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A novel prostate cancer biomarker

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BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

A NOVEL PROSTATE CANCER BIOMARKER

by

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B.S., Boston College, 2014

Submitted in partial fulfillment of the
requirements for the degree of
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DEDICATION

I would like to dedicate this work to my grandfather, Frank Thomas Piraneo, and to all men who have lost their battle to prostate cancer.

ACKNOWLEDGMENTS

I would like to thank Dr. Srinivas Pentyala for allowing me to join his lab and contribute to his intriguing, thought-provoking projects. Dr. Pentyala has been both a great mentor and friend and I am deeply thankful for his guidance.

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Finally I would like to thank my family. Their love and support has motivated me to be the best that I could be and this opportunity would not have been feasible without them.

A NOVEL PROSTATE CANCER BIOMARKER

JOHN NICHOLAS MULLER JR.

ABSTRACT

Prostate cancer is the most common non-cutaneous malignancy in men and is the second leading cause of cancer death in American men, trailing only lung cancer. About 1 man in 7 will be diagnosed with prostate cancer and about 1 in 38 will die of prostate cancer. Prostate cancer does not usually present any symptoms until it has advanced or metastasized and thus screening for prostate cancer is an arduous task. Three of the most common techniques used to screen for prostate cancer includes digital rectal exam, transrectal ultrasound, and the use of biomarkers, specifically Prostate Specific Antigen (PSA), which has proven controversial. Due to the need for a more rapid, specific marker for the early detection of prostate cancer, this study aims to identify a new biomarker for prostate cancer.

A novel strategy to identify a protein biomarker for prostate cancer was explored, a highly specific hybridoma against the novel biomarker was generated, the efficacy of the biomarker detection tools in prostate cancer was observed and an attempt to identify the biomarker protein sequence was made.

Every time a prostate cancer specimen was tested, it was found that the clone 164 antibody that was generated was able to identify unique antigens in the prostate cancer tissue that were not evident in normal tissue. In addition, it was noticed that the clone 164 antibody could identify the marker protein in urine as well. It is believed that the clone

164 antibody is highly specific for early stage prostate cancer diagnosis. Finally, using mass spectrometry, four candidate protein biomarkers that clone 164 recognizes were isolated, with the closest match being Ig alpha-1 chain C region.

It is believed that the antigens recognized by clone 164 promises great potential as a future biomarker for prostate cancer. Since the protein is only seen in the urine of patients with prostate cancer, it appears that the clone 164 antibody is suitable to include in a device that can be used in a urine-based, rapid diagnostics point of care kit.

Future steps involve animal studies before proceeding to the next step of clinical trials. If the clone 164 antibody identified biomarker proves successful, the respective biomarker protein can be analyzed in detail. Once the expression profile of this biomarker is elucidated, it can be compared to the normal prostate DNA and may help in determining the location in the DNA, which may eventually lead to the idea of treating prostate cancer through gene therapy or the possibility of preventing or curing prostate cancer. Also, the specific antibody against this biomarker can be used as a preventive agent by humanizing this antibody and using it as a therapeutic vaccine.

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LIST OF ABBREVIATIONS

ACT.....	a ₁ -antichymotrypsin
AMG.....	a ₂ -macroglobulin
BPH.....	Benign Prostatic Hyperplasia
DHT.....	Dihydrotestosterone
DRE.....	Digital Rectal Exam
ECE.....	Extra Capsular Extension
EPCA.....	Early Prostate Cancer Antigen
hK2.....	Human Kallikrein 2
hK3.....	Human Kallikrein 3
PAP.....	Prostatic Acid Phosphatase
PSA.....	Prostate Specific Antigen
TRUS.....	Transrectal Ultrasound

INTRODUCTION

Prostate cancer is the most common noncutaneous malignancy in American men (Figure 1.). The most recent numbers available show that about 220,800 men in the United States were diagnosed with prostate cancer while about 27,540 men in the United States died from prostate cancer (American Cancer Society, 2015). In fact, the estimated new cases and estimated deaths from prostate cancer in 2015 are 220,800 and 27,540, respectively (Siegel et al., 2015).

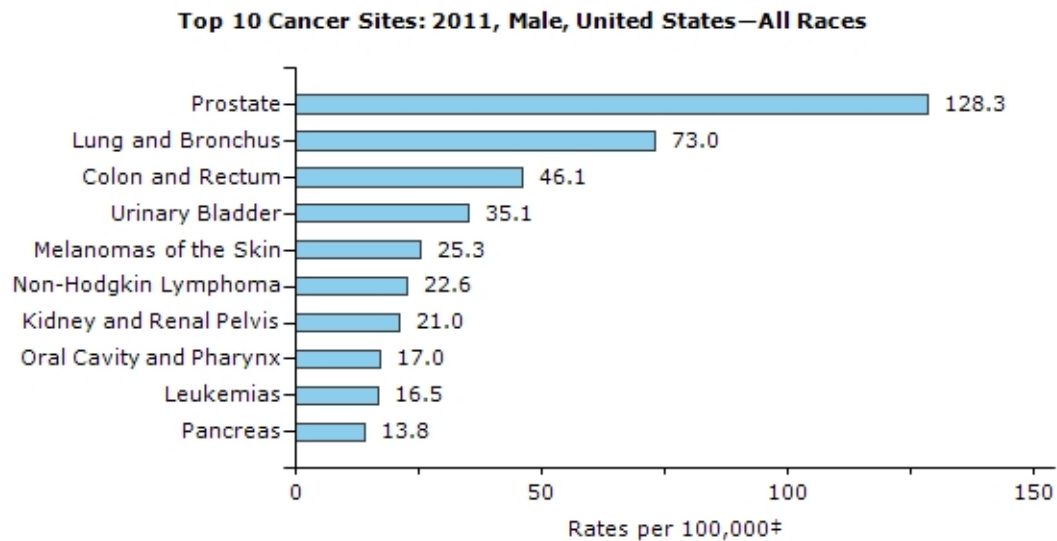


Figure 1. Top 10 Male Cancer Sites 2011. Prostate Cancer has the highest rate of cases in men of all races in the United States. Adapted from the National Program of Cancer Registries (NPCR), 2011.

The prostate is a small, soft gland located in front of the rectum and beneath the bladder, with the urethra running through it (Prostate Cancer Foundation, 2015). It is an androgen-regulated organ. Testosterone freely diffuses into prostate cells where it is rapidly reduced to dihydrotestosterone (DHT), its metabolically active form (Ross et. al, 1998). DHT and testosterone, to a lesser extent, bind to the androgen receptor which then

translocates to the nucleus for DNA binding and transactivation of genes, especially those controlling cell division.

Prostate cancer is a condition of the prostate gland where cells lose the ability to regulate cell growth or cell death and accumulate uncontrollably. According to Prostate Cancer Foundation (2015), more than 65% of all prostate cancers are diagnosed in men over the age of 65. For men under the age of 40, only about 1 in 10,000 men are diagnosed, however, this rate dramatically increased to 1 in 38 for ages 40 to 59, and 1 in 14 for 60 to 69. The incidence of prostate cancer is higher in African American men than with Caucasian men, while Asian men who live in Asia have the lowest risk. Interestingly, men in the United States who live north of 40 degrees latitude have the highest risk of dying from prostate cancer than other men in the United States (Prostate Cancer Foundation, 2015). It is believed that this effect is due to the meager exposure to sunlight during three months of the year, which reduces ones vitamin D levels.

The microscopic diagnosis of prostate adenocarcinoma is based primarily on certain features of glandular formation and pattern. In fact, 98% of prostate cancers are glandular in origin (Greenberg, 2003). Prostate cancers are also multifocal in nature with most cases presenting an average of at least two geographically distinct foci of varying histological pattern. The most accepted and implemented grading protocol is the Gleason score. Prostate adenocarcinoma can be stratified on a histological basis using this classification, thus providing meaningful prognostic information for urologists.

Since most malignancies arise in the peripheral portion of the gland away from the prostatic urethra, prostate cancer is seldom symptomatic early in its course. The

presentation of symptoms, however, often implies local extension or metastatic disease. Obstructive voiding symptoms often develop as the cancer begins to involve the urethra and/or bladder neck. These voiding symptoms include slowing of the urinary stream, hesitancy, and intermittent flow. Although irritative voiding symptoms, like urgency and frequency, may also occur, these are more difficult to attribute to cancer since they are also associated with benign prostatic hyperplasia (BPH). Patients may also notice sexual symptoms with tumor progression. This includes hematospermia and/or decreased ejaculatory volume secondary to ejaculatory duct obstruction. If there is local encroachment on neurovascular bundles, erectile dysfunction may be seen.

Bony pain is often a sign of metastatic involvement of the skeleton. Human prostate cancer is one of the rare cancers that repeatedly produce osteoblastic metastases to bone, an ability that may be governed by the localization and functionality of connexin43 (Lamiche et al., 2012). Other signs of metastasis include anemia secondary to bone marrow involvement and lower body edema due to local lymphatic and vein obstruction. Importantly, however, in the last 27 years the percentage of patients presenting symptomatically has decreased in proportion to patients being diagnosed with prostate cancer largely due to the use of prostate specific antigen (PSA) screening (Moul, 2003).

The most important indicator regarding course of action and survival of the patient is the stage of the cancer at diagnosis. Staging is imperative because the appropriate therapy is directly related to it (Fielding et al., 1992). There are two main classification systems used to stage tumors. The first is the Jewett system, which was

described in 1975 and since modified (Jewett, 1975). This system uses the classification of stage A through stage D. Adopted in 1997 by the American Joint Committee on Cancer, the second system is the TNM system, which was further revised in 2002 (American Joint Committee on Cancer, 2002). The T in the TNM system is based on tumor size and grade, the N is based on detection in lymph nodes and the M is for any other possible metastasis. Precise staging and early diagnosis is crucial for proper treatment in cancer. In fact, erroneous staging may result in inappropriate treatment and a substantial decrease in the patient's chance of survival (Yano et al., 2007). Thus is the need for an efficient and accurate biomarker for the screening and staging of prostate cancer.

Major techniques used in assessing prostate cancer

Prostate cancer does not usually present any symptoms until it has become advanced or has metastasized. Screening for prostate cancer is a difficult task that requires an assortment of methods. Three of the most common techniques used are the digital rectal exam (DRE), the transrectal ultrasound (TRUS), and the use of biomarkers.

DRE is estimated to have about a 59% overall accuracy (Basler & Thompson, 1998). Although DRE is regarded as having poor sensitivity, it is widely used because it frequently detects cancers that may be neglected by other testing methods (Mahon, 2005). The main advantage of DRE is that it is able to identify cancer in men who may have normal PSA levels and have small and well-differentiated tumors (Basler & Thompson, 1998). DRE can also be used to examine other abnormal conditions of the prostate such as BPH. The main disadvantage of DRE is that most palpable cancers are later stage

cancers. Furthermore, many prostate cancers may be located in regions of the gland that are distant and therefore evasive to digital palpation.

TRUS is a procedure where a probe is inserted into the rectum against the prostate gland. This probe sends out high-energy sound waves, which allows for the imaging of the entire gland (Prostate Cancer Foundation, 2015). One advantage of using TRUS is that it has high sensitivity, which allows for it to play an important role in the early detection of prostate cancer. TRUS is also used as a guide for needle biopsies of the prostate. The main disadvantage of TRUS is that it has poor specificity if used as the only screening method.

The use of biomarkers in the detection of prostate cancer began in 1938 and has since then been a field of great interest. What exactly a biomarker is and the history of biomarkers in prostate cancer will be discussed below.

History of diagnostic and prognostic markers

As defined by the National Cancer Institute, a biomarker is “a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease.” Biomarkers are often used as a means to assess biological criterion for therapeutic analysis as well as to further the development and assessment of therapies. As seen in Table I, adapted from Madu and Lu’s 2010 review, there are diverse uses for biomarkers, with its corresponding clinical goals, in patient care.

Table I. Use of Cancer Biomarkers. This table shows the diverse uses of biomarkers with the respective clinical goal (Madu et al., 2010).

Use of Biomarker	Clinical Goal
Screening	To identify early-stage cancers in the general population and deliver early treatment.
Diagnosis and Classification	To reliably determine and distinguish the presence and type of cancer.
Prognosis	Helps in estimating the outcome of the disease, without considering treatment, to establish the intensity of treatment.
Chemoprevention	To determine and target the cellular and molecular mechanisms of carcinogenesis in preneoplastic tissues.
Prediction of treatment	To foresee the response to treatments and select the therapy with the highest probability of being effective in a particular patient.
Risk Stratification	To evaluating the probability of the occurrence or recurrence of cancers
Therapy Tracking and Post-treatment Surveillance	To assess the effectiveness and adverse effects of a treatment and to provide early determination and treatment of recurrent disease.

Prostatic acid phosphatase

The first biochemical marker to be used routinely in the diagnosis and staging of prostate cancer was prostatic acid phosphatase (PAP). PAP hydrolyzes esters under acidic conditions to yield inorganic phosphates. The greatest concentration of PAP is found in the prostate; however, PAP is observed in many organs including the liver, brain, and lung. Prostate epithelial cells secrete PAP into the glandular lumen. The concentration of PAP can be measured via immunoassay or enzymatic assay.

A 1938 study showed PAP to be elevated in the prostates of patients with metastatic prostate cancer (Gutman & Gutman, 1938). However, a subsequent study

showed that PAP levels in prostate cancer tissue was only a fraction of those found in patients with BPH (Heller, 1987). Furthermore, in a study that evaluated PAP levels in 102 patients, 84% patients that had elevated PAP levels were subsequently found to have extracapsular extension (ECE) or metastatic disease (Bahnon & Catalona, 1987). With the advent of serum prostate specific antigen (PSA) testing, usage of PAP as a biomarker for prostate cancer has become less and less informative. In fact, in a study involving 460 consecutive patients referred to Johns Hopkins, PAP was found to be elevated in only 4.6% of patients and provided useful staging information amounting to only 0.9% of cases (Burnett et al., 1992).

Prostate Specific Antigen

Prostate specific antigen (PSA), identified in the 1970s, is a serine protease that consists of 237 amino acids, as well as a carbohydrate chain and has a molecular weight of 28,430 (Stenman et al., 1999). Belonging to the kallikrein family of proteases, prostate specific antigen is also called human kallikrein 3 (hK3). It is produced in the prostatic epithelium and it is primarily secreted into seminal fluid at concentrations of 0.5 – 2 g/l, where it digests the gel forming after ejaculation (Wang et al., 1981). Hence, the physiologic function of PSA is to liquefy seminal coagulum in the human ejaculate. Although often considered prostate-specific, PSA is also expressed at low concentrations in various other tissues such as in breast tissue and the periurethral glands (Diamandis & Yu, 1997). PSA in the glandular ducts of the prostate, however, are one million-fold the concentration of plasma (Stenman et al., 1999). In the normal prostate and in cases of BPH, PSA only enters circulation by leaking ‘backwards’ into the extracellular fluid.

From here it then diffuses into circulation. In prostate cancer, the abnormality of the epithelial cells causes a loss of normal secretory pathways into the prostatic ducts and thus PSA is actively secreted into the extracellular space and hence into circulation (Stenman et al., 1997). Most PSA circulates in serum bound to protease inhibitors such as α_1 -antichymotrypsin (ACT) and α_2 -macroglobulin (AMG), while the rest exists unbound or free.

PSA is used for early detection and screening for prostate cancer since it is expressed in more than 99% of all prostate cancers, however, there is much controversy about the reliability of this screening. Serum PSA is often used as a sensitive marker for the early detection of prostate cancer. The specificity of this testing is limited by the fact that elevated levels of PSA are also seen in benign prostatic conditions such as BPH and prostatitis. Nevertheless, a PSA level of 4 $\mu\text{g/l}$ is traditionally used as the cut-off for possible cancer (Stenman et al. 1999). It has been observed that 10% of all men over the age of 50 have PSA levels that exceed this limit. Of these men, around 30% have a prostate cancer that is found by ultrasound-guided biopsy. The issue arises when one understands that in order to find one case of prostate cancer, three men must be biopsied. Thus about two-thirds of elevated values in men over 50 are due to BPH and not prostate cancer.

PSA, although the most popular biomarker for prostate cancer, is one of the most controversial. A recent study recognized the disadvantage of PSA for the early detection of prostate cancer. It was observed that many men must be screened, biopsied, and diagnosed to only prevent one death (Stattin et al., 2015). This study sought to increase

the specificity of screening for lethal prostate cancer at an early stage. The results showed that screening for prostate cancer using PSA in men at ages 50-60 should focus on those with PSA levels in the top quartile. It was noted that men in this group comprised the majority of subsequent cases of metastasis. Furthermore, it was recommended to test men with elevated PSA levels for four kallikrein markers in order to aid in biopsy decision-making. This is one of many studies that seek to find a way to make PSA screening more accurate. The vast amount of literature on this subject has resulted in many differing opinions on the proper use of PSA and thus a more reliable marker is suggested.

Human Kallikrein-2

Human kallikrein-2 (hK2) hails from the same family as PSA and in fact shows approximately 80% homology in amino acid sequence with PSA. Unlike PSA, hK2 exists mostly in a free, unbound state in serum. It has been documented that in patients with PSA levels between 4 and 10 ng/mL, a higher percentage of free PSA usually means that the elevation in total PSA is likely due to BPH and not prostate cancer (Catalona et al., 1995). Furthermore, it was found that higher hK2 levels associated with lower free PSA levels increased the probability of finding prostate cancer (Partin et al., 1999). Specifically, in men with free PSA less than 25% and a hK2/free PSA ratio of greater than 0.18, there was an increase of 13-62% in detection of prostate cancer. Recent studies have sought a more advanced and accurate platform to use hK2 in the diagnosis of prostate cancer.

Early Prostate Cancer Antigen (EPCA)

Early Prostate Cancer Antigen (EPCA) is a nuclear structural protein that is expressed in prostate cancer but not in other normal tissues or cancer types. It was proposed that an immunohistochemical test for EPCA could serve in addition to the current diagnostic approach of using PSA. Furthermore, it is believed that EPCA could identify patients with prostate cancer as much as 5 or more years earlier than the current diagnostic approaches (Dhir et al., 2004). EPCA staining was found to have 84 percent sensitivity and 85 percent specificity in detecting prostate cancer.

Current and Future Prostate Cancer Biomarkers

Although many prostate cancer biomarkers have been explored thus far, the search for a more rapid and accurate marker continues. Recent research has turned to genomic testing as a biomarker for aggressive prostate cancer. A recent discovery of the SChLAP1 long noncoding RNA in the prostate has provided a novel biomarker that not only adds to the ability to identify prostate cancer, but also to conventional risk stratification (Prensner et al., 2015). Furthermore, this group has clinically validated SChLAP1 for the prognosis of aggressive prostate cancer and they suggest that integration of genomic tests may advance the diagnosis of prostate cancer through early identification of high-risk patients.

Exosomes have also appeared to be a non-invasive cancer biomarker since tumor-specific molecules can be found in exosomes isolated from biological fluids. One recent study has investigated the proteome of urinary exosomes by using mass spectrometry to identify proteins that are expressed differentially in prostate cancer patients (Øverbye et

al., 2015). This study found that when comparing normal and prostate cancer samples of urinary exosomes, 246 proteins were differentially expressed and of these, 221 were up-regulated in exosomes from the prostate cancer patients. Although many of the proteins showed high specificity and sensitivity as individual biomarkers for prostate cancer, when combined in a multi-panel test they had the potential for full differentiation of prostate cancer from non-disease controls. In conclusion, this study presents the potential of using urinary exosomes in the diagnosis and clinical management of prostate cancer.

Specific Aims and Objectives

Because of the large, and rising, rates of prostate cancer in men, and because of the need for a more rapid, specific marker for early detection of prostate cancer, this study aims to identify a new biomarker for prostate cancer. Although there is not a lack of research on this topic, the discovery of a novel biomarker that may have the advantage of being more specific and effective warrants scientific inquiry. It should be further understood that it is recommended to not solely use PSA as a biomarker because it misses about 20% of cases. Additionally, the benefits and new applications of said biomarker will be observed.

The specific aims of the study are:

1. To examine a novel strategy to identify a protein biomarker for prostate cancer
2. To generate highly specific hybridomas against the novel biomarker
3. To observe the efficacy of the biomarker detection tools in prostate cancer.
4. To identify the biomarker protein sequence.

This study seeks to develop a more comprehensive understanding about the field of biomarkers in regards to prostate cancer. Furthermore, this study hopes to identify a more rapid, effective biomarker test for prostate cancer.

METHODS

Acquisition of cancer tissue from a human subject

Upon Stony Brook Medical Center IRB approval, cancer tissue from radical prostatectomy from a patient with PSA levels of 87 and Gleason Grade 5 was used, after confirmation by the pathologist, as antigenic material in the generation of clone 164. The tissue, when excised, had a visible tumor region. From this region a 3 square mm block was isolated, homogenized, and used to inoculate mice.

Generation of monoclonal antibodies

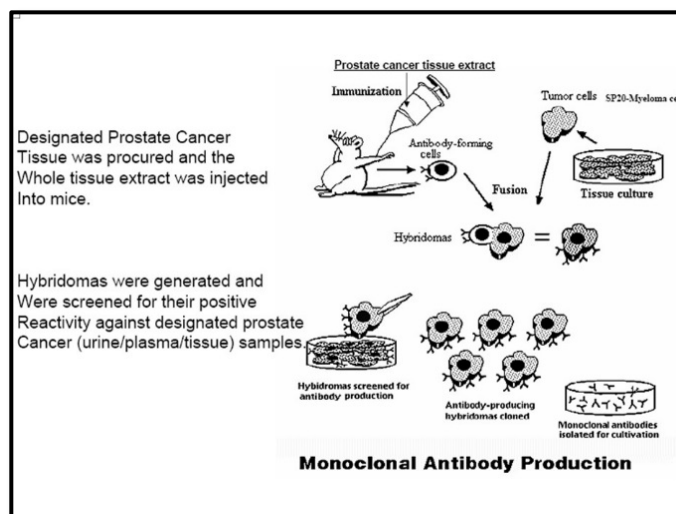


Figure 2: Monoclonal antibody production

One Balb/c mouse was immunized with prostate cancer tissue extract by four biweekly injections and serum was tested using ELISA. Isolated spleen cells were fused with mouse myeloma cell line SP2/O at a ratio of 10:1 spleen cells:myeloma cells by pelleting them together at 1000 rpm for five minutes in DMEM medium (Gibco), supplemented with 10% Fetal Clone I (HyClone), non-essential amino acids (Gibco),

penicillin and streptomycin (Gibco). The pellet was resuspended in 35% PEG 1500 (Roche) in DMEM medium, and the cells were immediately centrifuged at 1000 rpm for five minutes. The PEG was aspirated, and the fused cells were suspended in DMEM glutamax medium (Gibco), supplemented with 15% Fetal Clone I, 10% NCTC109 (Gibco), non-essential amino acids, penicillin, streptomycin, 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, 1.6×10^{-5} M thymidine, and 10% macrophage conditioned medium, and plated in ten 96 well plates. Macrophage conditioned medium was prepared as follows: J774.A1 (American Type Culture Collection) was cultured in a spinner flask in DMEM medium supplemented with 10% horse serum (HyClone). Lipopolysaccharide (E. coli LPS 055:B5, Cal Biochem) was added at a concentration of 5 ug/mL, and the cells were incubated for 20 hours. Cells were then harvested at 1000 rpm for 10 minutes and washed in one-half volume of PBS. Following centrifugation for 10 minutes at 1000 rpm, the supernatant was discarded and the cells resuspended in Iscoves-modified Dulbecco's medium (IMDM, Gibco) without horse serum and transferred to a spinner flask. The cells were then incubated for 48 hours at 37°C. The medium was harvested by pelleting the cells out of the medium by centrifugation at 1500 rpm for 10 minutes. The macrophage conditioned medium was then filtered and stored at 4°C. Two weeks following fusion, wells were screened using ELISA against prostate cancer tissue extract. The day before screening, 0.1 mL of medium was removed from each well of the ten 96-well fusion plates and replaced with 0.1 mL per well of fresh medium. To confirm the response, the following day wells exhibiting both cell growth and a positive response by ELISA were rescreened using ELISA. Cells in wells exhibiting a positive response on

retest were expanded and grown up to 30 mL in culture. Three 10-mL aliquots were pelleted and resuspended in freezing medium (10% dimethyl sulfoxide, 90% Fetal Clone I) for cryostorage. Positive samples were screened by dot blot against prostate cancer tissue extracts and biopsies, and clones were chosen for subcloning by limiting dilution. Subclones were screened by ELISA and were chosen for further study, expanded, and grown up to 1000 mL volume in DMEM supplemented with 10% fetal calf serum and antibiotics in T-175 flasks. Subclones were transferred to DMEM without serum and continued to incubate for three days. Supernatant was harvested by pelleting out the cells at 1500 rpm for 10 minutes and stored at 4°C.

After isolating a panel of stable hybridomas, antibodies were purified from these hybridomas and were tested for their production of IgG class or IgM class molecules. Antibodies were purified from the conditioned medium of the hybridomas using protein G Superose column chromatography linked to an FPLC to provide purified immunoglobulins.

The antibodies were screened for positive markers in prostate cancer samples. Initial screening methods involved the techniques of ELISA, western blotting, immunoprecipitation and immunocytochemistry. Different antigenic sources were employed such as urine and tissue from designated prostate cancer subjects. After screening hundreds of clones, a unique clone (named Clone 164) that produces antibodies specific to prostate cancer was identified. Cultured cells and prostate cancer tissue slices were probed with antibodies generated, and an immunocytochemistry method was employed to identify the specificity of the antibodies.

Table II: Approach to generate and screen monoclonal antibodies.

Starting Material to generate monoclonal antibodies	Screening method	Source for screening
Whole cell extract from prostate cancer tissue inoculated into mice and screened several hundreds of hybridomas.	1. Western Analysis 2. Immunoprecipitation 3. Immunocytochemistry	1. Cultured cell lines -whole cell extract 2. Prostate cancer tissue-whole cell extract 3. Tissue slices of prostate 4. Urine sample from prostate cancer subjects

Large scale purification of antibodies from clone 164

Clone 164 was scaled up and antibodies were purified and tested for the production of IgG class molecules. Antibodies were purified from the conditioned medium of the hybridomas or from the ascites fluid. Protein G Superose column chromatography linked to a FPLC was used to obtain purified immunoglobulins.

Screening of hybridomas for cancer specific antigens in prostate cancer cell lines, tissues and fluids

Clone 164 antibodies were screened for positive markers in different cell, tissue and fluid sources. Initial screening methods involved the techniques of ELISA, western blotting and immunocytochemistry. Different antigenic sources were employed such as conditioned media (CM) from cultured cells (LNCaP, PC-3 and Du-145), whole cell extracts and membrane and cytosolic fractions. Concurrently cancer and normal tissue (Source: Cooperative Human Tissue Network, NCI, and National Disease Research Interchange, NDRI), plasma and urine samples were also screened in the same manner. Antigens unique to prostate cancer were identified by differential analysis using normal tissue/cells and the results were compared with cancer tissue and cancer cells.

Immunocytochemistry was used to probe cultured cells and prostate tissue slices with antibodies to validate the findings observed with biochemical techniques.

Isolation of the antigens recognized by anti-cancer tissue specific antibodies

The antigens that are specific for clone 164 were identified and isolated. As proposed, screening by SDS-PAGE western analyses using different sources of material like cultured cells, serum samples and tissues identified the apparent molecular weights of the antigens that recognized the clone 164 antibodies. The polyacrylamide gels were run both under denaturing and non-denaturing conditions prior to blotting to ascertain whether some antigens are composed of subunits. Immunoprecipitation of cultured cell and tissue extracts was performed using anticancer antibodies as the primary antibody and protein-A sepharose beads as the immunoprecipitating agent. The immunoprecipitates were analyzed by reducing and non-reducing SDS-PAGE. This analysis identified antigens in their native confirmation. Comparing the results from western blot immunoprecipitation analysis provided confirmatory molecular weights and subunit structures of the antigens. Antigens recognized by anti-cancer antibodies using western and immunoblot techniques were also isolated by immuno-affinity chromatography. The purified monoclonal antibodies were covalently linked to activated sepharose beads, and CM, lysates and detergent-solubilized membranes from cancer cells and tissue were passed onto the affinity column. The column was extensively washed with different salt conditions to remove non-specifically bound proteins and then antigen-antibody interaction was disrupted using 0.1 M glycine under acidic conditions to elute the specific antigen and then it was analyzed by SDS-PAGE and western analysis. The

possibility that all or most of the identified anti-cancer antibodies recognized the same antigen was not likely as CM, membrane and cytosolic fractions of cells and tissues as different immunogenic starting material was used.

Identification and characterization of the antigen(s) by 2D gel electrophoresis and nanoelectrospray ionization tandem mass spectrometry (ESI-MS/MS)

Following antibody affinity column fractionation or immunoprecipitation, the eluted or gel resolved proteins were subjected to either two-dimensional (2-D) gel electrophoresis or straight in situ gel digestion and protein spots/bands were marked. These candidate proteins were first proteolyzed. Trypsin was preferred, since most of the product peptides will have a C-terminal Arg or Lys residue suitable for positive ion MS analysis. The protein spots/bands from the gels were digested and the resulting peptides were separated by reverse phase high performance liquid chromatography (RP-HPLC) and each was subjected to nanoelectrospray ionization tandem mass spectrometry (ESI-MS/MS). This is the most successful method for peptide-mass fingerprinting, the partial sequencing of proteins and determination of their covalent modifications. The MS spectrum was used to interrogate sequence databases using powerful search algorithms.

The amino acid sequence information was searched against the human protein database for identifying the antigen. The Pro ID search engine from Applied Biosystems was used to interrogate sequence databases to identify proteins from the MS/MS and precursor ion results. Generally, a confidence of > 99% (ProtScore > 2.00) is required before considering the candidate. Proteins cross-identified in the screens were further studied for relevancy. Commercially available antibodies against identified proteins were

used when possible. It is possible to construct corresponding oligonucleotides for probing cDNA libraries or designing primers for PCR based cloning in the future to further analyze and functionally characterize the recognized cancer specific antigen.

Identification of Protein

To characterize the specific protein detected by Clone 164 antibody in cancer tissue, immuno-precipitation, antibody affinity column isolation and 2D gel electrophoresis were performed. The antibody identified protein bands/spots were subjected to sequence analysis to obtain N-terminal sequence. *In situ* proteolysis digestion of the isolated proteins was performed and internal sequences of the antigen was performed using Mass-SPEC analysis in order to identify the protein.

RESULTS

Generation of monoclonal antibodies

The results indicate that cancer-specific monoclonal antibodies can be generated using the whole prostate cancer tissue as an immunogenic starting material. The results with Clone 164 revealed that this unique antibody was able to identify a specific protein species every time a designated prostate cancer specimen was tested. Several biopsy samples procured from designated prostate cancer subjects were tested and showed both 100% sensitivity and specificity in the western blot and immunocytochemistry methods. Preliminary studies to identify the specific protein to which clone 164 reacted indicated that it is a unique protein expressed in prostate cancer tissue. Additionally, initial testing demonstrated that the clone 164 antibody could identify the marker protein in urine as well as in tissue material.

The 49 kDa protein targeted by the clone 164 antibody was identified through standard protein chemistry and biochemical techniques. This was accomplished by using the clone 164 antibody as a bait to isolate the specific protein that was being detected by clone 164. Once isolated, the protein sequence was identified by ESI-MS/MS

Screening for cancer specific antigens in prostate cancer cell lines, tissues, and fluids

The results with cell culture showed that LNCaP (androgen sensitive) cells have a protein species of the same molecular weight as prostate cancer tissue extract when analyzed with clone 164 antibody by SDS-PAGE and immunoblotting. Results show that androgen insensitive cells (PC-3 and Du-145) may not express the protein identifiable by

Clone 164 antibody. It is believed that the clone 164 antibody is highly specific for early stage prostate cancer diagnosis as prostate cancer cells respond to androgen (testosterone) before metastasis and becoming androgen insensitive.

When analyzed by immunoblotting with purified IgG antibodies from Clone 164, protein extract from prostate cancer tissue revealed two bands with molecular weight of approximately 49,000 and approximately 129,000 daltons (Figure 3).

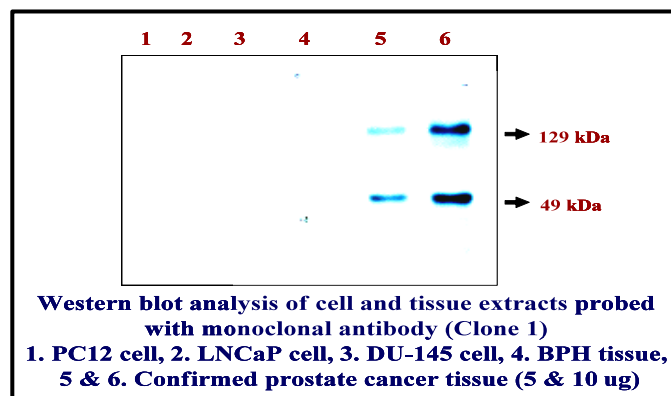


Figure 3. Immunoblot of cancer tissue extracts. This figure presents the protein extract from prostate cancer cell lines, prostate cancer tissue, and BPH tissue being analyzed by Immunoblotting. Purified IgG antibodies from clone 164 were used. Two bands with molecular weight of 49,000 and 120,000 daltons were observed.

Immunocytochemistry results also showed positive reactivity with prostate cancer tissue (Figure 4).

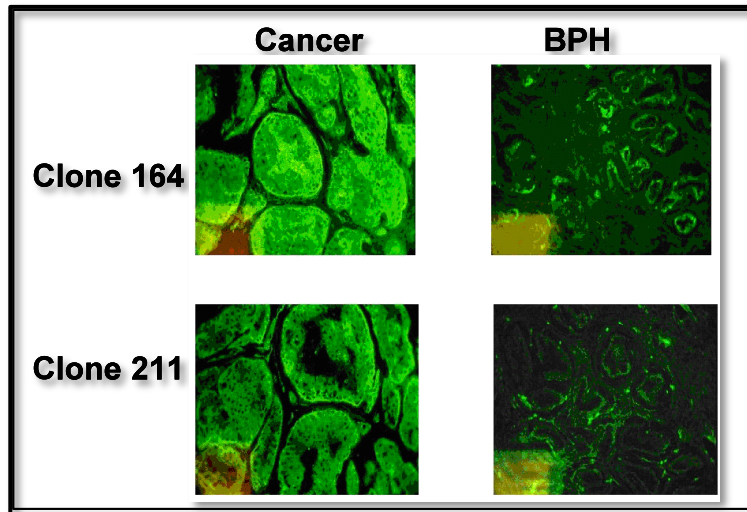


Figure 4. Cancer and BPH tissue slices stained with antibodies from clones 164 and 211. Identified prostate cancer and BPH tissue were probed with monoclonal antibodies (generated by injecting prostate cancer tissue in mice). The samples were probed with FITC labeled secondary antibody and viewed under a fluorescent microscope.

Urine specimens from a cancer patient, when subjected to immunoblotting, revealed a band in about the same location as the lower band (molecular weight of approximately 49,000) (Figure 5).

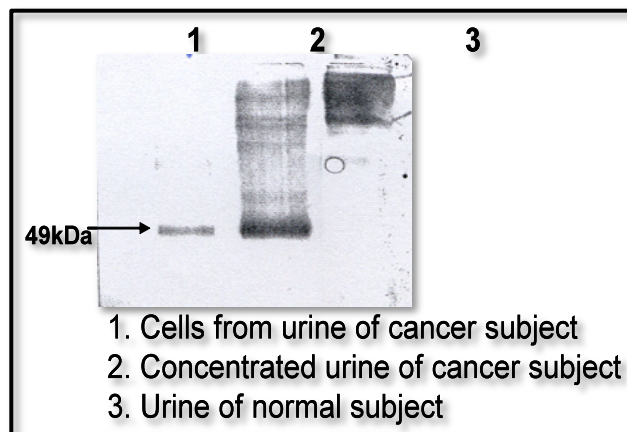


Figure 5. Urine analysis analyzed with antibody 164. A urine specimen from a cancer patient, when subjected to immunoblotting, revealed a band in about the same location as the lower band. Clone 164 was also able to identify a protein with a molecular weight of 49kDa in the urine sample of a prostate cancer patient

When cell extracts of cultured LNCaP cells were subjected to immunoblotting, clone 164 was able to identify a protein band of high molecular weight in soluble membrane extract but not in other fractions. Clone 164 was also able to identify a protein with a molecular weight of approximately 49kDa in the urine sample of a prostate cancer patient.

In view of Clone 164's strong reactivity with different prostate cancer samples and its ability to identify two protein bands by immunoblotting, the clone was further tested for its specificity. A commercially available immunoblot of different cancer tissue extracts (purchased from ProSci Inc, Poway, CA –containing human cancer tissues from a human bladder, breast, kidney, ovary, testis, cervix, uterus, and prostate) when analyzed with clone 164 antibody, showed strong reactivity only with the prostate cancer tissue and not with other cancer tissues (Figure 6). Prostate cancer tissue extract was subjected to 2D gel electrophoresis and immunoblotted with antibody (clone 164). Foci of reactivity of the resolved cancer tissue proteins on a 2D gel were identified.

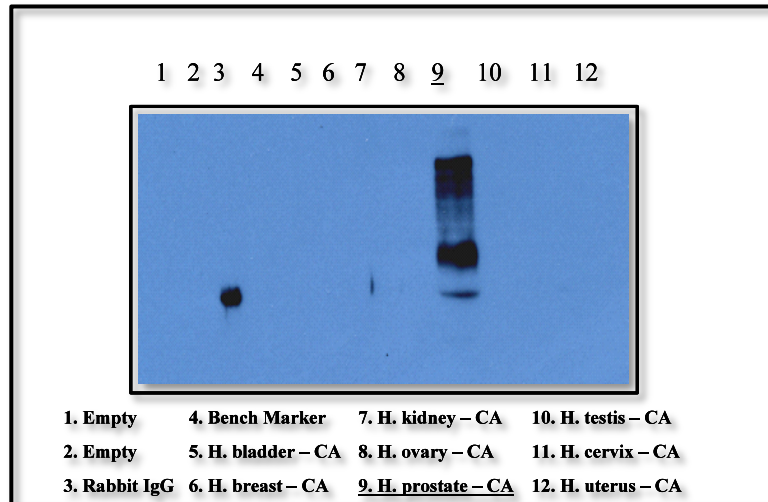


Figure 6. Clone 164 identifies a protein band in prostate cancer but not in other cancers. When analyzed with clone 164 antibody, a commercially available immunoblot of different cancer tissue extracts (purchased from ProSci Inc, Poway, CA – containing human cancer tissues from a human bladder, breast, kidney, ovary, testis, cervix, uterus, and prostate) showed strong reactivity only with the prostate cancer tissue but not with other cancer tissues. Lane 3 is an internal control of Rabbit antibody.

Identification and characterization of the antigen(s) by 2D gel electrophoresis and nanoelectrospray ionization tandem mass spectrometry (ESI-MS/MS)

Results analyzing prostate cancer tissue extracts with 2D gel electrophoresis revealed a cluster of protein spots as identified by western blot analysis of the 2D gel using clone 164 antibodies. The specific spots on 2D gels were characterized. Often, relative intensity of the protein spot on the 2D gel protein might cover many adjacent protein spots with the same isoelectric characters. Hence, the clone 164 specific antigen was isolated by immunoprecipitation or affinity chromatography which resolved the issue of multispotting analysis on 2D gels.

Identification of Protein

The results with whole tissue extract and MS with clone 164 revealed a cluster of protein spots in the excised gel piece as the spot location was picked based on western blot analysis and the relative intensity of the protein spot against a particular single marker protein that might cover many adjacent protein spots with the same isoelectric characters. The amino acid sequence information was searched against a human protein database for identifying the antigen. The Pro ID search engine from Applied Biosystems was used to interrogate sequence databases to identify proteins from the MS/MS and precursor ion results. A confidence of $\geq 99\%$ (ProtScore ≥ 2.00) was accepted before considering the candidate. The following table (table III) reveals high hit protein candidates that were obtained from the MS data.

Table III: Clone 164 antibody identified proteins by Mass-SPEC.

Protein	Peptides	Spectra	Coverage
sp P01876 HUMAN Ig alpha-1 chain C region	5	11	17.80%
sp P01011 HUMAN Alpha-1-antichymotrypsin	3	11	11.10%
sp P01019 HUMAN Angiotensinogen	3	7	8.90%
sp P02787 HUMAN Serotransferrin	3	3	5.00%

DISCUSSION

It was revealed that the clone 164 antibody was able to identify unique antigens in the prostate cancer tissue, which were not evident in the normal tissue every time a designated prostate cancer specimen was tested. Importantly, it was observed that the clone 164 antibody could identify the marker protein in urine as well as in prostate tissue. This is of importance for a future biomarker detection assay, because the availability of the protein in urine will allow for rapid and non-invasive diagnosis. The results showed that this protein is only seen in the urine of patients with prostate cancer. Thus, the clone 164 antibody appears to be suitable to include in a device that can be used as a urine-based, rapid diagnostics point of care kit.

It is believed that the clone 164 antibody is highly specific for early stage prostate cancer diagnosis. The results showed that that androgen sensitive (LNCaP) cells have a protein species of the same molecular weight as prostate cancer tissue extract whereas androgen insensitive cells (PC-3 and Du-145) may not. Thus it is believed that the clone 164 antibody will recognize the androgen sensitive cells but not the androgen insensitive cells. This translates into clone 164 antibody being highly specific for early stage prostate cancer diagnosis since the prostate cancer cells respond to androgens such as testosterone. Later stage prostate cancer and metastasis cause the prostate cancer cells to become androgen insensitive and thus may not respond.

Clone 164 identified two unique protein bands. Since the clone 164 antibody is monoclonal, the two bands may originate from the same protein and it is thus speculated that the lower (49,000 Da) band may be a breakdown product of the protein. In using

mass spectrometry we were able to isolate four candidate protein biomarkers that clone 164 recognizes. MS data reveals with confidence that one of these may be the protein in question. One of the four may be specifically interacting with the clone 164 antibody since the MS data was obtained by the protein band specifically identified by clone 164. Of the four (Ig alpha-1 chain C region, Alpha-1-antichymotrypsin, angiotensinogen, and serotransferrin), Ig alpha-1 chain C region was the closest matching with five peptides and a 17.80% coverage. According to the Toxin and Toxin Target Database, Ig alpha-1 chain C region is the major immunoglobulin class in body secretions. It is speculated to both prevent access of foreign antigens to the general immunologic system and to defend against local infection. Further studies are needed to identify the specific protein recognized by clone 164.

The primary function of the study after generating a monoclonal antibody that is highly specific to cancerous conditions was to isolate and characterize the antigens recognized by anti-cancer tissue antibodies. The antigen that was found in the tissue and detected by the antibodies promises great potential as a future marker for prostate cancer, as it is evident after running these tests that it is not present in any other cancerous tissues.

Future Steps

The results of this study will advance our understanding of prostate cancer specific proteins and the ability to develop antibodies to detect these proteins. Generating these cancer-specific antibodies will provide unique probes for detecting both diagnostic as well as prognostic prostate cancer markers. These antibodies will be put to further use

in animal studies before proceeding to the next step of clinical trials. Furthermore if the antibodies prove to be successful in both animal testing and clinical trials, the respective protein can be sequenced. As the protein that was found is present only in the prostate cancer tissue, once sequenced it can be compared to the normal prostate DNA and may be able to help determine the location in the DNA resulting in the generation of this particular protein. Discovering the loci that causes the up-regulation of this particular protein in prostate cancer cells may eventually lead to the idea of treating prostate cancer through gene therapy or the possibility of preventing or curing prostate cancer in the future.

With only unreliable prostate cancer biomarkers available, the need for a more rapid, specific marker is undisputable. The markers used today are both non-specific to prostate cancer and often produce misleading results. A marker that is better able to detect the presence of prostate cancer will greatly help in accurately detecting prostate cancer at any stage. The protein that was found in the prostate cancer tissue and detected by the clone 164 antibody promises great potential as a future marker for prostate cancer. This is especially true in the fact that after running our tests it was not found to be present in any other tissue. It is possible that the protein found in the prostate cancer tissue is a variation of a protein found in normal prostate cancer tissue. It could, however, be a completely different protein altogether. Nonetheless, this finding is of extreme significance because it is possible that a more reliable and accurate prostate cancer biomarker may soon be available. Furthermore, in time the protein could be sequenced and compared to the normal prostate DNA that would help to determine the location of

the gene resulting in the creation of this particular protein. Application of this knowledge in the form of gene and cellular therapy is showing promise and it may become a means for cure and prevention of prostate cancer.

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CURRICULUM VITAE

