suggesting an inherited cancer syndrome, and thus genetic testing had not been done. To our knowledge, this is the only case of a transplanted kidney from an LFS donor. Although anonymity is maintained by the transplant organization, the kidney is functionally normal after eight years and no malignancy has been detected within the donor organ. Kidney cancer is not currently considered to be a core malignancy in LFS patients, thus the risk of the recipient developing a malignancy, over and above the rates of non-LFS donor kidneys is unknown.

The discovery of a genetic cancer predisposition after kidney donation raises numerous questions about transplantation care by urologists. First, the importance of a family history and pedigree development cannot be overstated in that such a history focused on cancer occurrence might have detected the presence of LFS or, at a minimum, a very strong family cancer history that indicated a referral for genetic counseling and evaluation. To date though, familial cancer history is not one of the core elements of evaluation practiced by U.S. transplant centers in the
evaluation of donors. In a national survey of donor evaluation practices, familial history mostly focuses on hypertension, renal disorders and heart disease (146).

All other variables being equal, kidney donors without known cancer predispositions would be preferred for elective anonymous transplants. However, given the 7,000 end stage renal patients waiting on transplant lists in the U.S. and the high overall mortality rate, a positive cancer predisposition is not an absolute contraindication to donation. The risk of subsequent cancer is far less than the mortality associated with living without a transplant. In fact, many centers encourage persons with a previously treated cancer to donate after some waiting period to ensure remission (147).

Transplant recipients are at increased risk for tumor development (148). Renal cancer risks are increased fifteen-fold in recipients compared to the general population, but this is still a small number. Approximately ten percent of kidney transplant recipients will develop cancer within a decade after their transplant (149). Many of the cancers will be dermatologic in nature, but solid organ cancers, like the kidney, are also increased. The majority of the increased risk above the population risk is thought to be associated with the immunosuppressive therapy prescribed to prevent host versus graft rejection (150). The additional risk of cancer transplanted directly from a donor or derived from donor tissue is quite low (151). In two large registry studies, donor transplanted cancer and donor derived cancer occurred in less than half a percent of recipients (152). Regardless of the source of subsequent cancers, it behooves all kidney recipients to undertake aggressive screening and preventive measures for cancer because of the risks over the ensuing decades after transplantation.
Conclusion

LFS is an inherited cancer syndrome with urologic cancers, adrenalcortical cancer and nephroblastoma, as core malignancies. The improved detection of LFS leading to more aggressive surveillance and successful therapy of cancer is allowing affected men to live longer lives and survive into the decades where prostate cancer is more prevalent. Although it is premature to determine if risk and pathogenesis of prostate cancer is different in LFS males, it is prudent to consider them as high risk and institute recommended screening at ages earlier than for the general population. The use of surgical intervention as opposed to radiotherapy and systemic chemotherapy is preferable for men with clinically localized disease. The role of p53 as the guardian of the genome, and the frequency of mutations in human prostate cancer is an active area of research with critical implications for prognosis and therapy.
Abbreviation Key:

Digital Rectal Exam (DRE)

International Agency for Research on Cancer (IARC)

Ionizing radiation (IR)

Li-Fraumeni Syndrome (LFS)

Li-Fraumeni Exploration International Consortium (LiFE)

National Comprehensive Cancer Network (NCCN)

Prostate Cancer (PCa)

Prostatic Intraepithelial Neoplasm (PIN)

Prostate Specific Antigen (PSA)

Tumor Protein 53 (TP53)

Tumor Suppressor Gene (TSG)
Chapter 3: p53 Expression by Immunohistochemistry in Human Prostate Cancer

Abstract

In the United States, prostate cancer remains a significant public health issue. It is estimated that in 2011, over 217,000 new cases of prostate cancer will be diagnosed with death rates exceeding 32,000 (153). Because prostate cancer is so biologically heterogeneous, characterizing subtypes of the disease would provide valuable diagnostic criteria to discriminate indolent versus aggressive tumors. Surprisingly, few standardized clinical biomarkers have been integrated into practice to inform clinical treatment decisions. In this study, we characterize p53 expression, via immunohistochemical staining, in both normal and malignant prostate tissue in a cohort of 80 prostate cancer patients nested within the Health Professional’s Follow-up Study (HPFS). In addition, we evaluate p53 immunostaining in relation to cancer grade in 500 cohort patients. Histologic prostate sections were immunostained with monoclonal mouse anti-human p53 (DO-7) to label wild type and mutant p53 protein. After a pathologist identified areas of malignant versus normal prostate, up to three non-overlapping (400x) fields within each section were evaluated for p53 immunostaining in terms of p53 immunoreactivity and mean percent nuclear staining. p53 expression was identified in areas of cancer more so than in areas of noncancer prostate (p<0.01). Staining intensity positively correlated with areas of cancer and histological grade (p<0.023). The mean percentage of nuclei expressing p53 correlated with areas of cancer versus noncancer and staining intensity (p<0.001) but did not significantly
correlate with Gleason score. Image analysis of p53 stained prostate tissue provides an objective and quantifiable tool for characterizing cancer versus noncancer in the prostate. Our results suggest that p53 immunostaining may serve as valuable biomarker to characterize malignant versus normal prostate tissue and within cancer, distinguish indolent biology from those with more aggressive potential.

**Introduction**

The cancer process is a multifaceted process in which genomic deviations drive alterations of normal cells to malignant tumor cells (1). Normal cell cycle division results in DNA duplication and division. Integral to this process are the central control mechanisms that drive the cellular replication. Cell cycle checkpoints dissect critical events and become activated in response to recognition of mutational errors or cell cycle disturbances (154). Once initiated, these checkpoints halt normal cell cycle proliferation until the error has been corrected (155). Unfortunately, cancerous cells evade these normal cell cycle checkpoints, thus resulting in exponential and uncontrolled cellular growth and proliferation (156). This vicious cycle drives checkpoint insensitivity that further evades mechanistic controls and promotes tumorigenesis.

Gain-of-function (GOF) mutations and loss-of-function (LOF) tumor suppressor genes (TSG) are all related to prostate carcinogenesis (22, 157). In terms of the cell cycle, TSGs, such as $TP53$, are critical in monitoring and maintaining cell cycle checkpoints. Loss of normal $TP53$ function relinquishes the cell cycle’s capacity to terminate mutational division upon insult. Wild type p53, termed the “Guardian of the Genome (44)” is a key tumor suppressor protein, encoded by the $TP53$ gene, that is deleted or mutated in over 50% of human cancers as an acquired mutation (158). Yet surprisingly, its role in prostate cancer (PCA) has yet to be clearly defined. Specific to PCA, p53 mutations appear highly correlated with hormone-refractory and
aggressive disease (159). During normal cellular conditions, p53 protein levels remains low within the nucleus. DNA damage and cellular stress signals trigger an exponential increase in p53 transcription that results in growth arrest, DNA repair, programmed cell death, or senescence (160)(Figure 3.1).
Figure 3.1. p53–MDM2 feedback is fundamental to the p53 pathway. Under unstressed cellular conditions, it maintains a continual, low steady-state p53 levels. Various stressors liberate p53 from MDM2 control and p53 is shuttled into the nucleus for transcription (2).
The literature deviates from studies confronting p53 dysregulation related to histopathologic and prognostic variables specific to PCa. Standardized classifications and analyses of p53 protein expression incidence could help identify patients harboring a more aggressive disease phenotype. Although the feasibility of using immunohistochemistry (IHC) to quantify measures of p53 remains controversial in some tumors, the vast majority of IHC studies report a strong association between p53 nuclear overexpression and advanced disease in PCa (62). Yet few prospective studies include enough cases to yield consistent and reliable results (46).

There are several issues to consider when utilizing p53 immunostaining as a standard diagnostic tool. The first issue relates to biological heterogeneity. p53 protein is completely undetectable or present at very low levels in resting cells. Conversely, p53 nuclear overexpression is apparent in a wide variety of transformed cells, as well as in many actively proliferating, and nontransformed cells (59). Thus, the potential variability in p53 nuclear stain intensity must be addressed. The second confounder relates to technical considerations. In order for any potential biomarker to be incorporated into clinical practice, standardization in biospecimen handling, processing, and analysis is required. It is established that variations in tissue fixation, antigen retrieval, antibody concentrations, and incubation times can impact staining quality and intensity (161). Indeed, studies utilizing IHC to assess biomarker potential must be tightly controlled and methodologically standardized to allow maximum precision. Consistent IHC techniques are critical for rigorous analysis necessary to appreciate and assess the inherent heterogeneity of PCa. Because gene sequencing is both expensive and time consuming, especially for large genes like TP53, immunostaining may provide a feasible
alternative to characterizing prostate tissue. Our statistically significant study provides clues into the molecular and biological signature of malignant versus normal prostate tissue and prostate cancer to histological grade.

*TP53* is frequently mutated in PCa, although the overall prevalence of mutational events in the prostate is reported at lower rates than in other solid tumors (24)(Figure 3.2). The reason for this disparity has yet to be elucidated. The vast majority of p53 mutations involve single nucleotide polymorphisms (SNPs) (97)(Figure 3.3). Disruptions in functional *TP53* result in transcriptional errors that alter purposeful responses to genomic stressors. The consequences of the cell’s inability to initiate DNA repair, apoptosis, senescence, or cell cycle arrest lead to gross proliferation of mutagenic cells (21). This decontrolled cellular proliferation is a recognized hallmark of tumorigenesis and eventual metastases (1). In tandem, mutant p53 protein may exhibit dominant gain-of-function (GOF) oncogenic properties and/or loss-of-function (LOF) tumor suppression properties (158). It is hypothesized that these disruptional events, taken together, contribute to the gross aggressiveness related to tumorigenesis in the prostate.
Figure 3.2. IACR’s TP53 database image depicting the prevalence of TP53 gene mutations by site of tumor (102). Note that PCa is reported at approximately 18%.
Figure 3.3. IARC TP53 pie chart displaying various tumor-derived mutations reported to the database. B. Reported missense mutation distributions along the p53 AA sequence with common DNA-binding domain hotspots (yellow), locally distorted mutations (green), and globally denatured mutations (blue). PR, proline-rich domain; Reg, carboxy-terminal regulatory domain; TA, transactivation domain; Tet, tetramerization domain (97).
During normal cellular function, p53 is remains unexpressed or is activated in response to perceived cell cycle stress. Wild-type p53 protein has a short half-life and thus, is present in minute amounts which is typically below immunoreactivity detection (162, 163)(Figure 3.4). Weak p53 nuclear staining may be a transitory phase in the cell cycle when p53 is approaching or has just surpassed its threshold. In advanced stages of PCa and metastasis, loss of chromosome 17p and deletion of the p53 locus occurs. Mutant p53 is much more stable than wild type p53 due to its conformational alterations. Numerous mutations in the expressed protein result in dysfunctional p53 that evades targeted degradation leading to nuclear p53 overaccumulation (163)(Figure 3.4). This exaggerated immunoreactivity is correlated with TP53 gene dysfunction (79).
Figure 3.4. p53 IHC in human prostate cancer the same case (GG=7) displaying nondetectable (negative) nuclear staining (on top) and detectable nuclear staining (on bottom). Original magnification 400x.
The aim of this study was to validate clinically relevant biomarkers of PCa aggressiveness in relationship to measures of p53 in a large and statistically powerful prospective nested cohort. Although the TP53 gene is central to cancer research, there remains a significant gap in the literature specific to p53 dysfunction relative to short and long term biomarkers of prostate carcinogenesis. Because the p53 gene encodes key cell cycle and survival regulatory proteins, mutations of this gene may play a central role in prostate tumorigenesis. We evaluated p53 status, by quantitative immunostaining technology, to define a molecular signature of p53 staining intensity and percent nuclear positivity with respect to current prognostic biomarkers.

Methods

Study Population

The Health Professionals Follow-up Study (HPFS) prostatectomy cohort is a sample of American and Canadian male health professionals who were diagnosed with clinically localized PCa from 1986 to 2000 and underwent a prostatectomy as curative therapy. A total of 51,529 men were participants in the ongoing prospective HPFS cohort consisting of dentists, optometrists, osteopaths, podiatrists, pharmacists, and veterinarians. Incident PCa as initially identified through self-report or confirmed by a medical records review, pathology reports and death certificates when PCa was mentioned on death certificates. Of the 1,593 men who elected a prostatectomy, we retrieved blocks for 1,023 (64%). Most treating hospitals and medical centers forwarded blocks for the entire case, although some chose blocks containing tumor tissue. Our current analysis is based upon 500 men with completed morphological assessments. The clinical characteristics for these cases are representative of the entire HPFS prostatectomy cohort (12). Information pertaining to age at diagnosis, clinical and pathologic stage was extracted from medical records and pathology reports when available. Information
on race and ethnicity was collected on the baseline HPFS questionnaire. To obtain medical and lifestyle data, the professionals completed standardized biennial questionnaires. All men were prospectively followed for clinical outcomes through 2004. The HPFS research protocol has been approved by the institutional review board (IRB) at the Harvard School of Public Health and Partners Healthcare and The Ohio State University.

**Identification and Procurement of Specimens**

On biennial follow-up questionnaires, each HPFS participant was asked whether he has been diagnosed with PCa during the prior two years. If affirmative, participants were asked to provide confirmatory medical records that are reviewed by Drs. Giovannucci (Harvard) and Clinton (The Ohio State University). The reported grade, clinical stage, and pathologic stage (if available) were determined. Formalin-fixed, paraffin-embedded tissue blocks and pathology reports from all prostatectomy cases were requested using established cohort procedures. Upon arrival in Boston, relevant data were recorded, including patient name, date of arrival, date of procedure, total number of blocks and individual block identification numbers. A numerical code, used to maintain confidentiality, was assigned to individual pathology sets (Figure 3.5).
Figure 3.5. Study Design for HPFS prostatectomy cohort.
Uniform Grade and Stage

Paraffin-embedded tissue blocks were sectioned and transferred to Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Following rehydration, slides were stained with hematoxylin and eosin (H&E) on an automated slide stainer (Leica Microsystems Inc. Deerfield, IL). The slides were removed from the autostainer and cover slipped. The H&E slides were reviewed independently using the American Joint Committee on Cancer (AJCC) and World Health Organization (WHO) criteria, and then compared with the original pathology report.

Immunohistochemistry Staining

All IHC staining was performed on an OptiMax Automated Cell Staining System (BioGenex Laboratories, San Ramon, CA), which allows for staining of up to 40 slides simultaneously under precisely controlled conditions, thus eliminating a significant amount of variation associated with manual staining. Antigen retrieval was accomplished by heating in Citra Plus Antigen Retrieval Solution (BioGenex, San Ramon, CA) for 30 minutes.Slides were washed with phosphate buffered saline (PBS) (3 replicates) between steps. Endogenous peroxidase activity is quenched with Peroxidase Block (DakoCytomation, Carpenteria, CA). Sections were incubated with monoclonal mouse anti-human p53 (M7001, Clone DO-7 from DAKO, Carpinteria, CA) for 60 minutes. Detection of p53 was performed using Labeled Polymer-HRP and DAB + Chromogen solution (EnvisionPlus, DakoCytomation, Carpenteria, CA). After DAB color reaction, all slides were counter-stained with Mayer’s hematoxylin (Sigma, St. Louis, MO) for 2 minutes. Up to three representative non-necrotic areas of cancer and non-cancer areas from each slide were selected for digital image capture and analysis.
Image Analysis

All sections were examined by a pathologist, under light microscopy, for characterization of tumor morphology and cytological features. Tumor and normal areas were identified and outlined in up to four representative sections per case. Images from H&E and immunostaining were captured at 400x magnification by a high resolution digital camera (Spot RT, Diagnostic Instrument, Inc., Sterling Heights, MI) using bright field microscopy (Nikon Eclipse E800, Tokyo, Japan) and transmitted to an image analysis workstation (6500 Pentium III WorkStation, Dell Computer Corp, Round Rock, TX). Captured images were manually counted accordingly to standardized and objective parameters. Specific outcomes for p53 included percent of nuclei stained per high power field (HPF) and categorical intensity of p53 nuclear staining (0 to 2 grade scale where 0 = no detectable stain; 1 = p53 nuclear stain present but with weak intensity; 2 = nuclear overexpression of p53 stain). Representative images demonstrating the categories of staining intensity are demonstrated in Figure 3.6. Slides were evaluated without knowledge of the Gleason or staging of the tumor.
Category “0” = No detectable p53 immunostaining

Category 1 = p53 is detectable but has weak intensity

Category 2 = Strong p53 immunostaining

Figure 3.6. We categorized each sample as 0, 1, or 2 based upon p53 immunostaining. A category of “0” means the p53 is nondetectable or the sample is “negative” for p53 stain (top image). A category of “1” means the p53 is detectable but nuclear stain is “weak” (middle image).
Statistics

Data from image analyses was performed with SAS version 9.1 (SAS Institute, Inc., Cary, NC) or SigmaPlot version 11.0 (Systat Software, Germany). Descriptive statistics were employed to summarize p53 expression among the HPFS p53 cohort. The mean percent positive p53 staining for each case was calculated. When more than one p53 intensity category was reported for the same case, the maximum was used. Fisher exact test was used to compare the categorical relationships of incidence of staining with cancer status. Multiple logistic regression analysis was used to compare categorical intensity to cancer status and Gleason score. The mean percent positive p53 staining was assessed by p53 intensity categories using a Wilcoxon Rank Sum Test. An analysis of variance model with repeated measures was used to compare p53 percent positive between p53 intensity category within each Gleason group.

Results

p53 Study Cohort

Of the 51,529 men in the HPFS cohort, we obtained tissue samples from 500 men who were diagnosed and underwent a radical prostatectomy. For this analysis, we assessed 500 cases. In this subpopulation of subjects, the mean age at the time of diagnosis was 65.8 years (SD ± 6.1 years). In the cases with available data, 273 (56.9%) subjects presented with a lower grade disease with a combined Gleason grade (GG) of 2-6 and 207 (43.1%) had tumors with a GG of 7 or higher. Thirty-eight (35.6%) of the men in the p53 cohort demonstrated advanced PCa T stage of 3 or greater. Sixty-six (63.4) have lower T stages as defined by T<3 (Table 3.1).
Table 3.1. Clinical and demographic characteristics of HPFS prostatectomy - p53 study cohort.

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p53 Incidence in Cancer Versus Normal Prostate

Of a subsample of 80 cases, p53 immunoreactivity was assessed in areas of both cancer and noncancer. Each area was subjectively assigned a category score based upon intensity of staining as demonstrated in Figure 3.6. Regardless of staining category, the noncancerous prostate had significantly fewer cases demonstrating nuclear staining than PCa tissue (both \( p<0.01 \); Table 3.2). The frequency of detectable p53 nuclear stain (categories “0” versus categories “1 and 2”) is dramatically greater in cancer than in noncancer samples (\( p<0.01 \); Table 3.2). The overall effect of p53 staining in malignant versus normal prostate when comparing nondetectable nuclear stain (category 0) versus any detectable stain (categories 1 and 2) was also significant (\( p<0.01 \)).

Consistent with reported frequencies of mutations in p53 as identified by genomic sequencing, 70-80% (\( N=56 \) in category 0; \( N=64 \) in category 0 and 1) of malignant tissues revealed no overexpression of nuclear p53. In all cancer cases where p53 was negative, regardless of staining category, the noncancer counterparts were also negative for p53 stain. Positive p53 nuclear expression was evident in 20-25% (\( N=20 \) in category 1 and 2; \( N=16 \) in category 2) of our cancer samples (41). Interestingly, only 0-5% (\( N=4 \) in category 1 and 2; \( N=0 \) in category 2) of the noncancerous prostate counterparts revealed any detectable nuclear staining. There were no cases (0%) where p53 staining was identified in the noncancerous prostate independent of the cancerous tissue. A frequency summary of this data is also defined in Table 3.3.

The frequencies of p53 immunostaining in noncancer versus cancer prostate (\( N=80 \)) by category are arranged differently and data shown in Table 3.3. Here, p53 negative (category 0) is compared to detectable p53 stain (category 1 and 2) corresponding to both weak and strong
staining. The frequency of detecting p53 positivity is significantly greater in cancer tissue than in noncancer (p<0.001). Likewise, p53 “negative” (category 0 and 1) corresponds to cases with no detectable or weak nuclear stain. This is compared to category 2, corresponding to strong p53 immunostaining. The overall effect of p53 in cancer versus noncancer for this group is also significant (p<0.001; Table 3.3).
Figure 3.7. p53 immunohistochemistry in noncancer (left) and cancer (right) in the same case of human prostate (GG=9). Original magnification 400x.
Table 3.2. Frequencies of p53 immunostaining in cancer versus noncancer prostate (N=80) in the same cohort but categorized differently using Fisher’s exact test. A. In the table at the top, p53 “negative” or category “0” is compared to p53 detectable or category “1 and 2” corresponding to both weak and strong staining. The frequency of detecting p53 positivity is significantly greater in cancer than in noncancer (p<0.01); B. In the table at the bottom, p53 “negative” or category “0 and 1” corresponds to cases with no detectable plus those with weak nuclear stain. These cases are compared to p53 category “2” corresponding to cases with strong p53 immunostaining. The overall effect of p53 in cancer versus noncancer for this group was also significant (p<0.01).
Table 3.3. Summary of the frequencies of p53 immunostaining in noncancer versus cancer prostate (N=80) by category and arranged differently than Table 3.2 using Fisher’s exact test . In the table at the top, p53 “negative” or category “0” is compared to p53 detectable or category “1 and 2” corresponding to both weak and strong staining. The frequency of detecting p53 positivity is significantly greater in cancer than in noncancer (p<0.001); B. In the table at the bottom, p53 “negative” or category “0 and 1” corresponds to cases with no detectable or weak nuclear stain. This is compared to p53 category “2” corresponding to strong p53 immunostaining. The overall effect of p53 in cancer versus noncancer for this group was also significant (p<0.001).
Figure 3.8. Summary of p53 staining categories (N=544) with the vast distribution of those cases staining negative (N=371; 68.2%); followed by cases displaying detectable weak stain (N=116; 21.3%) and strong stain (N=57; 10.5%).
p53 in Relation to Pathological Grade

There was a significant overall effect of Gleason scores in relation to the presence of p53 staining categories within prostate cancer. Both means of categorical staining distribution (0 vs. 1 and 2; 0 and 1 vs. 2) demonstrated significant differences. Yet, the effect is stronger in p53 staining category 2 (p=0.023 vs. p=0.004, respectively; Table 3.4).
Table 3.4. Summary tables comparing the relationship of p53 staining categories to Gleason score groups. A. p53 staining categories are segregated between no detectable immunostaining (category 0) and any detectable staining (categories 1 and 2). There is a significant overall Gleason score effect when comparing GG groups to p53 staining categories (0 vs. 1 and 2; p=0.023); B. p53 staining categories are segregated between no detectable p53 staining and weak p53 staining (categories 0 and 1) versus strong p53 staining (category 2). There is a more dramatic overall Gleason score effect when comparing GG groups to these p53 staining categories as well (p=0.004).
Percent p53 Nuclear Staining

To better understand the relationship between the observed differences in p53 staining category, the percentage of detectable positive nuclear staining within staining category 1 versus category 2 was accessed. Of the 456 cases with adequate sample quality, 116 displayed weak staining (category 1) and 57 displayed strong staining (category 2). Mean percent p53 positive nuclear staining in those with weak staining (category 1) was 5.3% (+/- 0.5%) while mean p53 positive staining was 30.8% (+/- 4.1%) in those displaying strong staining (p<0.001) (Table 3.5).

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<th>N</th>
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<th>Min</th>
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Table 3.5: Nuclear p53 staining by category when all nonstained samples (category 0) are removed, and we limit our analyses to the categories “1” and “2” with a total of 173 cases. The table above shows that cases in category “2” display a dramatic and significant increase in mean percent nuclear p53 staining when compared to category “1” using Wilcoxon Rank Sum Test (p<0.001).
The percentage of p53 nuclear stain in the subset of those cases demonstrating immunoreactivity (staining categories 1 and 2) was stratified by Gleason score in cancer samples. There was no significant differences in the percentage of p53 nuclear staining as compared to the overall effect of Gleason score or the interaction of Gleason score and staining category (0 and 1 versus 2 difference; p=0.08; Table 3.6).

Of the cases where p53 staining was detected in cancer, mean percent p53 nuclear staining (category 1 vs. 2) was compared to Gleason groups (2-5, 6, 7, 8-10). There is a significant IHC effect evident in mean percent p53 stain by category 1 versus category 2 within Gleason score groups <8 (p<0.001). A trend towards significance is noted in Gleason group 8-10 (p=0.06). The overall p53 category (1 vs. 2) effect is significant (p<0.001), but no significance is reported when assessing the overall Gleason score effect (p=0.90) or the Gleason interaction effects (p=0.56; Table 3.7).
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Table 3.6. This table is limited to the 173 cases where p53 staining was detected in cancer and presents mean detectable percent p53 nuclear staining (category 1 and 2) in relation to Gleason groups in malignant prostate (2-5, 6, 7, 8-10). ANOVA was used to compare detectable mean p53 percent stain to overall Gleason scores within groups (p=0.08).
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Table 3.7. This table is limited to cases where p53 staining was detected in cancer and presents mean percent p53 nuclear staining (category 1 and 2) in relation to Gleason score groups (2-5, 6, 7, 8-10). ANOVA with repeated measures are used to compare p53 percent positive nuclear staining within groups. There is a significant IHC effect noted in mean percent p53 stain between p53 categories 1 versus 2 within Gleason scores (p<0.001) in Gleason groups <8. Near significance is noted in Gleason group 8-10 (p=0.06). An overall p53 category (1 vs. 2) effect is significant (p<0.001), but no significance is reported when assessing the overall Gleason score effect (p=0.904) or the Gleason interaction effects (p=0.561).
Discussion

Previous reports examining p53 immunostaining in prostate tissue primarily focus on malignant, not normal tissue. Thus, to our knowledge, this is the first study to characterize p53 nuclear protein accumulation in a multi-institutional cohort in both normal and malignant human prostate. Additionally, our study examined 80 cases of human prostate tissue to evaluate and characterize both cancer and noncancerous prostate microbiology via p53 immunostaining. We then extended our study to assess 500 cases of human prostate relative to stage and grade. We proposed that p53 IHC, coupled with image analysis, could provide a valuable tool to elucidate insights into PCa progression and serve as a solid biomarker for the assessment of indolent versus aggressive solid tumors.

As hypothesized, there were significant differences in the incidence of p53 nuclear staining between the noncancerous versus the cancerous prostate. The ratio of cases presenting with strong positive staining is consistent with the reports of p53 mutations in prostate cancer identified by sequencing (Olivier, 2010 #638). This finding supports the premise that strongly stained and over-accumulated nuclear p53 is serving as a biomarker of dysfunctional p53 status. Certainly, sequencing of the same prostate tissues must be conducted to validate this hypothesis; hence the focus of future studies.

For these analyses, we chose to categorize the data in two ways. First, we separated nondetectable or “negative” p53 stain (category 0) versus any detectable stain (categories 1 and 2). Next, we separated the staining categories into categorical distributions where no detectable or weak stain (categories 0 and 1) were compared to strong “positive” stain (category 2). We opted to evaluate our results in this manner to minimize subjective intensity
grading associated with IHC. There remains variability in the literature on IHC quantification methods and analyses. Strong positive staining (our category 2) is typically hypothesized to correlate with p53 biological dysfunction in the literature (164). This novel approach to categorization of p53 by presence of staining alone could contribute to the widespread application of this assessment tool and translate to clinical settings where automated quantification tools are unavailable. However, the potential to develop a standardized system taking into consideration all of the technical factors influencing staining intensity, as well as development of an image analysis system that can objectively measure intensity, would be of value yet remains beyond the scope of this project.

As anticipated, statistical significance was observed between malignant versus noncancerous prostate for p53 staining incidence, intensity, and percentage of p53 staining. In those cases demonstrating strong intensity (category 2), the percent staining was significantly greater (30.8%). This suggests that a biological relationship exists between p53 stain intensity and mean percent of nuclear staining. Our observations confirm that p53 staining in normal prostate tissue is non- to barely detectable due to its presumed functional status and short half-life as opposed to malignant and presumed dysfunctional p53 status (164). These results also demonstrate that p53 IHC analyses are able to detect molecular differences between cancerous and normal prostate tissue that are also evident in grade and stage.

While we hypothesized that the percentage of nuclei staining would correlate with Gleason score, this in fact was not the case. There were simply too few cases of p53 staining in areas of noncancer to assess significant stratification by grade. Among all positively stained cases in areas of cancer, the mean percentage does not demonstrate a significant pattern with
respect to grade. It is again, the p53 staining category that correlates with grade. Gleason score is a clinically relevant diagnostic biomarker; however, it is not, in isolation, the most informative (165).

We aimed to assess p53 by stage, but the number of cases with positive staining is limited in the cases where TNM information was available for this cohort. Due to the age and multi-institutional nature of this cohort, there are variations in how this information was initially recorded. In addition, many of these patients were diagnosed prior to the “PSA era”.

These direct observations have encouraged our further investigation into the relationship between p53 and angiogenesis. There is a growing body of literature establishing a biological relationship between p53 and angiogenesis (166). There is currently no study with the size of this cohort that has addressed this relationship. Preliminary data with respect to the interaction is addressed in Chapter 5. In concert, ongoing studies are underway to assess the relationship between markers of p53 and pathological recurrence of disease and risk of death similar to what we have assessed with respect to measures of tumor vasculature (12).

The efforts described within are directed towards the ultimate goal of defining histopathologic and molecular biomarkers associated with p53 dysfunction in prostate carcinogenesis that may serve as surrogate endpoint biomarkers and provide important information regarding clinical outcomes that can impact evidence based treatment options. To our knowledge, this study represents the first attempt to evaluate p53 as related to characterization of tumor biology as well as a biomarker of tumorigenesis. Such data will provide a mechanistic basis for future large scale clinical trials to assess the role of p53 for human prostate cancer prevention.
Abbreviation Key:

Body mass index (BMI)
Gain of function (GOF)
Gleason grade (GG)
Health Professionals Follow-Up Study (HPFS)
Immunohistochemistry (IHC)
Loss of function (LOF)
Prostate cancer (PCa)
Prostate specific antigen (PSA)
Tumor protein 53 (TP53)
Tumor suppressor gene (TSG)

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Chapter 4: Vascular Architecture as a Biomarker of Angiogenesis in Human Prostate Carcinogenesis

Abstract

Prostate cancer is an incredibly complex disease characterized by diverse clinical behavior that ranges from indolent to aggressive progression. In addition to the current biomarkers used to predictive disease progression, such as Gleason score and cancer staging, more advanced molecular signatures are needed to help inform clinical treatment decisions and more personalized medical management. Tumor angiogenesis plays a key role in the hallmarks of cancer. Evidence from recent studies suggest that angiogenesis encourages solid tumor growth, invasion, and metastases. The present study was conducted to assess the relationship of vascular density and architecture in areas of cancer and noncancer in the human prostate. We examined blood vessel count and vessel architecture (size and shapes) in a prostatectomy cohort of prostatectomy patients nested within the Health Professionals Follow-up Study. This prospective epidemiologic study of over 50,000 men began in 1986 at the Harvard School of Public Health. We received archived tissue blocks with adequate tissue specimens from over 500 men that elected a prostatectomy. Histologic sections were immunostained for endothelial cell marker Factor VIII. In both areas of cancer and noncancerous tissue, three to four non-overlapping images (200x) were captures in each field of vision. Each image was evaluated for vascular density, vessel size, and vascular shape using quantitative analyses. Signed Rank Test, paired t-test and Spearman Correlation Coefficients were used to assess angiogenesis in relation
to architectural variables, tumor grade and tumor stage. Cancer tissue showed more angiogenic features such as greater microvessel density, smaller vessels, and more irregularly shaped vessels based upon Factor VIII immunostaining (all p<0.001). In addition, more tortuous vasculature was related to higher Gleason grades and stages in malignant prostate. Our study has shown that digital image analysis of Factor VIII stained prostate tissue provides predictive value for quantifying pro-angiogenesis characteristics in both cancer and noncancer prostate. Vascular density, as well as vessel size and shape, may serve as solid biomarkers to differentiate cancer with an indolent biology from those solid tumors with an aggressive potential. To our knowledge, this is the first study to characterize angiogenic biomarkers between cancer and normal prostate tissue via FVIII immunostaining.

Introduction

Prostate cancer (PCa) is a heterogeneous disease in both biology and clinical behavior. Indeed, PCa ranges from indolent disease to rapidly progressing disease with few biomarkers to discriminate between the two. The development of PCa is a multistep process that progresses over decades until tumors acquire a variety of characteristics, such as limitless proliferation, evasion of apoptosis and ability to secrete their own growth factors (19)(Figure 4.1). Solid tumors depend on the formation of new blood vessels (BV) for continued growth and to allow invasion of surrounding tissues leading to distant metastasis (167, 168).
Figure 4.1: Hallmarks of Cancer (1).
Angiogenesis is a process that involves the formation of new BVs which, under noncancerous conditions, occurs in the body during normal stages of embryogenesis, cellular reproduction, and wound healing (6, 169). This process is tightly orchestrated by a balance of pro- and anti-angiogenic factors. Neoangiogenesis is one of the critical steps necessary for progression of solid tumor development (1). Without angiogenesis, the solid tumors cannot exceed growth above 1-2 mm$^3$ in diameter (1, 167). Indeed, tumor size constraints are limited by the amount of available nutrients and oxygen diffusing within the tumor tissue. It is hypothesized that tumors gain the ability to secrete pro-angiogenic factors and induce secretions in the surrounding stromal tissues (167, 170), thus altering the microenvironment towards one favoring neovascularization. This process, referred to as the “angiogenic switch” (4, 170, 171)(Figure 4.2), stimulates new vascularity to provide cancer cells with the necessary substrates and adequate oxygenation, as well as the means for removal of toxic metabolites.
Figure 4.2: The “angiogenic switch” is a distinct process in tumorigenesis whose behavior is driven by oncogenic pathways and the tumor microenvironment. Solid tumors begin via avascular nodule growth (A). Continued growth leads to enhanced proliferation and necrosis. The angiogenic switch, or initiation of angiogenesis, must occur for metastases. Neoangiogenesis begins with perivascular detachment and BV dilation (B). This is followed by neoangiogenic sprouting (C), new BV formation and eventual maturation, and recruitment of surrounding vascular cells (D). BV formation will continue in concert with continued cancer proliferation, and the BVs feed the hypoxic and necrotic areas of the tumor with essential nutrients and oxygen (E)(82).
Angiogenesis is believed to be necessary for tumor invasion and formation of distal metastases, thus affecting progression of disease to a more lethal phenotype. Hence, assessment of angiogenesis and vascularity of solid tumors has been proposed as a potential predictor of cancer development, progression, and patient survival in various types of malignancies, including PCa (172-178).

Despite recent advances, there remain few concrete methods for assessing angiogenesis for use as a predictive model of disease progression. To date, the most frequently cited method for quantifying human angiogenesis is via microvessel density (MVD). This technique involves counting the number (or count) of vessels per given area. A landmark study by Weidner, et al (7) revealed that greater MVD was a prognosticator of local invasiveness and distal metastasis in PCa. It has also been reported that PCa tissue displays a significantly higher number of microvessels as compared to noncancerous tissue post prostatectomy. This correlation was the strongest between MVD and GG in poorly differentiated tissues (7, 159, 179, 180). MVD and total vascular area appear most predictive of tumorigenesis and metastases in tumors associated with significant neoangiogenesis, including the prostate (181).

Immunohistochemistry (IHC) has been utilized to characterize tumor tissue architecture and appears to be a dependable technique for quantifying MVD (88, 182). Antibodies used to detect blood vessels may target Factor VIII (FVIII), also known as von Willebrand factor (vWF), a clotting factor found on the surface of the endothelial cells that detect more mature BVs (183-185). Previous reports examining angiogenesis in human prostate tissue has focused primarily on PCa, not on normal (noncancerous) tissue. The goal of the present study is to characterize vascular molecular signatures of both malignant and normal human prostate.
Methods

Study Population

The Health Professionals Follow-up Study (HPFS) is a prospective study, started in 1986, of 51,529 male health professionals in the United States and Canada who were aged 40-75 at the time of recruitment. Participating men were voluntarily asked to submit information on age, marital status, height and weight, disease history, medications, smoking, physical activity and diet. Every other year, questionnaires solicited further information pertaining to recent diagnoses, including PCa. Individuals diagnosed with PCa were asked to provide associated medical records with grade, clinical stage, pathologic stage, and lethality. Paraffin-embedded tissue blocks and all pathology reports were requested from the prostatectomy cohort. Immediately upon arrival at The Ohio State University (OSU), all cases were recoded into a computerized database and housed in a tissue bank until further analyses. Of the 1,000-plus samples received, the present study focused on analyses of a 100 randomly selected cases with total Gleason scores between 4 and 10. The HPFS research protocol has been approved by the institutional review board (IRB) at the Harvard School of Public Health and Partners Healthcare and The Ohio State University.

Antibodies and Reagents

Rabbit polyclonal antibody against FVIII (# N1505) was used for IHC and purchased from Dako DakoCytomation Carpinteria, CA. Other reagents used for immunostaining include DakoCytomation EnVision + Dual Link System Peroxidase (cat # K4061), DakoCytomation Dual Endogenous Enzyme Block (S2003), DakoCytomation Liquid 3,3’-diaminobenzidine (DAB) + Substrate Chromogen System (K3468), Mayer’s Hematoxylin Solution (MHS32-1L) from Sigma-Aldrich Inc. (St. Louis, MO), Surgipath Micromount Mounting media from Surgipath Medical
Industries, Inc. (Grayslake, IL), Antigen Retrieval Citra Plus Solution and concentrated Reagent Diluent were purchased from Biogenex (San Ramon, CA). Unless otherwise stated, all reagents were purchased from Dako DakoCytomation (Carpinteria, CA).

**H&E Staining**

Paraffin-embedded tissue blocks were sectioned and transferred to Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). After rehydration, slides were stained with hematoxylin and eosin (H&E) on an automated slide stainer (Leica Microsystems Inc. Deerfield, IL) and cover slipped. The H&E slides were independently reviewed using the American Joint Committee on Cancer (AJCC) and World Health Organization (WHO) criteria, and compared with the original pathology reports.

**Immunohistochemistry**

IHC staining was performed on an OptiMax Automated Cell Staining System (BioGenex Laboratories, San Ramon, CA), which allows for staining of up to 40 slides concurrently under tightly controlled conditions, thus eliminating variations inherently associated with manual immunostaining. We then examined tumor microvessel density (MVD) with antibodies directed against FVIII. Here, sections were deparaffinized, rehydrated, and washed before staining. Sections were pre-treated with heated citrate buffer for antigen retrieval (AR). To eliminate endogenous peroxidase activity, sections were treated with peroxide for 5 minutes. Sections were exposed to primary antibodies directed against FVIII (1:2000) for 30 minutes (DakoCytomation) followed by a peroxidase labeled polymer conjugated to goat anti-rabbit immunoglobulins (DakoCytomation). To visualize FVIII stain, sections were treated with DAB chromagen solution (DakoCytomation) and counterstained with hematoxylin (Sigma-Aldrich)
Sections were then dehydrated and cover slipped. During the staining process, sections were washed with phosphate buffered saline (PBS) three times before and after each of staining steps. Unless otherwise noted, incubations were completed at room temperature.

**Digital Imaging and Analysis**

All tissue sections were examined by a pathologist, under light microscopy, for characterization of tumor tissue morphology and cytological features. Malignant and noncancerous areas were identified and outlined in up to four representative sections per case. On each of the prostate tissue sections, normal stroma and glandular tissues were localized, if possible, at least 5mm from the cancer area outlined by the pathologist. Tissue areas were visualized under bright field microscopy using a Nikon Eclipse E800 microscope (Nikon Instruments, Melville, NY). Representative images of random, non-overlapping high power fields (200x) were captured from each section using a RT Slider Spot Camera (Diagnostic Instruments, Sterling Heights, MI). Captured images were analyzed using Image ProPlus 4.5 software (Media Cybernetics, Silver Spring, MD) designed specifically for histological analyses (Figure 4.3). Images were quantified by the presence of angiogenic markers (FVIII) as demonstrated in Figure 4.4. Branching structures were counted as one single vessel, the lumen did not have to be visible for a structure to be considered a vessel, and red blood cells (RBCs) were not used to categorize a structure as a BV. In addition, slides were evaluated without knowledge of GG or staging of the tumors.
A. Paraffin-embedded radical prostatectomy tissues were sectioned. H&E and IHC was performed.

B. Areas of cancer versus noncancer were identified and outlined by a pathologist.

C. A Nikon Eclipse E800 microscope equipped with a Spot RT Slider digital camera was used to capture images of non-overlapping high power fields

Figure 4.3. Images depicting methods involved in study design.
Figure 4.4. Representation of digital image analysis for vascular architecture using ImagePro Plus digital analyses tools. A. Original image demonstrating FVIII immunostaining (brown; hematoxylin counterstain). B and C. Selection of vessels using separation by color. D. Analysis extrapolates each object (vessel) to assess count, area, perimeter, and diameter.
Statistics

Data from image analyses performed with Image ProPlus 4.5 were exported into an Excel spreadsheet and transferred into SAS version 9.1 (SAS Institute, Inc., Cary, NC). The mean for each case was calculated and utilized for further analysis. Vasculature in normal prostate and cancer tissue were compared using a Signed Rank Test on MVD and paired t-test on total vascular area, area per vessel, vessel diameter, vessel perimeter, vessel roundness, and percent area per vessel (Table 4.1). Spearman Correlation Coefficients were performed to examine the relationship between clinical features of the tissue with measured biomarkers of angiogenesis. Vascular architecture was compared in cancer and noncancer tissue within tumor stage and grade using repeated measure analysis of variance models.
<table>
<thead>
<tr>
<th>ANGIOGENESIS VARIABLES</th>
<th>MEASUREMENTS</th>
<th>DESCRIPTIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microvessel Density (MVD)</td>
<td>sum</td>
<td>Number of BVs in 2x2mm high power field (HPF)</td>
</tr>
<tr>
<td>Total Vascular Area</td>
<td>sum, um²</td>
<td>Sum of all vessels in 2x2mm HPF</td>
</tr>
<tr>
<td>Area Per Vessel</td>
<td>mean, um²</td>
<td>Mean area per vessel within 2x2mm HPF</td>
</tr>
<tr>
<td>Vessel Diameter</td>
<td>mean, um</td>
<td>Mean vessel diameter per case within 2x2mm HPF</td>
</tr>
<tr>
<td>Vessel Perimeter</td>
<td>mean, um</td>
<td>Mean vessel perimeter per case within 2x2mm HPF</td>
</tr>
<tr>
<td>Vessel Roundness</td>
<td>mean, um</td>
<td>Mean vessel roundness per case within 2x2mm HPF; 1.0 = perfect round circle; higher numbers indicate greater irregularity in shape</td>
</tr>
<tr>
<td>Percent Area Per Vessel</td>
<td>mean, um²</td>
<td>Area within % of total area in 2x2mm HPF occupied by 1 vessel</td>
</tr>
<tr>
<td>Percent All Vessels</td>
<td>mean, um²</td>
<td>Mean percent of 2x2mm area that is vascular (eg: 12 vessels/2x2mm area = %)</td>
</tr>
</tbody>
</table>

Table 4.1: Summary of FVIII angiogenesis variables, corresponding units of measure, and lay descriptors.
Results

Study Cohort

Of the 51,529 men in the HPFS prospective cohort, we obtained tissue samples from over 500 men who were diagnosed and underwent a radical prostatectomy between 1986 and 2002. For FVIII analysis, we assessed 100 cases. In this subpopulation of subjects, the mean age at the time of diagnosis was 65.3 years (SD ± 6.1 years). Forty-seven subjects (49%) presented with a lower grade disease with a combined GG of 2-6 and 49 (51%) had tumors with a GG of 7 or higher. Sixty-two (64.6%) men presented with a T stage of 1 or 2 and 34 (35.4%) of men demonstrated more advanced PCa T stage (3 or 4; Table 4.2).
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total, N</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>65.3</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>51-79</td>
<td></td>
</tr>
<tr>
<td>Gleason Grade</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>2-5</td>
<td>12</td>
<td>12.5</td>
</tr>
<tr>
<td>6</td>
<td>35</td>
<td>36.5</td>
</tr>
<tr>
<td>7</td>
<td>35</td>
<td>36.5</td>
</tr>
<tr>
<td>8-10</td>
<td>14</td>
<td>14.6</td>
</tr>
<tr>
<td>Tumor Stage</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>T1b</td>
<td>4</td>
<td>4.2</td>
</tr>
<tr>
<td>T2</td>
<td>11</td>
<td>11.5</td>
</tr>
<tr>
<td>T2a</td>
<td>11</td>
<td>11.5</td>
</tr>
<tr>
<td>T2b</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>T2c</td>
<td>35</td>
<td>36.5</td>
</tr>
<tr>
<td>T3</td>
<td>2</td>
<td>2.1</td>
</tr>
<tr>
<td>T3a</td>
<td>25</td>
<td>26.0</td>
</tr>
<tr>
<td>T3b</td>
<td>7</td>
<td>7.3</td>
</tr>
</tbody>
</table>

Table 4.2: Clinical and demographic characteristics of HPFS FVIII study cohort
Vascular Architecture and Prostate Cancer

FVIII IHC identified BVs in both cancerous and noncancerous tissues (Figure 4.5). Based upon our findings, normal prostate had significantly lower MVD than PCa tissue. Specifically, mean MVD in the normal prostate was 12.1 (SD + 6.0) while mean MVD in cancer tissue was 32.0 + 17.8 (p<0.0001; Table 4.3; Figure 4.6). We then evaluated the mean vessel area and observed that normal prostate had significantly larger mean area per vessel (5742.1 µm² + 4185.0 µm²) than PCa tissue (1866.6 µm² + 1422.1 µm², p< 0.0001; Figure 4.6). Likewise, the mean percent area per vessel in normal prostate was significantly greater (0.38 % + 0.27%) than in PCa (0.12 % + 0.09%, p<0.0001; Figure 4.6). Based on our analyses, there was no difference in the total percent area comprised by vessels between normal and PCa tissue (47,686.6 µm² + 24,063.0 µm² and 43,419.0 µm² + 17,725.8 µm², respectively). We observed a difference between the mean vessel diameter and perimeter between normal prostate and cancer tissue. Specifically, normal prostate was characterized by vessels of larger diameter (72.7 µm + 23.8 µm) and perimeter (303.5 µm + 103.3 µm) than vessels observed in PCa (40.9 µm + 12.2 µm in diameter and 182.7 µm + 54.8 µm in vessel perimeter, p<0.0001 for both parameters measured). A comparison of these two parameters is shown in Figure 4.7.

Lastly, the shape of the vessels was evaluated using a computerized scoring system which assumes that a perfectly round vessel is scored as 1 and the more irregular the shape of the BVs, the higher the score. The shape of the vessels observed in normal prostate was significantly more regular (2.3 + 0.4) than the shape of vessels in PCa (2.6 + 0.4; P<0.0001; Figure 4.6). Overall, these data indicate that PCa tissue is characterized by significantly higher MVD and that vessels are significantly smaller (diameter, perimeter and area) and more irregular in shape than vessels observed in normal prostate (Figure 4.6).
Figure 4.5. Representative images (2 each) of FVIII BVs in areas of noncancer and cancer in human prostate. Note FVIII immunostaining displays vasculature that is more orderly and larger in overall vascular architecture in the noncancer prostate (left) as opposed to smaller and more tortuous vascularity in cancer tissue (right). Original magnification 200x.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Variable</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>SE</th>
<th>Med</th>
<th>Min</th>
<th>Max</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MVD</strong></td>
<td>Noncancer</td>
<td>99</td>
<td>12.1</td>
<td>6.0</td>
<td>0.60</td>
<td>11</td>
<td>3</td>
<td>38</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>Cancer</td>
<td>99</td>
<td>31.9</td>
<td>17.9</td>
<td>1.8</td>
<td>28.8</td>
<td>5</td>
<td>123</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Difference</td>
<td>98</td>
<td>19.8</td>
<td>17.1</td>
<td>1.7</td>
<td>16.8</td>
<td>-8</td>
<td>111</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>Mean Total Vascular Area</strong></td>
<td>Noncancer</td>
<td>99</td>
<td>47765</td>
<td>24104</td>
<td>2423</td>
<td>43184</td>
<td>4333</td>
<td>126183</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cancer</td>
<td>99</td>
<td>43429</td>
<td>17820</td>
<td>1791</td>
<td>39547</td>
<td>11541</td>
<td>88662</td>
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</tr>
<tr>
<td></td>
<td>Difference</td>
<td>98</td>
<td>-4514</td>
<td>28227</td>
<td>2851</td>
<td>-4588</td>
<td>-71525</td>
<td>64252</td>
<td>0.1166</td>
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<tr>
<td><strong>Mean Area Per Vessel</strong></td>
<td>Noncancer</td>
<td>99</td>
<td>5740</td>
<td>4183</td>
<td>420</td>
<td>4548</td>
<td>973</td>
<td>23388</td>
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<tr>
<td></td>
<td>Cancer</td>
<td>99</td>
<td>1870</td>
<td>1426</td>
<td>143</td>
<td>1503</td>
<td>331</td>
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<td></td>
<td>Difference</td>
<td>98</td>
<td>-3903</td>
<td>4349</td>
<td>439</td>
<td>-2779</td>
<td>-19793</td>
<td>6827</td>
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</tr>
<tr>
<td><strong>Mean Vessel Diameter</strong></td>
<td>Noncancer</td>
<td>99</td>
<td>73</td>
<td>29</td>
<td>2.4</td>
<td>69</td>
<td>31</td>
<td>153</td>
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<tr>
<td></td>
<td>Cancer</td>
<td>99</td>
<td>41</td>
<td>12</td>
<td>1.2</td>
<td>38</td>
<td>20</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Difference</td>
<td>98</td>
<td>-32</td>
<td>25</td>
<td>2.5</td>
<td>-30</td>
<td>-115</td>
<td>33</td>
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<tr>
<td><strong>Mean Vessel Perimeter</strong></td>
<td>Noncancer</td>
<td>99</td>
<td>304</td>
<td>103</td>
<td>10</td>
<td>284</td>
<td>122</td>
<td>650</td>
<td></td>
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<tr>
<td></td>
<td>Cancer</td>
<td>99</td>
<td>183</td>
<td>55</td>
<td>6</td>
<td>167</td>
<td>99</td>
<td>368</td>
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</tr>
<tr>
<td></td>
<td>Difference</td>
<td>98</td>
<td>-122</td>
<td>110</td>
<td>11</td>
<td>-108</td>
<td>-479</td>
<td>182</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>Mean Vessel Roundness</strong></td>
<td>Noncancer</td>
<td>99</td>
<td>2.263</td>
<td>0.437</td>
<td>0.044</td>
<td>2.215</td>
<td>1.363</td>
<td>3.934</td>
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<tr>
<td></td>
<td>Cancer</td>
<td>99</td>
<td>2.582</td>
<td>0.418</td>
<td>0.042</td>
<td>2.521</td>
<td>1.78</td>
<td>4.176</td>
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</tr>
<tr>
<td></td>
<td>Difference</td>
<td>98</td>
<td>0.321</td>
<td>0.541</td>
<td>0.055</td>
<td>0.277</td>
<td>-1.735</td>
<td>1.857</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>Mean Percent Area Per Vessel</strong></td>
<td>Noncancer</td>
<td>99</td>
<td>0.375</td>
<td>0.274</td>
<td>0.028</td>
<td>0.3</td>
<td>0.064</td>
<td>1.541</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cancer</td>
<td>99</td>
<td>0.123</td>
<td>0.094</td>
<td>0.009</td>
<td>0.099</td>
<td>0.022</td>
<td>0.694</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Difference</td>
<td>98</td>
<td>-0.254</td>
<td>0.285</td>
<td>0.029</td>
<td>-0.183</td>
<td>-1.304</td>
<td>0.45</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>Mean Percent All Vessels</strong></td>
<td>Noncancer</td>
<td>99</td>
<td>3.147</td>
<td>1.588</td>
<td>0.16</td>
<td>2.845</td>
<td>0.286</td>
<td>8.314</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cancer</td>
<td>99</td>
<td>2.854</td>
<td>1.178</td>
<td>0.118</td>
<td>2.586</td>
<td>0.739</td>
<td>5.642</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Difference</td>
<td>98</td>
<td>-0.305</td>
<td>1.862</td>
<td>0.188</td>
<td>-0.302</td>
<td>-4.713</td>
<td>4.233</td>
<td>0.1084</td>
</tr>
</tbody>
</table>

Table 4.3: Mean differences in VIII cancer versus noncancer variable in cohort.
Figure 4.6. Graphical representation of data presented in Table 4.3.
Figure 4.6. Graphical representation of data presented in Table 4.3.
Figure 4.6. Graphical representation of data presented in Table 4.3.
For visual representation, data is presented as scatterplots to assess more complex comparisons and cluster effects of selected variables. In cancer tissue, vessel diameter, area per vessel, and perimeter were inversely related to mean vessel diameter (Figure 4.7). In terms of vessel shape, MVD and roundness of shape were related (Figure 4.7).
Figure 4.7. Scatterplots showing angiogenic patterning in cancer versus noncancer FVIII immunostaining.
Figure 4.7. Scatterplots showing angiogenic patterning in cancer versus noncancer FVIII immunostaining.
Discussion

In 1889, Stephen Paget proposed the “seed and soil” theory. In short, Paget hypothesized that when cancerous cells metastasize, their “seeds” travel and root in a compatible microenvironment, or “soil,” that is supportive of their continued cellular growth (186). Much of Paget’s premise can be explained by a tumor’s ability to recruit its own continued vasculature, invade and take residence at distant sites, and colonize via oncogenic alterations within the circulatory system (8, 182). Angiogenesis plays a central role in PCa carcinogenesis and progression (8, 182), and several studies have documented a significant correlation between MVD with GG, pathological stage, and lethality of disease (12, 187).

Because PCa is notoriously recognized for its biological heterogeneity, molecular biomarkers are essential for informing feasible treatment options. Angiogenesis appears to serve as a key biological factor in the initial development of solid tumors and one that is essential for cancer progression and metastasis. Historically, investigators have focused on the number of vascular structures (MVD) observed in a given histopathologic section as the sole vascular biomarker of angiogenesis. Our study examined 100 cases of human prostate tissue and currently represents the largest study known to evaluate and characterize both cancer and noncancerous prostate via FVIII immunostaining. We not only measured MVD, but extended our study to examine other parameters of vascular architecture such as vessel area, diameter, perimeter, and shape. We propose that the assessment of vascular architecture, coupled with quantitative image analysis, can provide a quantitative tool that elucidates insights into prostate carcinogenesis.

We noted that normal (noncancer) prostate tissue is characterized by lower MVD, with BVs that are larger in area, diameter and perimeter. Normal prostate is also characterized by more regularly shaped vessels than those observed in malignant prostate. These observations
confirm that BVs, in normal tissues, typically display more orderly and organized vasculature architecture than is observed in tumor tissue (167, 188) (Table 4.4). These findings support the hypothesis that advancing prostate tumors form numerous small, archaic, and more irregularly shaped vessels resulting in a primitive and possibly dysfunctional vasculature (12, 189, 190). These data reveal that vascular architecture analyses are capable of detecting differences between malignant and normal human prostate. These findings have encouraged additional investigations into the relationship between vascular architecture and tumor stage and grade in our laboratory.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Cancer</th>
<th>More Angiogenic Phenotype</th>
<th>Noncancer</th>
<th>Less Angiogenic Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microvessel Density (MVD)</td>
<td>32 (18/1.8)</td>
<td>Higher number</td>
<td>12 (6/0.6)</td>
<td>Lower number</td>
</tr>
<tr>
<td>Mean Area Per Vessel</td>
<td>1870 (1426/143)</td>
<td>Smaller vessels</td>
<td>5740 (4183/420)</td>
<td>Larger vessels</td>
</tr>
<tr>
<td>Mean Vessel Diameter (um)</td>
<td>41 (12/1.2)</td>
<td>Smaller diameter</td>
<td>73 (29/2.4)</td>
<td>Larger diameter</td>
</tr>
<tr>
<td>Mean Vessel Perimeter (um)</td>
<td>183 (55/6)</td>
<td>Smaller perimeter</td>
<td>304 (103/10)</td>
<td>Larger perimeter</td>
</tr>
<tr>
<td>Mean Vessel Roundness (um)</td>
<td>2.6 (0.4/.04)</td>
<td>Irregular shape</td>
<td>2.3 (0.4/.04)</td>
<td>More regular shape</td>
</tr>
</tbody>
</table>

Table 4.4: Angiogenic characteristics in the FVIII cohort. Data represented as mean (SD/SEM).
Assessment of various angiogenic factors revealed that reduced mean vessel area, smaller diameter, and more irregularly shaped BVs were associated with cancerous tissue. Analyses of correlations among our selected parameters confirm the relationship between area, diameter, and perimeter. It also revealed that more irregularly shaped vessels were associated with smaller mean vessel area, diameter, and perimeter. Based on our data, PCa is characterized by smaller vessels (diameter, perimeter, and mean vessel area) and more irregularly shaped vessels. This may be secondary to the vessels undergoing more rapid growth and assertive biology in an effort to meet the demands of the cancerous growth (6, 169).

Quantification of various aspects of BV architecture, in addition to MVD, provides a more thorough characterization of PCa tissue behavior relative to angiogenesis.

The current study utilizes a random subset of the >500 available cases from the HPFS prostatectomy cohort and was designed to detect biological differences between cancer and noncancer. Based upon the observed differences, we hypothesize that there is a relationship between quantifiable vascular variables, Gleason score and TNM stage. Expansion of this analysis is underway to assess this hypothesis and compare the results to additional biomarkers of disease progression such as p53 expression. Within our subset, descriptive data is presented in Figure 4.5. There is a growing body of literature suggesting a mechanistic relationship between p53 and neoangiogenesis. We have generated preliminary data that confirms this relationship in Chapter 5.
| Total Gleason Scores | 2 to 5  
(N=12) | 6  
(N=35) | 7  
(N=34) | 8 to 10  
(N=14) |
|---------------------|---------|---------|---------|-------------|
| Microvessel Density  
(MVD) | 30  
(22/6) | 34  
(22/4) | 31.3  
(14.4/2.5) | 32  
(12/3) |
| Total Vessel Area  
(sum, um²) | 53174  
(20692/5973) | 42050  
(18294/3092) | 41393  
(15240/2614) | 46063  
(20599/5505) |
| Area Per Vessel(mean,  
um²) | 2934  
(2736/790) | 1633  
(1104/187) | 1761  
(1158/199) | 1737  
(910/243) |
| Vessel Diameter  
(mean, um) | 50  
(20/6) | 39  
(9/2) | 40  
(11/2) | 41  
(11/3) |
| Vessel Perimeter  
(mean, um) | 224  
(84/24) | 172  
(42/7) | 181  
(52/9) | 182  
(50/13) |
| Vessel Roundness  
(mean, um) | 2.8  
(0.7/0.2) | 2.5  
(0.3/0.1) | 2.6  
(0.4/0.1) | 2.7  
(0.3/0.1) |
| Percent Area Per  
Vessel (mean, um²) | 0.2  
(0.2/0.1) | 0.1  
(0.1/0.0) | 0.1  
(.1/0.0) | 0.1  
(0.1/.02) |
| Percent All Vessels  
(mean, um²) | 3.5  
(1.4/0.4) | 2.8  
(1.2/0.2) | 2.7  
(1.0/0.2) | 3.0  
(1.4/0.4) |

Table 4.5. Total Gleason scores in relation to mean FVIII variables. Data represented as mean (SD/SEM).
While this large prospective cohort of PCa patients and the associated pathologic data is extremely valuable, future studies in cohorts of patients with available PSA, TNM, and clinical recurrence would provide even greater insights into the ability of angiogenic biomarkers to detect differences between indolent and aggressive prostatic disease. Due to the fact that this prospective cohort study was initiated in 1986, information on PSA was not available as this was prior to the introduction and widespread utilization of PSA screening. There is also limited information available for extensive biochemical and/or clinical recurrence of disease in this cohort. In addition, for FVIII to be recognized as a valid prognostic biomarker, standardization of IHC methods must be employed and validated. Likewise, standardization of digital quantification is needed.

These data show that digital image analysis of BVs is a feasible and objective approach to the quantification of architectural alterations in vascular structures during prostate carcinogenesis. Smaller vessels of greater number and more irregularly shaped BVs are characteristic of human PCa angiogenesis. In addition, higher tumor grade display even greater biological changes in PCa vascular architecture. These biomarkers may be of utility in the assessment of presumed preventive and therapeutic agents that act to target angiogenesis.

This study was distinctive in approach in terms of characterizing angiogenesis in cancer versus noncancer prostate tissue. In addition, we assessed quantitative measures of angiogenesis as a potential biomarker to predict biological subtypes of PCa that may inform clinical and medical management. It is apparent that genetic alterations, including tumor suppressor mutations and vascular architectural changes influence PCa progression and disease
aggressiveness. Our efforts will provide data to support future studies intended to assess environmental and genetic interactions that are critical to our understanding of human prostate carcinogenesis and mortality.
Abbreviation Key

Blood vessels (BVs)
Factor VIII (FVIII)
Health Professionals Follow-up Study (HPFS)
Hematoxylin and eosin (H&E)
Immunohistochemistry (IHC)
Microvessel density (MVD)
Prostate cancer (PCa)
Prostate specific antigen (PSA)
Transurethral resection of the prostate (TURP)
von Willebrand Factor VIII (FVIII)
CHAPTER 5: Integration

Introduction and Overview

The public health burden associated with prostate cancer (PCa) is troubling. With over 240,000 new cases of prostate cancer (PCa) and more than 33,000 deaths from the disease anticipated in the U.S. in 2011, it remains the most frequent nondermatologic cancer among males (2). Treatment strategies for PCa include watchful waiting, surgery, radiation, or androgen deprivation therapy, all with possible consequences.

There is a dire need to characterize sluggish versus hostile prostate carcinogenesis, so clinicians and patients may make evidence-based and individualized treatment decisions. We have yet to identify key molecular signatures discriminating indolent versus aggressive disease. It is essential that we fully realize the complexity of prostate carcinogenesis and its inherent heterogeneity. An understanding of these mechanistic and molecular nuances will assist in establishing rational approaches to disease prevention, interventions, and subsequent treatments. Here we have presented a comprehensive effort to fill a gap in the literature and answer key research questions pertaining to biomarkers of disease (p53 and angiogenesis) utilized to decipher and characterize malignant prostate versus normal prostate tissue.

Our first goal was to assess the interrelationship between p53 and established diagnostic biomarkers of PCa progression. It is well established that prostate carcinogenesis is incredibly multifaceted and impacted by numerous variables. p53 is a central regulator of normal cellular function and any event leading to p53 dysfunction dramatically impacts cancer
progression in any tissue of origin. To our knowledge, the literature is void of any studies investigating treatment regimens specific to PCa patients affected by germline TP53 gene mutations. The critical objective of this aim was to thoroughly examine such relationships by utilizing a case study and focused literature review to inform clinical considerations and recommendations. This case highlights the critical need for greater awareness of cancer genetics in those treating urologic cancers. This field presents incredible opportunities to investigate the relationship of p53 dysfunction to PCa risk and the impact of behaviors and interventions on this risk.

The Health Professionals Follow-Up Study (HPFS) prostatectomy cohort provides a unique opportunity for which to assess p53 as a biomarker of cancer progression and a means in which to characterize malignant versus normal cancer in prostate tissue. Although p53 protein is central to current research, there still remains a void in the literature specific to p53 protein dysfunction relative to biomarkers of prostate carcinogenesis. H&E staining is adequate for the diagnosis of many genitourinary cancers, yet the more challenging and heterogeneous solid tumors, such as PCa, require more extensive pathologic tools in which to adequately diagnose and inform optimal treatment.

We evaluated the relationship between p53 expression by immunostaining and cancer status and have established promising diagnostic biomarkers. Our results demonstrate that there is a significant relationship between the incidence of p53 immunoreactivity and cancer status as well as the intensity of staining by cancer status (cancer versus noncancer) and Gleason score. The percentage of nuclear staining positively correlates with cancer status and intensity category but surprisingly, does not
correlate with grade. Our results confirm that p53 IHC may serve as a viable molecular marker for predicting more aggressive disease in PCa and that presence of staining or staining category may be more informative than percentage of p53 stain.

Our third aim was to characterize the profiles of the vascular architecture in the cancerous and noncancerous areas of the prostate as visualized by FVIII immunostaining. In addition, this study supports the development of image analysis of FVIII immunostained PCa tissue as an objective and quantitative tool for analysis of tumor vasculature. Despite numerous publications evaluating MVD in relation to PCa, the use of this biomarker has still not been adequately validated as a predictor of PCa progression and a means to characterize malignant versus normal tissue in the prostate. The novel aspects of this aim are the use of the additional measures of vasculature (vessel diameter, perimeter, roundness, etc.) in addition to MVD and their biological profile characterizing noncancerous and cancerous areas of the prostate.

Our study demonstrates that prostate tumor vasculature can be quantitated in an objective manner and predicts more aggressive tumor biology and higher grade disease. We recognize that there are different antigens (CD31, CD34, and FVIII) that can be used to identify vasculature and there are differences in the resulting MVD score between each (191). A more comprehensive understanding of the variations in vasculature, especially with respect to BV maturity that may impact tumor metastasis, is warranted.

Indeed, p53 dysfunction and neovascularization are likely interrelated in the complex series of events leading to malignant disease. Within the HPFS cohort, preliminary analysis indicates that the presence of p53 expression correlates with greater MVD and vascular area, smaller vessels, and more irregular vessels identified by CD34 positive endothelial cells (Table 5.1). These preliminary results support the growing evidence that wild type p53 exerts an anti-
angiogenic effect by interfering with hypoxic regulators, proangiogenic factors, and endogenous angiogenesis inhibitors (166)(Figure 5.1). Further analysis is ongoing to determine the differences in the vascular profile identified by CD34 and FVIII in the areas of cancer and noncancer as well as the relationship between p53 and these biomarkers and their association to cancer grade and stage.
<table>
<thead>
<tr>
<th>CD34 Variable</th>
<th>p53 Variable</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>SE</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area Per Vessel</td>
<td>p53 negative</td>
<td>309</td>
<td>551.5</td>
<td>274.0</td>
<td>15.6</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>p53 positive</td>
<td>185</td>
<td>486.6</td>
<td>246.8</td>
<td>18.1</td>
<td></td>
</tr>
<tr>
<td>Diameter</td>
<td>p53 negative</td>
<td>309</td>
<td>25.4</td>
<td>5.1</td>
<td>0.3</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>p53 positive</td>
<td>185</td>
<td>24.0</td>
<td>5.1</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Perimeter</td>
<td>p53 negative</td>
<td>309</td>
<td>128.5</td>
<td>24.4</td>
<td>1.4</td>
<td>0.132</td>
</tr>
<tr>
<td></td>
<td>p53 positive</td>
<td>185</td>
<td>125.1</td>
<td>24.6</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>Roundness</td>
<td>p53 negative</td>
<td>309</td>
<td>4.0</td>
<td>1.1</td>
<td>0.1</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>p53 positive</td>
<td>185</td>
<td>4.3</td>
<td>1.0</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Percent Area Per Vessel</td>
<td>p53 negative</td>
<td>309</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>p53 positive</td>
<td>185</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Percent All Vessels</td>
<td>p53 negative</td>
<td>309</td>
<td>2.4</td>
<td>1.4</td>
<td>0.1</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>p53 positive</td>
<td>185</td>
<td>2.1</td>
<td>1.2</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>MVD</td>
<td>p53 negative</td>
<td>309</td>
<td>75.7</td>
<td>38.8</td>
<td>2.2</td>
<td>0.033</td>
</tr>
<tr>
<td></td>
<td>p53 positive</td>
<td>184</td>
<td>69.2</td>
<td>28.4</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>Total Vessel Area</td>
<td>p53 negative</td>
<td>309</td>
<td>37,444.7</td>
<td>21,843.0</td>
<td>1,242.6</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>p53 positive</td>
<td>184</td>
<td>31,982.2</td>
<td>18,991.0</td>
<td>1,396.2</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.1. p53 in relation to CD34 immunostaining.
Figure 5.1. p53 limits angiogenesis by obstructing HIF, a key regulator of hypoxia. Under normal conditions, levels of HIF-1α remain. Conversely, under stressful hypoxic conditions, levels of HIF-1α become stable and permit HIF target gene activation to facilitate angiogenesis. Upon oncogenic initiation, p53 binds to HIF-1α to inhibit angiogenesis. VHL, von Hippel-Lindau protein; Pro, proline (166).
We anticipate that, ultimately, the ability to identify indolent and aggressive cancers will require the integration of multiple biomarkers and that p53 and vascular architecture are valuable components. Proliferative and apoptotic indices are plausible candidates and have correlated with standard diagnostic biomarkers in pre-clinical models of PCa (9, 191). In fact, we have a growing body of evidence evaluating p53, proliferative, and apoptotic indices in the novel Rbf/APT121 murine models of prostate carcinogenesis and the impact of androgen deprivation (Appendix A). As part of a highly collaborative study, there is evidence that multiple genes involved in the p53 signaling pathway are altered early during the carcinogenesis process (unpublished observations). This preliminary data must be validated in a future cohort, of which, assessment of molecular signatures, along with data of disease progression, recurrence, and survival are available.

Cumulatively, these results demonstrate that p53 and vascular architecture are valuable molecular markers of an aggressive phenotype in prostate carcinogenesis and as such, may provide the basis for further inquiry evaluating effects of therapy and/or chemopreventive agents. Future studies are needed to assess the effects of genetics and nutrition on both p53 expression and vasculature.

Impact and Future Directions

This novel project continues a unique collaboration that leverages expertise in the fields of nutrition, genetics, and cancer. Although promising gene-diet interactions have been observed for lycopene/carotenoids, vitamin E, and cruciferous vegetables (192, 193), the science of nutritional genomics remains in its infancy. Widespread collaborative research efforts must focus on the discovery of modifiable interactions to
reduce disease risk or progression of disease. The efforts described within are directed towards the ultimate goal of defining histopathologic and molecular biomarkers associated with p53 dysfunction and angiogenesis in prostate carcinogenesis that may serve as surrogate endpoint biomarkers and provide important information regarding clinical outcomes that can impact evidence based treatment options. To our knowledge, our data represent the first attempt to evaluate p53 as related to biomarkers of angiogenesis in both cancer and noncancerous prostate. Such data will provide a mechanistic basis for future large scale clinical trials to assess the role of p53 and angiogenesis for human PCa prevention.
Appendix A: Characterization of p53 Expression during Transgenic Mouse Prostate Carcinogenesis and Its Response to Testosterone

Abstract

Objectives

Well characterized rodent models of prostate carcinogenesis are critical for the preclinical evaluation of dietary and chemopreventative interventions. p53 signaling is a target for genetic dysregulation in transgenic murine models of prostate cancer. Our objective was to examine p53 expression by immunohistochemistry, temporally and spatially in the prostate, as a potential biomarker of efficacy for chemopreventative interventions.

Methods

p53 immunohistochemical staining in wild type mice and two murine models (TRAMP and Rbf/TgAPT121) of prostate carcinogenesis was evaluated. Prostates from male TRAMP (up to 28 weeks) and Rbf/TgAPT121 mice (up to 32 weeks) were histopathologically evaluated. Normal epithelium, prostatic intraepithelial neoplasia (PIN) and adenocarcinoma was evaluated by p53 (CM5) staining, proliferation index (Ki67) and apoptotic index (ApopTag). Digital image analysis was employed to assist in quantification of biomarkers.
Results

p53 nuclear staining is rare in wild type mouse prostate epithelium. In TRAMP, p53 accumulation is first seen in the nuclei of young mice in areas of hyperplasia and low grade PIN (11%), with greater staining as carcinogenesis progresses through invasive cancers (59%). In the Rbf/TgAPT121 model, a less aggressive model, the PIN can be further subclassified and the percentage of p53 stained epithelial cells in the PIN lesions increases with advanced grade (PIN I: 3%; PIN II: 9%; PIN III: 17%; PIN IV: 32%). p53 staining also correlates with the apoptotic index in the PIN lesions (PIN I: 7%; PIN II: 9%; PIN III: 12%; PIN IV: 13%). Androgen dependency of p53 staining was demonstrated in TRAMP mice, as castration attenuates and repletion of testosterone restores the p53 nuclear staining. p53 staining is predominately located in the basal layer of the glandular epithelium and similar indices were observed in the dorsal and lateral lobes of the prostate.

Conclusions

p53 nuclear staining in TRAMP and Rbf/TgAPT121 models of prostate carcinogenesis increases as a function of lesion grade and stage. The early expression of p53 in murine prostate carcinogenesis is in parallel with other biomarkers of progression, such as proliferation and apoptotic index.

Significance

p53 immunostaining may serve as an early indicator of effective chemopreventative agent intervention in prostate carcinogenesis.

Introduction

The molecular mechanisms leading to the development and progression of human prostate cancer are heterogeneous and likely account for the dramatic variability in clinical course between men. Thus, our ability to personalize prostate cancer prevention and therapy
will require the use of objective biomarkers allowing for the discrimination between aggressive
versus indolent biology at each stage of the carcinogenic process. Well characterized rodent
models of prostate carcinogenesis may provide insight into this effort. In addition, biomarkers
associated with each stage of prostate cancer progression will be valuable for the preclinical
evaluation of dietary and chemopreventative interventions. Because TP53, a tumor suppressor
gene, is central to metabolic and apoptotic pathways in the cancer cascade process, the present
study was designed to more completely characterize histopathological p53 expression during
prostate carcinogenesis in the TRAMP (1) and Rbf/TgAPT121 (2) murine models.

The TRAMP mouse is a widely used transgenic model of prostate cancer that rapidly
develops invasive adenocarcinoma and distant metastases with neuroendocrine features. The
transgenic Rbf/TgAPT121 model is a variation of the TRAMP model in that it uses a SV40
transformation system and is driven by the androgen-responsive probasin promoter. However,
only the 121 N-terminal amino acids of the large T-antigen are expressed resulting in a less
aggressive model of prostate carcinogenesis, characterized by progression through PIN and
locally microinvasive cancer.

The SV40 large T antigen impacts the Rb and p53 regulatory network to impact cell cycle
control and DNA replication. In this study, we focus upon p53 expression during TRAMP and
Rbf/TgAPT121 prostate carcinogenesis in relation to stages of progression and anatomical
location of cells expressing p53. We also examine the role of testosterone as a modulator of
prostate p53 expression. Finally, we examine the spatial and temporal relationship between p53
expression and proliferative index.
Materials & Methods

Animal Models

The TRAMP (transgenic adenocarcinoma mouse prostate) mouse is a widely used transgenic model of prostate cancer and rapidly develops invasive adenocarcinoma and distant metastases with neuroendocrine features by 30 weeks of age. The transgenic Rbf/TgAPT121 model is a variation of the TRAMP model in that it uses a SV40 transformation system, and is driven by the androgen-responsive probasin promoter. However, in this model, only the 121 N-terminal amino acids of the large T-antigen are expressed. This results in specific inactivation of the Rb family proteins in androgen responsive prostate epithelium, without altering the activity of p53 and other targets of the SV40 large T antigen. These mice demonstrate enhanced prostatic epithelial proliferation and apoptosis resulting in PIN and adenocarcinoma. For these studies, prostate tissues from TRAMP and Rbf/TgAPT121 transgenic mice, 8-32 weeks of age and their respective non-transgenic counterparts, (WT; wild type), were analyzed for histopathologic biomarkers.

Histopathology

At sacrifice, the prostate was removed and fixed overnight in 10% neutral buffered formalin and then transferred to 70% ethanol. The tissues were processed and embedded in paraffin using standard techniques. Tissues were sectioned at 3.5-4.0 micron thickness, mounted on charged barrier slides (BioGenex, San Ramon, CA) and stained with H&E by an automated slide stainer (Leica Autostainer XL, Leica Microsystems Nussloch GmbH, Heidelberger, Germany) for histopathologic examination.

Immunohistochemistry

Immunohistochemical analysis was performed on FFPE tissues sections to visualized p53 expression and localization and proliferation (Ki67). Briefly, tissues were deparaffinized and
rehydrated and antigen retrieval was performed using Citra Plus Antigen Retrieval Solution (BioGenex, San Ramon, CA) for 35 minutes. Endogenous peroxidase activity was quenched with Peroxidase Block (DakoCytomation, Carpenteria, CA). Tissues were incubated with anti-mouse p53 (NCL-CM5, rabbit polyclonal, 1:500; DAKO, Carpentry, CA, 1h); anti-human p53 (DO-7, mouse monoclonal, 1:75, DAKO, 1h), anti-Ki67, (clone TEC-3, rat monoclonal,1:50, DAKO, Carpenteria, CA, 1h). Antibody binding was visualized using the Labeled Polymer-HRP and DAB + Chromogen solution (EnvisionPlus, DakoCytomation, Carpenteria, CA). After DAB color reaction, all slides were counter-stained with hematoxylin. Apoptosis was visualized using ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore, Bellerica, MA).

**Image Analysis**

Three representative areas from each slide were selected for image capture and analysis. Images from H&E and immunostaining were captured at 400x magnification by a high-resolution digital camera (Spot RT, Diagnostic Instrument, Inc., Sterling Heights, MI) using bright field microscopy (Nikon ECLIPSE E 800, Tokyo, Japan) and transmitted to an image analysis workstation (6500 Pentium III WorkStation, Dell Computer Corp, Round Rock, TX). Specific outcomes were analyzed using image analysis software (Image-Pro Plus 7.0/4.1, Media Cybernetics, Silver Spring, MD). The number of positive (brown) and total (blue / green + brown) epithelial nuclei were quantified and data is represented as the percent positive of total.

**Results**

Representative images demonstrating a range of PIN lesions (I-IV; H&E) and corresponding proliferation (Ki67), apoptosis (ApopTag), and p53 expression in Rbf/TgAPT121 murine models. Proliferative index drops throughout PIN grades I to III with an increase in proliferative index noted in PIN IV lesion (Figures A.1, A.2). This response may be due to
secondary increases in cellular metabolic stressors noted with advancing PIN grades until a vascular supply is established or a p53 threshold is reached activating further proliferation. Both ApopTag and p53 staining increase in relation to advancing PIN grades.
Figure A.1. Representative images demonstrating a range of PIN lesions (I-IV; H&E) and corresponding proliferation (Ki67), apoptosis (ApopTag), and p53 expression in Rbf/TgAP121 murine models. Original magnification 400x.
Figure A.2. p53 Expression Increases in Relation to Prostatic Intraepithelial Neoplasia (PIN; Grades I-IV) in Rbf/Tgapt121. Figures demonstrating a range of PIN lesions (I-IV; H&E) and corresponding proliferation (Ki67), apoptosis (ApopTag), and p53 expression in Rbf/TgAPT121 murine models. Proliferative index drops throughout PIN grades I to III with an increase in proliferative index noted in PIN IV lesion. Data represented as the mean +/- SEM.
Mean % ApopTag Staining in PIN Grades
p53 Expression in PIN and Invasive Cancer

Representative images of p53 immunostaining demonstrates little to no p53 in the normal (wild type) murine prostate, detectible levels in the early stages of PIN formation, and a dramatic response in the most advanced lesions identified in both Rbf/TgAPT121 and TRAMP transgenic murine models (Figure A.3).

Figure A.3. Representative images of p53 immunostaining demonstrates little to no p53 in the normal (wild type) murine prostate, detectible levels in the early stages of PIN formation, and a dramatic response in the most advanced lesions identified in both Rbf/TgAPT121 and TRAMP transgenic murine models. Original magnification 400x.
p53 Response to Testosterone in the Prostate of TRAMP Mice

Representative images demonstrating the p53 response to androgen depletion and repletion in 8 week old TRAMP mice where hyperplasia and low grade PIN are the predominant lesions. The percentage of p53 expressing prostatic epithelial cells in the dorsal and lateral lobes of wild type and TRAMP mice were quantified in mice that underwent sham operation (intact), castration, or castration followed by 2.5 or 5.0 mg/kg body weight/mouse/day testosterone repletion (Figures A.4, A.5). p53 expression also responds to androgen depletion and repletion in relation to proliferation and apoptosis in 10 week old Rbf/TgAPT121 mice where PIN is the predominant lesion(Figure A.6).
Figure A.4. Representative images demonstrating the p53 response to androgen depletion and repletion in 8 week old TRAMP mice where hyperplasia and low grade PIN are the predominant lesions. The percentage of p53 expressing prostatic epithelial cells in the dorsal and lateral lobes of wild type and TRAMP mice were quantified in mice that underwent sham operation (intact), castration, or castration followed by 2.5 or 5.0 mg/kg body weight/mouse/day testosterone repletion. Original magnification 400x.
Figure A.5. Representative images demonstrating the p53 response to androgen depletion and repletion in 8 week old TRAMP mice where hyperplasia and low grade PIN are the predominant lesions. The percentage of p53 expressing prostatic epithelial cells in the dorsal and lateral lobes of wild type and TRAMP mice were quantified in mice that underwent sham operation (intact), castration, or castration followed by 2.5 or 5.0 mg/kg body weight/mouse/day testosterone repletion. Original magnification 400x.
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<th>Rbf/Tg APT121</th>
<th>Intact</th>
<th>Castrate</th>
<th>Castrate + Testosterone 2.5</th>
<th>Castrate + Testosterone 5.0</th>
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Figure A.6. p53 Response to Androgens in Rbf/TgAPT121 Prostate. Original magnification 400x.
Conclusions

Immunohistochemical expression of p53 is present in murine models of prostate carcinogenesis (TRAMP and Rbf/TgAPT121). p53 nuclear stain is initially detected by 8 weeks of age is localized to the areas of hyperplasia and PIN. The intensity and mean percentage of p53 expression increases with advancing grade of PIN lesions and is most abundant in the advanced carcinoma for each model. Accumulation of p53 stain decreases after castration and increases when testosterone is exogenously replenished. Taken together, p53 expression may be a useful biomarker of disease progression and serve as an indicator of responses to dietary patterns that either promote or inhibit prostate cancer progression. The induction of p53 may suggest genomic stresses upon cells within the tumor microenvironment, perhaps related to unregulated proliferative signaling and cell growth coupled with nutrient and/or oxygen depletion.
References


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