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Award Number: W81XWH-12-1-0072

TITLE: Molecular Heterogeneity in Primary and Metastatic Prostate Tumor Tissue

PRINCIPAL INVESTIGATOR: Dr. Julie Kasperzyk

CONTRACTING ORGANIZATION: Harvard College,
BOSTON MA 02115-6028

REPORT DATE: October /2013

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2013	Annual Summary	30September2012-29September2013

4. TITLE AND SUBTITLE

Molecular Heterogeneity in Primary and Metastatic Prostate Tumor Tissue

5a. CONTRACT NUMBER
W81XWH-12-1-0072

5b. GRANT NUMBER
W81XWH-12-1-0072

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

HARVARD COLLEGE,
677 HUNTINGTON AVE
BOSTON MA 02115-6028

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Purpose: The overarching goal of the grant is to characterize molecular heterogeneity in multi-focal and metastatic prostate cancer. Aim 1 focuses on a 4-gene signature of prostate cancer prognosis, and whether the signature differs across within-patient tumor nodules. Aim 2 compares gene expression profiles between primary and lymph node metastases in order to identify genes involved in metastatic progression of prostate cancer. Scope: In year 1, Dr. Kasperzyk has received IRB approval, completed a series of courses to augment her expertise in prostate cancer epidemiology, has coordinated meetings to discuss the study progress with collaborators, and has begun specimen and data collection for the proposed work. In the upcoming year 2, Dr. Kasperzyk will complete data collection, lead the statistical analyses, and publish the findings in peer-reviewed journals. Major Findings: To date, the proposed biomarkers are in the process of being measured. In a related analysis of tumor expression of prostate-specific membrane antigen (PSMA) and prostate cancer-specific mortality, Kasperzyk et al. found that PSMA was positively correlated with Gleason score and tumor angiogenesis, but was not an independent predictor of prostate cancer survival (*Cancer Epidemiol Biomarkers Prev,* in press). Significance: The clinical significance of the project is to better characterize putative prognostic markers for prostate cancer, as well as identify potential therapeutic targets for secondary prevention.

15. SUBJECT TERMS
Prostate cancer, metastases, prognosis, heterogeneity, gene expression

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	36	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

Since approximately 1 in 8 men with prostate cancer in the US will die of their disease, it is critical to identify early in the disease course those men who are likely to progress in order to administer appropriate therapies. Several tumor-derived RNA expression signatures have been developed to improve upon the prognostic value of known clinical parameters (e.g. Gleason score, tumor stage, PSA levels) to predict prostate cancer recurrence or death. However, hundreds of genes have been identified in the current signatures, and it is unclear which ones are biologically relevant for metastatic spread due, in part, to the difficulty in obtaining metastatic specimens and inherent tumor heterogeneity. The focus of this proposal is to assess tumor heterogeneity and prognostic value of a 4-gene signature of prostate cancer prognosis, and to identify genes involved in metastatic progression to the lymph nodes.

BODY

Task 1. Aim 1: Characterize heterogeneity of a 4-gene signature across prostate tumor nodules and validate its prognostic potential

IRB approval was obtained for this project at Harvard School of Public Health in October 2012. Dr. Kasperzyk has taken several courses to enrich her knowledge of pathology, molecular epidemiology, and biostatistics (see Task 4 below) to be prepared for the statistical analyses for Aim 1. Also, the tissue microarray of 228 prostate cancer patients who underwent radical prostatectomy, including 75 with multi-focal disease, has been constructed and is readily available for immunohistochemistry (see Table 1 in Supporting Data).

Since the original grant proposal, the funding source to measure the 4-gene signature in these tumor specimens is no longer be available to perform the assay.[1] Dr. Kasperzyk has identified an alternative solution, and the new technology is currently undergoing pilot testing to multiplex dozens of protein markers using a single section of archival tumor tissue. The pilot study should be completed in November 2013, at which point, the 4-gene signature will be measured using the tissue microarrays for Aim 1. Statistical analyses and manuscript preparation will proceed immediately following the data acquisition.

While awaiting the data for the 4-gene signature, Dr. Kasperzyk has tested the prognostic potential of another protein highly expressed in prostate tissue: prostate specific membrane antigen (PSMA). Utilizing archival prostate tumor tissue from two US-based cohort studies, Kasperzyk et al. found that PSMA protein expression measured in prostate tumor tissue was associated with progression to lethal disease, but not independent of clinical predictors.[2] Increasing tumor expression of PSMA was correlated with higher Gleason score and increased tumor angiogenesis. Overall, these findings support a do not support the clinical utility of tumor PSMA expression as a predictor of lethal disease among patients who undergo radical prostatectomy. A copy of the manuscript (in press) is included in the appendix.

Task 2. Aim 2: Identify genes critical for metastatic progression to lymph nodes in prostate cancer

IRB approval was obtained at Harvard School of Public Health in October 2012. Dr. Kasperzyk has begun a literature review detailing current studies that have compared molecular differences in metastatic versus primary prostate cancer. She has also taken several courses to enrich her knowledge of pathology, molecular epidemiology, and biostatistics (see Task 4 below). These courses have aided Dr. Kasperzyk in the study design of the project, and will also be key to the statistical analysis. Currently, our collaborator Dr. Andren is organizing the collection of within-person primary and lymph node-positive archival tumor specimens in Sweden. The collection is expected to be completed in December 2013, at which point, the mRNA expression profiling will be performed. Following the expression profiling analysis, Dr. Kasperzyk will begin the statistical analysis and manuscript preparation in year 2.

Task 3. Mentored training with Dr. Mucci

Drs. Mucci and Kasperzyk have completed this task by meeting regularly to discuss progress on the specific aims of the project, as well as evaluating short- and long-term goals.

Task 4. Coursework

In year 1, Dr. Kasperzyk has taken several courses in pathology, molecular epidemiology, and biostatistics. In September 2012, Dr. Kasperzyk attended a 2-hr course on "Introduction to Microarrays and Affymetrix Data analysis using R/Bioconductor" at Harvard Medical School where she familiarized herself with the R programming language. In October 2013, Dr. Kasperzyk attended a 2-hr course on "Whole Transcript Expression analysis using Gene and Exon 1.0 ST arrays" at Harvard Medical School. The course further developed her knowledge of the R programming language and techniques for analyzing expression array data. In January 2013, Dr. Kasperzyk completed EPI508 (Pathology for Epidemiologists; 1-week course) with a grade of 'Pass' at Harvard School of Public Health. The objective of the course was to provide an overview of tumor classification systems, histology, immunohistochemistry, and other molecular techniques used in epidemiologic research involving tumor specimens. From January-May 2013, Dr. Kasperzyk completed BIO508 (Genomic Data Manipulation; semester-long course) at Harvard School of Public Health with a grade of "A." The course taught computational methods for genomic data analysis using the Python programming language and online, publically available research tools.

Task 5. Meetings and seminars

Dr. Kasperzyk has attended numerous meetings and seminars as planned in year 1. She has attended two bi-weekly meetings, including a prostate cancer epidemiology meeting and pathology-epidemiology working group. Monthly meetings that Dr. Kasperzyk attends include meetings for the Prostate Cancer SPORE at Dana-Farber/Harvard Cancer Center and for prostate cancer journal club at Harvard School of Public Health. In March 2013, Dr. Kasperzyk presented an abstract at the Multi-Institutional Prostate Cancer Program Retreat in Ft. Lauderdale, Florida. Dr. Kasperzyk was also accepted to take part in a special week-long workshop entitled "Integrative Molecular Epidemiology Workshop" in July 2013 in Boston, MA, sponsored by the American Association of Cancer Research. This workshop addressed the

challenges faced when integrating high-dimensional data from multiple sources in order to inform disease etiology and outcomes.

KEY RESEARCH ACCOMPLISHMENTS

- Publication of a manuscript entitled "Prostate-specific membrane antigen protein expression in tumor tissue and risk of lethal prostate cancer" in *Cancer Epidemiology, Biomarkers, and Prevention* [2]
- Completion of a tissue microarray with prostate tumor specimens representing patients with multi-focal disease
- Literature review of current studies comparing molecular differences in metastatic versus primary prostate cancer
- Initiation of a prostate tumor tissue resource that utilizes patient-matched primary and lymph node-positive prostate cancer specimens

REPORTABLE OUTCOMES

- Dr. Kasperzyk was promoted to Instructor in the Department of Medicine at Harvard Medical School/Brigham and Women's Hospital (July 2013)
- Manuscript (Kasperzyk et al.) accepted to *Cancer Epidemiology, Biomarkers, and Prevention* entitled "Prostate-specific membrane antigen protein expression in tumor tissue and risk of lethal prostate cancer" (October 2013)[2]
- Applied for NIH-NCI R03 award in July 2013 entitled "Dairy intake and tumor markers of prostate cancer progression"; awaiting grant review.
- Dr. Kasperzyk presented an abstract at the Multi-Institutional Prostate Cancer Program Retreat in Ft. Lauderdale, Florida (March 2013)
- Completion of a tissue resource by colleague (Dr. Ove Andren) that utilizes tissue microarray technology to catalog >200 prostate cancer patients with single and multi-focal prostate tumor specimens. The tissue microarrays that have been generated during year 1 will be used for the analyses in Aim 1, and are available as a resource for any of our collaborators who wish to study protein expression and histological differences across tumor foci in this patient population. (Spring 2013)
- Development of a tissue resource that combines within-person primary and lymph node-positive prostate cancer specimens. This resource is currently being initiated in Sweden (Dr. Ove Andren) where archival tumor specimens are readily available for research purposes.

CONCLUSION

Dr. Kasperzyk has made significant progress on the current Career Development Award through coursework, organizational meetings, and completing many research-related tasks. Regarding career accomplishments, Dr. Kasperzyk was promoted to Instructor in the Department of Medicine at Harvard Medical School/ Brigham and Women's Hospital in July 2013. Dr. Kasperzyk has worked to overcome the challenge of finding an alternative means of performing the assays for Aim 1, and is making great progress to develop the tissue resource for Aim 2. She has also published a paper on a putative prognostic marker for prostate cancer, and has presented her findings at several scientific meetings/conferences. In year 2, Dr. Kasperzyk will continue to make significant progress on Aims 1 & 2, and will conclude by submitting her findings to peer-reviewed journals. In summary, this project is well underway toward making important contributions to the understanding and characterization of molecular heterogeneity in prostate cancer.

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APPENDICES

Prostate-specific membrane antigen protein expression in tumor tissue and risk of lethal prostate cancer

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Running Title: Tumor PSMA expression and lethal prostate cancer

Key Words: prostate-specific membrane antigen, prostate cancer, tumor biomarkers, prognosis, angiogenesis

Financial Support: This work was supported by the National Institutes of Health [grant numbers CA34944, CA40360, CA097193, CA055075, CA131945, CA136578, CA141298, CA09001 to J.L.K., HL26490, HL34595]; the American Institute for Cancer Research, CDMRP Department of Defense [grant number W81XWH-12-1-0072], and Dana-Farber Cancer Institute Mazzone Awards Program to J.L.K.; Dana-Farber/Harvard Cancer Center SPORE in Prostate Cancer [grant number 5P50CA090381-08]; and the Prostate Cancer Foundation to L.A.M. and S.P.F.

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Conflict of Interest: The authors declare no conflict of interest.

Word Count: abstract: 235; text: 3761; tables: 3; figures: 1

Abstract

Background: Over-expression of prostate-specific membrane antigen (PSMA) in tumor tissue and serum has been linked to increased risk of biochemical recurrence in surgically treated prostate cancer patients, but no studies have assessed its association with disease-specific mortality. **Methods**: We examined whether high PSMA protein expression in prostate tumor tissue was associated with lethal disease, and with tumor biomarkers of progression, among participants of two US-based cohorts (n=902, diagnosed 1983-2004). We used Cox proportional hazards regression to calculate multivariable hazard ratios (HR) and 95% confidence intervals (CI) of lethal prostate cancer, defined as disease-specific death or development of distant metastases (n=95). Partial Spearman rank correlation coefficients were used to correlate PSMA with tumor biomarkers. **Results**: During an average 13 years of follow-up, higher PSMA expression at prostatectomy was significantly associated with lethal prostate cancer (age-adjusted $HR_{Quartile(Q)4vs.Q1}$=2.42; p-trend<0.01). This association was attenuated and non-significant (multivariable-adjusted $HR_{Q4vs.Q1}$=1.01; p-trend=0.52) after further adjusting for Gleason score and PSA at diagnosis. High PSMA expression was significantly (p<0.05) correlated with higher Gleason score and PSA at diagnosis, increased tumor angiogenesis, lower vitamin D receptor and androgen receptor expression, and absence of ERG expression. **Conclusions**: High tumor PSMA expression was not an independent predictor of lethal prostate cancer in the current study. PSMA expression likely captures, in part, malignant features of Gleason grade and tumor angiogenesis. **Impact**: PSMA is not a strong candidate biomarker for predicting prostate cancer-specific mortality in surgically treated patients.

Introduction

Prostate-specific membrane antigen (PSMA) is a type II transmembrane glycoprotein that is highly expressed in the normal prostate epithelium, and to a lesser extent in other tissues such as brain, liver, and kidney (1, 2). PSMA expression is higher in primary prostate tumors and metastatic lesions compared to benign tissue, and is positively associated with tumor grade and stage (3-7). Due to its high expression in malignant prostate tissue, PSMA has been utilized in immunoscintigraphy to monitor metastatic disease and as a target antigen for immunotherapy (8, 9).

PSMA may also have prognostic utility. Three studies of surgically treated prostate cancer patients showed that high PSMA protein expression in tumor tissue was associated with biochemical recurrence (5-7). Two of these studies found that PSMA overexpression was predictive of biochemical recurrence after multivariable adjustment for clinical parameters such as tumor stage, grade, and preoperative prostate specific antigen (PSA) levels (5, 6). However, Minner et al. did not find PSMA to be an independent predictor after adjusting for clinicopathological features (7). High PSMA mRNA expression in preoperative peripheral blood cells, possibly detecting micrometastatic disease, similarly showed a positive association with biochemical recurrence in four prospective studies (10-13), a relationship not observed in a fifth study (14). No studies to date have investigated PSMA expression in relation to prostate cancer-specific mortality.

PSMA functions as a peptidase with both N-acetylated α-linked acidic peptidase and folate hydrolase activity (15, 16). *In vitro* and *in vivo* experiments have shown that high PSMA expression activates signaling pathways that promote tumor cell survival and proliferation (17).

The association of PSMA with anaphase-promoting complex disrupts cell cycle checkpoints, induces chromosomal instability, and contributes to aneuploidy (18). In addition, PSMA is negatively regulated by 1α,25-dihydroxyvitamin D_3(19), a nutrient associated with reduced proliferation in animal models and prostate cancer cell lines (20, 21). Interestingly, androgen deprivation enhances PSMA expression (1, 22), and a role in the development of castration resistance has been hypothesized. Androgens stimulate *TMPRSS2:ERG* expression, a gene fusion mutation common in human prostate cancer (23), as the TMPRSS2 promoter has an androgen responsive element, thus providing a potential link between inhibition of PSMA by androgen and *ERG* expression in fusion-positive prostate cancer cells (24). PSMA has also been identified as a regulator of new blood vessel formation (i.e. angiogenesis) in mouse models (25, 26). While virtually absent from non-prostatic normal tissues, PSMA is expressed in the neovasculature of many solid tumors, thus underscoring its importance in tumor angiogenesis (27-30).

In this prospective study, our main objective was to determine whether tumor PSMA protein expression from primarily radical prostatectomy specimens was an independent predictor of prostate cancer-specific mortality in 902 participants of the Physicians' Health Study (PHS) and Health Professionals Follow-up Study (HPFS). To identify potential mechanisms of PSMA in disease progression, we also evaluated correlations between PSMA expression and measures of cell proliferation, apoptosis, angiogenesis, and protein expression of vitamin D receptor (VDR), androgen receptor (AR), and ERG in prostate tumor tissue.

Materials and Methods

Study population

This study population of prostate cancer patients is drawn from participants of the prospective PHS and HPFS studies for whom archival prostate tumor tissue, primarily from radical prostatectomy, was available for biomarker analysis. PHS I and II were randomized, placebo-controlled, double-blind trials for the prevention of cardiovascular disease and cancer. PHS I began in 1982 and evaluated aspirin and ß-carotene among 22,071 U.S. male physicians (31); in 1997 PHS II randomized 7,641 physicians from PHS I and 7,000 new physicians to ß-carotene, vitamin E, vitamin C, and multivitamins (32). All arms of the PHS I and II have been terminated (33-35), and the PHS continues to be followed annually. The HPFS began in 1986 with 51,529 U.S. male health professionals (dentists, veterinarians, pharmacists, optometrists, osteopathic physicians, and podiatrists) who are prospectively followed on biennial questionnaires to collect lifestyle and medical information (36). This study was approved by the Partners Healthcare and Harvard School of Public Health Institutional Review Boards.

Clinical data and prostate cancer outcomes

Self-reported, incident cases of prostate cancer arising in the PHS (1983-2004) and HPFS (1986-2001) were confirmed by medical record and pathology report review by study investigators. In rare cases, prostate cancer diagnoses were identified on death certificates and confirmed by medical record, pathology report, and death certificate review. To ascertain clinical characteristics and disease-specific treatments or outcomes, information on tumor stage, PSA at diagnosis, body mass index (BMI), and metastases events during follow-up was collected from medical record and pathology report review, and from questionnaires sent to prostate cancer survivors (2004 onward). Pathological tumor stage was available for 90% of patients, while the remaining had clinical stage information (n=89) or were missing (n=2).

Greater than 97% of tumor specimens were re-reviewed by a study pathologist (M.F. and R.F.) to achieve uniformity of scoring, and the remaining assigned clinical Gleason score. Cause of death was assigned via review of medical records and death certificates for the vast majority of participants, and secondarily via information from family. We defined lethal disease as death from prostate cancer or distant metastases (to bone or other organs, excluding lymph nodes) during follow-up. A total of 95 lethal events occurred: 29 in PHS and 66 in HPFS. We analyzed a composite of biochemical recurrence and lethal prostate cancer (n=231) as a secondary endpoint, using the first recorded event as the event date. Biochemical recurrence was defined as PSA above 0.2 ng/mL after surgery sustained over 2 measures (when abstracted from medical records), or a report of biochemical recurrence by the participant or treating physician.

Tumor biomarker measurements

Tissue microarray construction. Formalin-fixed, paraffin-embedded archival tumor tissue specimens were obtained from the hospital pathology departments: 95% were from radical prostatectomy procedures and the remainder from transurethral resection of the prostate (TURP). Our pathologist reviewed all available slides to provide standardized Gleason grading and for identification of the areas of tumor tissue for tissue microarray construction blinded to outcome status (37). For this project, we used 9 tissue microarrays constructed from areas of the dominant tumor nodule or highest Gleason grade, with at least 3 tumor cores (0.6 mm) sampled from each patient.

PSMA immunohistochemistry. Protein expression of PSMA was ascertained on 5 µm sections of the tissue microarrays (pathologist: S.P.F.). Antigen retrieval was by microwave in citrate buffer (3x5 minutes). We used a primary mouse monoclonal antibody (Clone E36,

M3620, Dako, Carpinteria, CA) with 1:100 dilution for 60 minutes after treatment with a peroxidase block (Dako, Carpinteria, CA). An anti-mouse secondary antibody was applied, followed by a counterstain with hematoxylin (Sigma-Aldrich, St. Louis, MO). PSMA expression was quantified using the Ariol platform (Genetix Corp., San Jose, CA), a semi-automated, quantitative image analysis system, and defined as staining intensity (scale: 0-255) multiplied by percent area staining positive (scale: 0-100%) for a given tumor field on each tissue microarray core. All 9 microarrays were stained in the same batch, and positive and negative controls were included according to the antibody manufacturer's instructions.

Proliferation and apoptosis indices. Cellular proliferation was assessed on 5 µm sections of the tissue microarrays using rabbit polyclonal anti-Ki67 antibody (Vector Laboratories, Burlingame, CA), diluted 1:2000 with citrate-based antigen retrieval solution (pathologist: S.P.F.). Ki67 staining was visualized using the Ariol platform (Genetix Corp., San Jose, CA), and quantified as the percent of positively stained nuclei in the tumor region of each core. Apoptosis was evaluated on 5 µm sections of the tissue microarrays using the ApopTag Peroxidase *In Situ* kit (Chemicon International, Temecula, CA) according to manufacturer's instructions, and defined as the percent of tumor cells undergoing apoptosis (pathologist: M.F.) (38).

VDR, AR, and ERG immunohistochemistry. VDR expression was calculated on 5 µm sections of the tissue microarrays using rabbit polyclonal anti-VDR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:600 as previously described (pathologist: R.F.) (37). VDR expression was quantified as a combination of percent area that was positively stained and staining intensity using CRi Vectra, a semi-automated, quantitative image analysis

system (CRi, Woburn, MA). AR expression was calculated on 5 µm sections of the tissue microarrays using rabbit polyclonal anti-AR antibody (Upstate/Millipore, Billerica, MA) at a dilution of 1:100 (pathologist: S.P.F). Mean intensity (scale: 0-255) of AR staining in the nucleus of tumor cells in a given core was calculated using the Ariol platform (Genetix Corp., San Jose, CA). ERG expression was calculated on 5 µm tissue microarray sections (91% of patients) and prostate tissue block sections (9% of patients), using rabbit monoclonal anti-ERG antibody (Clone ID: EPR3864, Epitomics, Inc., Burlingame, CA) at a dilution of 1:100. Tumor specimens were evaluated individually by a study pathologist (R.L.). The presence of ERG staining in the vascular endothelium served as a positive internal control, with ERG assessment restricted to cores in which the positive internal control was observed. A patient was considered positive for tumor ERG expression if ERG staining was observed within prostate cancer epithelial cells of at least one tissue microarray core.

Biomarkers of angiogenesis. Angiogenesis markers were assessed on 5 µm serial sections of the individual prostate tissue blocks in the HPFS cohort only. One to nine blocks with cancer were evaluated per case by a study pathologist as previously described by Mucci *et al*. (39). Endothelial cell marker CD34 protein expression was visualized using immunohistochemistry (QBEND10 primary mouse monoclonal antibody; Biogenex, San Ramon, CA) and imaged using Image ProPlus 4.5 software (Media Cybernetics, Bethesda, MD), a semiautomated image analysis platform. Angiogenesis markers were defined as the following: microvessel density, i.e. the number of vascular structures in a high-powered field (x200); vessel area in μm^2; vessel diameter in µm; and vessel irregularity, i.e. the irregularity of the vessel lumen calculated as the $perimeter^2/4 \cdot \prod$ X area, where a value of 1.0 indicates a perfect

circle and values >1.0 indicate increasing irregularity. Measurements were averaged over the total tumor area evaluated for each patient. Smaller vessel area and diameter, and less regular vessel shape were associated with development of lethal prostate cancer in this cohort (39).

Statistical analysis

Analyses were based on the 902 men (n=346 from PHS, n=556 from HPFS) for whom PSMA expression was measured. The average value of each biomarker was calculated across all cores or tumor sections for a given patient. We compared age at diagnosis, clinical parameters, and BMI across quartiles of PSMA expression using analysis of variance (ANOVA) for normally-distributed continuous variables, Kruskal-Wallis test for non-normally distributed continuous measures, and Chi-square tests for categorical variables.

Cox proportional hazards regression was used to calculate multivariable hazard ratios (HR) and 95% confidence intervals (CI) for the association between PSMA expression and lethal prostate cancer. Follow-up time was calculated from the date of diagnosis to development of distant metastases, death from prostate cancer, or censored at death from another cause or end of follow-up (January 2009 or last date of contact for PHS; April 2012 for HPFS), whichever occurred first. We adjusted for tissue microarray (indicator variables) to account for staining variation across microarrays, and age at diagnosis (continuous), in all models. We further adjusted for Gleason score (2 to 6, 3+4, 4+3, 8 to 10) and PSA at diagnosis (<4, 4 to <10, ≥10 ng/ml, missing) to test whether PSMA expression was an independent predictor of lethal prostate cancer risk. We also examined these associations stratified by tumor stage (T1-T2, N0-Nx, M0-Mx vs. T3-T4 or N1 or M1), Gleason score (2 to 7 vs. 8 to 10), and ERG expression (absent, present). Violation of the proportional hazards assumption was tested by creating

interaction terms between PSMA quartiles and follow-up time; the addition of the interaction terms to the model including PSMA quartiles, age at diagnosis, and tissue microarray, was not statistically significant (Wald test p-value=0.21; 3 degrees of freedom), thus the assumption was satisfied. Since PSMA is negatively correlated with androgen levels (1, 22), we also performed a sensitivity analysis excluding the 57 patients who received any type of neoadjuvant or adjuvant hormone therapy ± 1 year from the date of radical prostatectomy or TURP. To test the association between PSMA expression and the composite endpoint of biochemical recurrence and lethal disease, follow-up time was calculated from date of diagnosis to date of recurrence, distant metastases, or death from prostate cancer; patients without a recurrence were censored at death from another cause or end of follow-up.

We examined correlations between PSMA expression and tumor biomarkers (proliferation index, apoptotic index, AR expression, VDR expression, and angiogenesis measures) using partial Spearman rank correlations, adjusted for age at diagnosis and tissue microarray. PSMA expression across categories of ERG expression (absent, present) was evaluated using analysis of covariance (ANCOVA), adjusted for age at diagnosis and tissue microarray.

Analyses were conducted using SAS system software (version 9.2; SAS Institute, Cary, NC). All p-values were two-sided and considered statistically significant if <0.05.

Results

Among the 902 prostate cancer patients, mean age at diagnosis was 65.8 years with an average follow-up time of 13.2 years (**Table 1**). Higher PSMA expression was associated

(p<0.01) with increasing age, higher Gleason score, and higher PSA at diagnosis, and modestly associated (p=0.07) with higher tumor stage. Mean tumor PSMA expression among all patients was 43.9 with an interquartile range of 10.5-70.7. PSMA expression (mean ± standard deviation) was similar between the cohorts (44.7 ± 36.8 for PHS and 43.5 ± 35.7 for HPFS), and between prostatectomy and TURP specimens (44.2 ± 36.1 and 39.6 ± 36.4, respectively). PSMA staining in the tumor was membranous and cytoplasmic (**Fig 1**).

PSMA protein expression in tumor tissue was associated with a 2.4-fold (95% CI: 1.3, 4.5) increased risk of lethal prostate cancer comparing the highest to lowest quartile, adjusting for age at diagnosis and tissue microarray (**Table 2**). This positive association was stronger among patients with non-advanced stage disease ($HR_{Quartile(Q)4vs1}$=4.3; p-trend<0.01), lower Gleason score ≤7 tumors (HR_{Q4vs1}=4.6; p-trend<0.01), as well as those with ERG-positive tumors (HR_{Q4vs1}=3.5; p-trend<0.01). No associations with lethal cancer were found in men with advanced stage disease (p-trend=0.27), poorly differentiated (Gleason score ≥8) tumors (p-trend=0.39), or ERG-negative tumors (p-trend=0.35). After further adjustment for Gleason score and PSA at diagnosis, the associations between PSMA expression and lethal prostate cancer were attenuated for overall (p-trend=0.76), non-advanced (p-trend=0.61), Gleason score ≤7 (p-trend=0.51), and ERG-positive (p-trend=0.88) prostate cancer, and all were non-significant.

Among all 902 patients, associations of clinical parameters and risk of lethal prostate cancer were: age at diagnosis (per 5-year increase, HR=1.2, 95% CI:1.0,1.4); Gleason score ($HR_{3+4vs2-6}$=1.4, 95% CI:0.5,4.5; $HR_{4+3vs2-6}$=4.1, 95% CI:1.4,12.0; $HR_{8-10vs2-6}$=7.7, 95% CI:2.7,21.9); PSA at diagnosis ($HR_{4-9.9vs<4}$=1.5, 95% CI:0.3,6.2; $HR_{\geq 10vs<4}$=2.8, 95% CI:0.7,11.8); tumor stage

($HR_{T3vsT1\text{-}T2}$=1.7, 95% CI:1.1,2.8; $HR_{T4/N1/M1vsT1\text{-}T2}$=5.1, 95% CI:2.9,9.1); mutually adjusted for all 4 parameters.

In the model adjusting for age at diagnosis and tissue microarray, effect estimates were slightly stronger after excluding patients who had received neoadjuvant or adjuvant hormone therapy: HR_{Q2vs1}=2.14 (95% CI: 1.03,4.44), HR_{Q3vs1}=2.01 (95% CI:0.96,4.21), HR_{Q4vs1}=3.20 (95% CI:1.60,6.39), p-trend<0.01. Similar to the main analysis, results were attenuated and non-significant after further adjusting for Gleason score and PSA at diagnosis: HR_{Q2vs1}=1.78 (95% CI: 0.84,3.80), HR_{Q3vs1}=1.72 (95% CI:0.80,3.72), HR_{Q4vs1}=1.38 (95% CI:0.67,2.86), p-trend=0.92.

Compared to the primary outcome of lethal prostate cancer, the association between PSMA expression and the composite outcome of biochemical recurrence and lethal disease was weaker and non-significant: HR_{Q2vs1}=0.90 (95% CI: 0.61,1.33), HR_{Q3vs1}=1.26 (95% CI:0.87,1.82), HR_{Q4vs1}=1.24 (95% CI:0.86,1.78), p-trend=0.09, adjusting for age at diagnosis and tissue microarray; and HR_{Q2vs1}=0.75 (95% CI: 0.50,1.12), HR_{Q3vs1}=0.89 (95% CI:0.61,1.31), HR_{Q4vs1}=0.68 (95% CI:0.46,1.01), p-trend=0.13, after further adjusting for Gleason score and PSA at diagnosis.

Tumors with high PSMA expression showed significantly lower protein expression of VDR and AR, and absence of ERG protein expression, among all patients (**Table 3**). High PSMA expression was also significantly correlated with markers of angiogenic activity, including higher microvessel density, smaller vessel area, smaller vessel diameter, and irregular shape. With the exception of ERG expression, the correlations between PSMA and other tumor biomarkers did not retain statistical significance in poorly differentiated tumors. No correlations were found for proliferation or apoptotic indices among all patients or within subgroups.

The association between PSMA expression and lethal prostate cancer among all patients, adjusted for age at diagnosis and tissue microarray, remained statistically significant after further adjustment for VDR (HR_{Q4vs1}=2.16; 95% CI: 1.14, 4.11; p-trend=0.03; n=812), AR (HR_{Q4vs1}=2.31; 95% CI: 1.25, 4.29; p-trend<0.01; n=860), or ERG expression (HR_{Q4vs1}=2.41; 95% CI: 1.28, 4.53; p-trend<0.01; n=880). Among HPFS patients with measured angiogenesis markers (microvessel density, vessel area, vessel diameter, and vessel irregularity), higher PSMA expression was non-significantly associated with lethal disease ($HR_{Q4vs.1}$=2.45; 95% CI: 0.92, 6.49; p-trend=0.19; n=414), adjusting for age at diagnosis and tissue microarray. This association was attenuated after further adjusting for all 4 markers (HR_{Q4vs1}=1.65; 95% CI: 0.60, 4.54; p-trend=0.75), or any of the markers individually (data not shown).

Discussion

In a large cohort of prostate cancer patients with over 13 years of average follow-up, PSMA protein expression in tumor tissue was positively associated with risk of lethal disease, but this association was not independent of clinical parameters. Thus, our study does not support the clinical utility of PSMA expression as a strong candidate biomarker for lethal prostate cancer among surgically treated patients. After considering additional markers of disease aggressiveness, we found that PSMA expression likely captures, in part, malignant features of Gleason grade and tumor angiogenesis.

Three prior studies of PSMA protein expression in prostate tumor tissue have reported positive associations with risk of biochemical recurrence (5-7). Minner et al. followed 1,426 prostate cancer patients for up to 12 years and noted a borderline significant association for high vs. low PSMA expression in radical prostatectomy tissue and PSA recurrence (7). Similar to

our study, the association did not remain statistically significant after multivariable adjustment for clinical parameters. A smaller study of 136 patients (61% with organ-confined tumors) who underwent radical prostatectomy found that PSMA overexpression was associated with biochemical recurrence, even after multivariable adjustment for clinicopathological parameters (6). A third study of 93 patients (43% with lymph-node positive disease at surgery) found a significant positive association between PSMA expression and biochemical recurrence after adjusting for extraprostatic extension, though the estimates adjusted for additional clinical parameters is not presented (5). Our results may differ from these studies since >70% of our patients were diagnosed with non-advanced stage tumors, our specimens were re-reviewed by a study pathologist for uniformity of Gleason score, and PSA levels at diagnosis were included in the multivariable models. Since PSMA expression has been positively correlated with these clinicopathological features, it is unclear whether the positive findings from other studies would persist after accounting for all these factors. Furthermore, Ross et al. used the 7E11 anti-PSMA antibody which recognizes the internal domain of PSMA (6), whereas the other two prior studies (5, 7) and our current study used clone 3E6 which recognizes the extracellular domain. Lastly, our results may differ since our study was the first to assess lethal disease as the primary endpoint, whereas all prior studies evaluated time to biochemical recurrence.

We previously showed that a greater number of smaller and more poorly formed vessels within the prostate tumor were strong predictors of lethal disease (39). Our current study supports that PSMA is indicative of increased tumor angiogenesis, and after adjusting for these markers, the association of PSMA expression with lethal prostate cancer was markedly attenuated. This is consistent with the prior observation of PSMA being expressed in the

endothelial cells of certain solid tumor neovasculature, including prostate cancer, renal cell carcinoma, transitional cell carcinoma of the bladder, gastric cancer, and colorectal cancer (27-30). Also, a small study of LNCaP tumors grown in nude mice found a strong positive correlation between protein expression of PSMA and vascular endothelial growth factor, a signal protein that stimulates angiogenesis (40).

PSMA appears to be regulated by androgens, in that PSMA expression in prostate tumors is highest in hormone-deprived states, and is repressed in response to testosterone (1, 22). We found that higher PSMA expression was correlated with lower AR expression in prostate tumor tissue, though we did not have a measure of circulating testosterone levels at the time of surgery in our study. We also found that PSMA expression was lower in tumors that expressed ERG, which is supported by the prior finding that *TMPRSS2-ERG* fusion negatively regulated PSMA expression in LNCaP cells (24). Additionally, the association between PSMA expression and lethal prostate cancer in our study was limited to ERG-positive tumors, suggesting that the link between PSMA and disease progression may depend on the molecular subtype of the tumor. Further studies are warranted to better understand the mechanisms by which PSMA, AR and the *TMPRSS2-ERG* fusion may interact to influence prostate carcinogenesis.

The negative correlation we observed between VDR and PSMA expression is consistent with Serda et al. who reported that 1α,25-dihydroxyvitamin D_3 down-regulated PSMA expression in LNCaP cells (19). We previously reported an inverse association between VDR expression and prostate cancer progression in this patient cohort (37). In the current study, PSMA expression was associated with lethal prostate cancer independently of VDR levels in the

age- and tissue microarray-adjusted models, suggesting that PSMA and VDR may act through different mechanisms to influence disease progression. Indeed, vitamin D has been shown to exert anti-proliferative and pro-apoptotic effects on prostate tumors (20, 21, 41), whereas we found no correlation between PSMA and indices of proliferation or apoptosis.

Limitations of our study include potential misclassification of PSMA protein expression due to assay and detection variability, though any bias is likely non-differential since study pathologists were blinded to outcome status. Also, we had low statistical power to detect associations among subgroups of patients with small numbers of outcomes. Furthermore, we used mainly prostatectomy tissue with the majority of patients having organ-confined disease, thus it is unknown whether our findings would be generalizable to PSMA expression measured in biopsy specimens. Our study has several notable strengths. We were the first to evaluate the association between PSMA expression and lethal disease within two large, established cohort studies with long-term and complete follow-up among prostate cancer patients. Additionally, the patients were well-characterized with respect to clinical and pathological measures, including re-review of Gleason scores.

In our study of 902 US-based prostate cancer patients, PSMA protein expression measured in prostate tumor tissue was associated with progression to lethal disease, but not independent of clinical predictors. Our results suggest that PSMA is an indicator of increased tumor angiogenesis, and through this pathway, increased risk of prostate cancer progression. Overall, our findings do not support the clinical utility of tumor PSMA expression as a predictor of lethal disease among patients who undergo radical prostatectomy, though it is unknown how

this biomarker may perform in biopsy specimens from patients who choose other treatment modalities such as active surveillance or radiation.

Acknowledgements

We are grateful to the participants and staff of the Physicians' Health Study and Health Professionals Follow-Up Study for their valuable contributions. In addition we would like to thank the following state cancer registries for their help: AL, AZ, AR, CA, CO, CT, DE, FL, GA, ID, IL, IN, IA, KY, LA, ME, MD, MA, MI, NE, NH, NJ, NY, NC, ND, OH, OK, OR, PA, RI, SC, TN, TX, VA, WA, WY. The Dana-Farber/Harvard Cancer Center Tissue Microarray Core Facility constructed the tissue microarrays in this project, and we would like to thank Chungdak Li for her expert tissue microarray construction.

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Table 1: Characteristics of 902 men with prostate cancer in the Physicians' Health Study and Health Professionals Follow-up Study according to prostate-specific membrane antigen (PSMA) expression in tumor tissue

		PSMA quartile (Q)				
	All patients	Q1 (low)	Q2	Q3	Q4 (high)	p-value
N cases	902	225	226	226	225	
Mean (SD) age at diagnosis, yrs	65.8 (6.3)	65.1 (6.4)	66.2 (6.3)	65.2 (6.7)	66.8 (5.6)	<0.01 [1]
Mean (SD) follow-up time, yrs	13.2 (5.0)	13.6 (5.1)	13.1 (4.9)	13.4 (5.0)	12.6 (4.8)	0.13 [1]
Tumor stage, N(%)						
T1-T2, N0-Nx, M0-Mx	640 (71.0)	173 (76.9)	166 (73.5)	144 (63.7)	157 (69.8)	0.07 [2]
T3, N0-Nx, M0-Mx	222 (24.6)	45 (20.0)	49 (21.7)	70 (31.0)	58 (25.8)	
T4 or N1 or M1	38 (4.2)	6 (2.7)	11 (4.9)	12 (5.3)	9 (4.0)	
missing	2 (0.2)	1 (0.4)	0	0	1 (0.4)	
Gleason score, N(%)						
2 to 6	178 (19.7)	70 (31.1)	57 (25.2)	36 (15.9)	15 (6.7)	<0.01 [3]
3+4	335 (37.1)	100 (44.4)	84 (37.2)	82 (36.3)	69 (30.7)	
4+3	223 (24.7)	29 (12.9)	52 (23.0)	64 (28.3)	73 (34.7)	
8 to 10	166 (18.4)	26 (11.6)	33 (14.6)	44 (19.5)	63 (28.0)	
PSA at diagnosis, ng/ml						
Median (IQR)	7.0 (5.0,11.0)	7.0 (4.8,9.9)	6.5 (4.7,9.3)	7.5 (5.0,12.5)	7.6 (5.5,13.0)	<0.01 [4]
Categories, N(%)						
< 4	87 (9.7)	26 (11.6)	28 (12.4)	21 (9.3)	12 (5.3)	0.01 [2]
4 to < 10	449 (49.8)	118 (52.4)	120 (53.1)	109 (48.2)	102 (45.3)	
≥ 10	231 (25.6)	47 (20.9)	48 (21.2)	65 (28.8)	71 (31.6)	
missing	135 (15.0)	34 (15.1)	30 (13.3)	31 (13.7)	40 (17.8)	
BMI at diagnosis, kg/m^2						
Mean (SD)	25.6 (3.4)	25.6 (3.3)	25.7 (4.2)	25.5 (3.0)	25.6 (3.1)	0.91 [1]
Categories, N(%)						
< 25	379 (42.0)	96 (42.7)	104 (46.0)	86 (38.1)	93 (41.3)	0.40 [2]
25 to < 28	276 (30.6)	73 (32.4)	64 (28.3)	81 (35.8)	58 (25.8)	
≥ 28	155 (17.2)	39 (17.3)	39 (17.3)	35 (15.5)	42 (18.7)	
missing	92 (10.2)	17 (7.6)	19 (8.4)	24 (10.6)	32 (14.2)	

SD: standard deviation; IQR: interquartile range; PSA: prostate specific antigen; BMI: body mass index.

[1] ANOVA test; 3 degrees of freedom. Excluded individuals with missing values.

[2] Chi-square test; 6 degrees of freedom. Excluded individuals with missing values.

[3] Chi-square test; 9 degrees of freedom.

[4] Kruskal-Wallis test; 3 degrees of freedom. Excluded individuals with missing values.

Table 2: Hazard ratios (HR) and 95% confidence intervals (CI) for the association between prostate-specific membrane antigen (PSMA) expression in tumor tissue and lethal prostate cancer

	PSMA quartile (Q)				
	Q1 (low)	Q2	Q3	Q4 (high)	p-trend [1]
All patients					
N lethal events	15	24	22	34	
N censored	210	202	204	191	
Person-time, yrs	3061	2971	3032	2825	
Model 1 [2]	1.00	1.64 (0.85,3.14)	1.55 (0.80,3.01)	2.42 (1.31,4.48)	<0.01
Model 2 [3]	1.00	1.17 (0.60,2.30)	1.11 (0.56,2.22)	1.01 (0.52,1.93)	0.76
Non-advanced stage[4]					
N lethal events	4	10	8	16	
N censored	169	156	136	141	
Person-time, yrs	2393	2277	1958	2007	
Model 1 [2]	1.00	2.43 (0.75,7.83)	2.42 (0.73,8.07)	4.34 (1.43,13.12)	<0.01
Model 2 [3]	1.00	1.86 (0.55,6.30)	2.06 (0.60,7.06)	1.74 (0.53,5.73)	0.61
Advanced stage[5]					
N lethal events	10	14	14	18	
N censored	41	46	68	49	
Person-time, yrs	661	693	1074	810	
Model 1 [2]	1.00	1.35 (0.59,3.09)	1.17 (0.50,2.74)	1.65 (0.74,3.64)	0.27
Model 2 [3]	1.00	0.90 (0.38,2.11)	0.85 (0.34,2.09)	0.78 (0.34,1.78)	0.55
Gleason score 2 to 7					
N lethal events	5	13	11	16	
N censored	194	180	171	146	
Person-time, yrs	2808	2591	2504	2109	
Model 1 [2]	1.00	3.05 (1.08,8.65)	2.62 (0.91,7.59)	4.63 (1.68,12.73)	<0.01
Model 2 [3]	1.00	2.64 (0.90,7.73)	2.02 (0.67,6.11)	2.11 (0.72,6.17)	0.51
Gleason score 8 to 10					
N lethal events	10	11	11	18	
N censored	16	22	33	45	
Person-time, yrs	253	380	528	716	
Model 1 [2]	1.00	0.51 (0.21,1.27)	0.60 (0.24,1.47)	0.53 (0.23,1.25)	0.39
Model 2 [3]	1.00	0.56 (0.22,1.45)	0.78 (0.30,2.03)	0.59 (0.24,1.40)	0.47

[1] Wald test modeling the median expression values for each PSMA quartile.

[2] Adjusted for age at diagnosis (continuous) and tissue microarray.

[3] Additionally adjusted for Gleason score (2 to 6, 3+4, 4+3, 8 to 10), and PSA at diagnosis (<4, 4 to <10, ≥10 ng/ml, missing).

[4] Tumor stage T1-T2, N0-Nx, M0-Mx.

[5]Tumor stage T3-T4, or N1 or M1.

Table 3: Correlation of PSMA protein expression in prostate tumor tissue with other tumor biomarkers

	All patients	Non-advanced stage[1]	Advanced stage[2]	Gleason score 2 to 7	Gleason score 8 to 10
Partial Spearman rank correlation coefficients[3]					
Proliferation index					
N	867	613	252	707	160
median [Q1, Q3]	0.13 [0,0.55]	0.14 [0,0.56]	0.12 [0,0.49]	0.11 [0,0.46]	0.23 [0.03,1.01]
r	-0.00002	-0.001	0.009	0.004	-0.127
p-value	1.00	0.98	0.89	0.93	0.12
Apoptosis index					
N	716	507	208	589	127
median [Q1, Q3]	0.50 [0,2.00]	0.50 [0,2.00]	0.50 [0,2.00]	0.50 [0,2.00]	0.50 [0,2.00]
r	-0.005	-0.004	0.015	0.038	-0.166
p-value	0.89	0.93	0.83	0.37	0.07
VDR protein expression					
N	812	567	243	658	154
median [Q1, Q3]	29.1 [13.0,45.4]	31.6 [14.9,47.7]	24.0 [8.9,42.8]	30.9 [14.3,47.7]	21.0 [7.0,37.7]
r	-0.084	-0.098	-0.010	-0.066	-0.049
p-value	0.02	0.02	0.87	0.09	0.56
AR protein expression					
N	860	612	246	704	156
median [Q1, Q3]	117.7 [112.3,123.0]	117.3 [112.3,123.0]	117.7 [111.0,123.0]	115.0 [111.0,123.0]	117.7 [112.3,123.0]
r	-0.103	-0.099	-0.123	-0.100	-0.144
p-value	<0.01	0.01	0.06	<0.01	0.08
Markers of angiogenesis[4]					
Microvessel density					
N	414	275	139	332	82
median [Q1, Q3]	67.1 [55.0,95.0]	65.3 [53.0,92.5]	74.3 [58.0,100.0]	66.6 [52.9,93.0]	75.5 [59.0,102.7]
r	0.162	0.165	0.168	0.167	0.011
p-value	<0.01	<0.01	0.05	<0.01	0.93
Vessel area					
N	415	276	139	332	83
median [Q1, Q3]	466.5 [357.7,654.7]	486.5 [370.5,664.4]	430.2 [304.6,648.7]	485.0 [371.9,671.6]	420.0 [301.3,567.4]
r	-0.168	-0.165	-0.198	-0.147	-0.150
p-value	<0.01	<0.01	0.02	<0.01	0.19
Vessel diameter					
N	415	276	139	332	83
median [Q1, Q3]	24.2 [21.4,27.8]	24.4 [21.9,27.7]	23.3 [20.3,27.9]	24.5 [21.8,28.3]	22.6 [19.8,26.2]
r	-0.141	-0.130	-0.192	-0.120	-0.119
p-value	<0.01	0.03	0.03	0.03	0.30
Vessel irregularity[5]					
N	415	276	139	332	83
median [Q1, Q3]	4.0 [3.2,4.8]	3.9 [3.1,4.7]	4.1 [3.3,4.9]	3.9 [3.2,4.7]	4.1 [3.4,5.1]
r	0.100	0.026	0.250	0.057	0.124
p-value	0.04	0.68	<0.01	0.31	0.28

ANCOVA [3]					
ERG expression					
Absent, N	434	322	111	348	86
Adjusted mean PSMA	64.2	67.5	64.8	59.0	72.6
Present, N	446	301	144	366	80
Adjusted mean PSMA	49.3	51.7	50.3	44.0	59.1
p-value	<0.01	<0.01	<0.01	<0.01	0.02

ANCOVA, analysis of covariance; AR, androgen receptor; ERG, ets-related gene; PSMA, prostate-specific membrane antigen; VDR, vitamin D receptor.

[1]Tumor stage T1-T2, N0-Nx, M0-Mx.

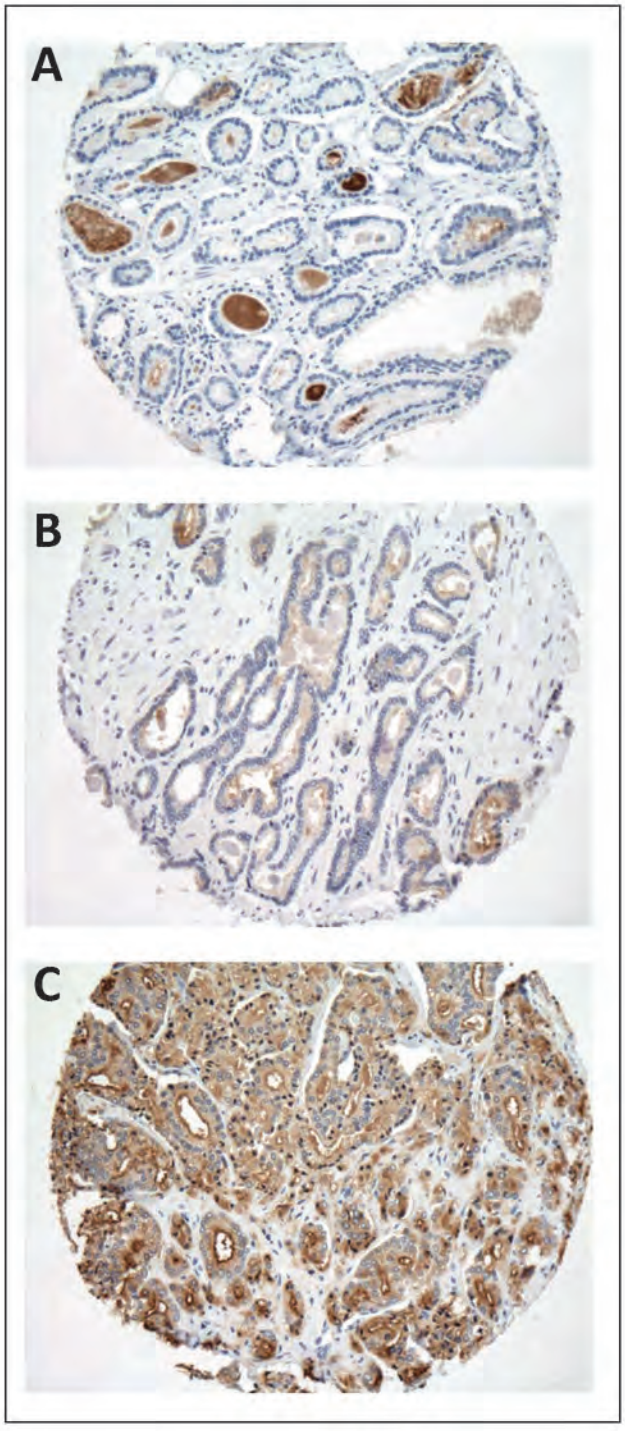
[2]Tumor stage T3-T4 or N1 or M1.

[3]Adjusted for age at diagnosis and tissue microarray.

[4]Measured in HPFS cohort only.

[5]Higher score indicates more irregularity.

Fig 1. Representative images of prostate specific membrane antigen (PSMA) protein expression in selected prostate tumor tissue microarray cores from the Health Professionals Follow-Up Study: (A) Weak staining in a patient with Gleason score 3+3 tumor; (B) moderate staining in a patient with Gleason score 3+4 tumor; and (C) strong staining in a patient with Gleason score 4+4 tumor. Images were taken at x20 magnification. The prostate tumor glands showed cytoplasmic and membranous staining.



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SUPPORTING DATA

Table 1: Characteristics of prostate cancer patients on tissue microarrays for multi-focal prostate cancer

Distribution of patients with 1-4 total tumor foci in radical prostatectomy specimens, N (%)	
1	153 (67%)
2	59 (26%)
3	14 (6%)
4	2 (< 1%)
Gleason score in across tumor foci, mean [range]	
Primary focus	6.9 [6, 9]
Secondary focus	6.7 [5, 9]
Tertiary focus	6.7 [6, 9]
Quaternary focus	7.0 [6. 8]