Published in final edited form as:
Hum Mutat. 2013 January ; 34(1): 149–156. doi:10.1002/humu.22176.

Investigation of the relationship between prostate cancer and *MSMB* and *NCOA4* genetic variants and protein expression

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Abstract

Two genome-wide association studies (GWAS) identified the *β-microseminoprotein* (*MSMB*) promoter SNP, rs10993994:C>T, as significantly associated with prostate cancer (PC) risk. Follow-up studies demonstrate that the variant allele directly affects expression of the *MSMB* encoded protein, PSP94, and also suggest that it affects mRNA expression levels of an adjacent gene, *NCOA4*, which is involved in androgen receptor transactivation. In a population-based study of 1,323 cases and 1,268 age-matched controls, we found the *NCOA4* SNP, rs7350420:T>C, was associated with a 15% reduction in PC risk, but the association was not significant after adjustment for the rs10993994:C>T genotype. Tumor tissue microarrays of 519 radical prostatectomy patients were used to measure PSP94 and NCOA4 protein expression. Taken together, these data confirm that the rs10993994:C>T variant allele is associated with decreased PSP94 expression, and the association is stronger in tumor compared to normal prostate tissue. No association was observed between rs10993994:C>T and NCOA4 expression, and only moderate associations were seen between two *NCOA4* SNPs, rs10761618:T>C and rs7085433:G>A, and NCOA4 protein expression. These data indicate that the increase in PC risk associated with rs10993994:C>T is likely mediated by the variant's effect on PSP94 expression; however this effect does not extend to NCOA4 in the data presented here.

Keywords

Prostate cancer; MSMB; PSP94; NCOA4; protein expression; genetic variants

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INTRODUCTION

In the past few years, over 40 prostate cancer (PC) risk loci have been identified through genome-wide association studies (GWAS), accounting for up to 25% of the hereditary component of this disease [Kote-Jarai et al., 2011]. One of the largest effect sizes, about a 25% per allele increase in the relative risk of PC, is associated with a variant on 10q11, denoted by rs10993994:C>T [Eeles et al., 2008; Thomas et al., 2008]. The SNP rs10993994:C>T lies in the proximal promoter region (−59 bp) of the *beta-microseminoprotein* gene (*MSMB*; MIM# 157145), which encodes for the prostatic secretory protein of 94 amino acids (PSP94). PSP94 is one of the major proteins secreted by the human prostate [Lilja and Abrahamsson, 1988] and both mRNA and protein expression are observed in varying levels in healthy pubertal and adult prostate tissue [Chan et al., 1999]. However, mRNA levels have been reported to decrease in both prostatic intraepithelial neoplasia (PIN) and prostate tumor tissue [Chan et al., 1999], and in a study of PC3 xenografts, cell growth was inhibited in a dose- and time-dependent manner by PSP94 [Garde et al., 1999]. Recently it was reported that total PSP94 protein levels in serum significantly predicted the presence of prostate cancer; also, in patients classified as low risk (PSA ≤20.0 ng/mL, normal DRE, and <70 years of age), PSP94 levels in serum distinguished between patients with higher-grade (Gleason score >7) and higher-stage (metastases) disease [Nam et al., 2006].

To determine if rs10993994:C>T directly affects PSP94 expression or is in linkage disequilibrium (LD) with a causal variant, two independent studies fine-mapped ~65kb of the surrounding region with tagSNPs [Chang et al., 2009; Lou et al., 2009]. Both studies confirmed rs10993994:C>T as being the most likely functional variant and demonstrated in reporter assays that the promoter activity of the risk-associated allele was significantly less than that of the wild-type allele. Lou et al (2009) further demonstrated that the risk allele disrupts CREB transcription factor binding and that mRNA expression levels of PSP94 were significantly lower in cell lines carrying the risk allele [Lou et al., 2009]. Of note, Chang and colleagues extended their fine-mapping across an adjacent gene *nuclear receptor coactivator 4* (*NCOA4*; MIM# 601984) and identified a second locus, rs10761581:T>G, to be independently associated with risk [Chang et al., 2009]. However, the association was only weakly confirmed in one of three independent study populations and the *NCOA4* risk allele had no effect on *MSMB* promoter activity. A more recent study investigating rs10993994:C>T in relation to RNA expression of PSP94 and NCOA4 isoforms, found that the *MSMB* risk allele was associated with reduced expression of PSP94 in both normal prostate and prostate tumor tissue [Pomerantz et al., 2010]. While the rs10993994:C>T risk allele was associated with increased mRNA expression of NCOA4 isoforms in normal prostate, there was no correlation with expression in prostate tumor tissue.

To evaluate the above findings in the context of a population-based study, we examined the association of *MSMB* and *NCOA4* genetic variants with risk of PC. The association between *MSMB* and *NCOA4* genetic variants and protein expression of these genes in normal prostate and prostate tumor tissue was also investigated.

MATERIALS AND METHODS

Study Subjects and Tumor Tissue

Study participants were incident PC cases and age-matched controls without a history of PC, all of whom were residents in King County, Washington. Patients were identified via the Seattle-Puget Sound SEER Cancer Registry and included men aged 35 to 74 years who were diagnosed with histologically confirmed PC during one of two 4-year ascertainment periods, 1993–1996 or 2002–2005. Controls were identified through random digit dialing [Agalliu et

al., 2008; Stanford et al., 1999]. The SEER registry provided information on Gleason score, stage of cancer, diagnostic PSA level and primary therapy. Vital status and underlying cause of death were also ascertained through the SEER cancer registry and collection of death certificates; November 30th, 2011 was the most recent vital status update. All participants signed informed consent and the study was approved by the Fred Hutchinson Cancer Research Center Institutional Review Board. Due to sample size and differences in minor allele frequencies (MAF), only Caucasian men were included in this investigation. A total of 1,323 cases and 1,268 controls had constitutional DNA available for analyses. At the time of interview, cases were also asked for consent to obtain copies of pathology reports and tumor tissue. For this study, formalin-fixed, paraffin-embedded prostate tumor tissue blocks were retrieved for 519 Caucasian cases, of which 473 (91%) had constitutional DNA available (Table 1).

SNP Genotyping

The Genome Variation Server (http://gvs.gs.washington.edu/GVS/) was used to identify seven SNPs tagging the genetic variation across *NCOA4* (Table 2). All genotype information has been submitted to the Leiden Open Variation Database (LOVD; www.LOVD.nl/MSMB and www.LOVD.nl/NCOA4). The NCOA4 tagSNPs and *MSMB* SNP, rs10993994:C>T, were genotyped in DNA isolated from peripheral blood samples using standard methods. The Applied Biosystems SNPlex™ Genotyping System (Applied Biosystems, Inc) was used for genotyping and proprietary GeneMapperR software® was used for allele calling (http://www.appliedbiosystems.com). Discrimination of the specific SNP allele was carried out on the ABI 3730*xl* DNA Analyzer and is based on the presence of a unique sequence assigned to the original allele-specific oligonucleotide. Quality control included genotyping of 59 blind duplicate samples distributed across all genotyping batches. There was 100% agreement between the blinded samples for each of the SNPs. Each 384-well batch of DNA aliquots genotyped incorporated similar numbers of case and control samples, and all laboratory personnel were blinded to the case-control status of samples.

Tissue Microarrays and Immunohistochemistry

H&E stained slides were reviewed for each patient by an experienced pathologist and areas of tumor were marked on both the slide and corresponding paraffin block. Two samples per patient were macro-dissected (at least 70% tumor) from a single tumor focus from 519 radical prostatectomy specimens. Core tissue specimens with a diameter of 1.0 mm were taken from the preselected region of the donor block and precisely arrayed into a new recipient paraffin block with a manual tissue arrayer (Beecher Instruments).

Immunohistochemistry was performed by the FHCRC Experimental Histopathology Laboratory. Four-micron TMA sections were cut, deparaffinized and rehydrated in dH_2O. PSP94 slides were antigen retrieved for 20 min in a Black and Decker steamer in preheated 6.0 pH Citrate Buffer (Dako) and cooled for 20 min at room temperature (RT). Slides were rinsed in wash buffer and all subsequent staining steps were performed at RT using the Dako Autostainer (Dako). Endogenous peroxide activity was blocked using 3% H_2O_2 for eight min and slides were then blocked in Tween, Cassin and TBS Buffer containing 1% BSA for 10 min. PSP94 antibody (ab49952; Abcam) was diluted to 10 μg/ml, incubated on the tissue for 30 min, followed by 30 min incubation with Mach 2 anti-mouse HRP-labeled polymer (MHRP520L; Biocare Medical). NCOA4 slides were antigen retrieved in a Russell Hobbs pressure cooker for 10 min in Trilogy (Cell Marque) and cooled for 20 min at RT. Slides were rinsed in wash buffer and all subsequent staining steps were performed at RT using the Dako Autostainer (Dako). Endogenous peroxide activity was blocked for eight min using 3% H_2O_2 followed by avidin/biotin blocking (Biocare). Slides were blocked in 15% horse serum and 5% human serum in TBS containing 1% BSA for 10 min. NCOA4 antibody

(H00008031-M03; Novus Biologicals) was diluted to 60 μg/ml, incubated on the tissue for 60 min and then rinsed with wash buffer. Antibody staining was detected using biotinylated horse anti-mouse (Jasckson ImmunoResearch) prepared at 1:200 dilution, for 30 min followed by HRP- labeled strep-avidin (Jackson ImmunoResearch) prepared at 1:2000, for 30 min. PSP94 and NCOA4 staining was visualized with 3,3′-diaminobenzidine (DAB; Dako) for eight minutes, and the sections were counter-stained with hematoxylin (Dako) for two minutes. Concentration matched isotype control slides were run for each tissue sample (Jackson ImmunoResearch Laboratories). The stained slides were scanned on the automated Tissuefax microscope (TissueGnostics). Resulting images were viewed by the pathologist (X.Z.) via an online web gallery.

Immunohistochemistry Evaluation

TMA tissue staining was scored by a pathologist blinded to clinical parameters. A number of cores in the TMAs were not able to be scored due to missing tissue cores, uncertain presence of cancer, or insufficient cancer cells. These cores were excluded from the results. There was excellent intra-observer concordance between 5% of cores that were randomly selected for rescoring by the study pathologist (X.Z.). Immunostaining was assessed using a quasi-continuous nuclear score, created by multiplying each intensity level (0 for no stain, 1 for weak stain, and 2 for intense stain) by the corresponding percentage of positive cells, and then summing the results. Each case was duplicated on the TMA and the average score was taken for each case. For statistical analyses, this score was then divided into three categories: <0.25 = no/weak staining; ≥0.25 – <1.25 = moderate staining; and ≥1.25 = intense staining. As benign prostate tissue was also present in some cores, immunostaining was assessed for both malignant and benign prostate epithelial cells separately.

Statistical Methods

Departure from Hardy-Weinberg equilibrium ($p<0.01$) was assessed for each SNP in controls. Linkage disequilibrium between SNP markers was calculated in our dataset using Haploview (www.broad.mit.edu/mpg/haploview/). Unconditional logistic regression was used to generate odds ratios (ORs) and 95% confidence intervals (95% CIs) to measure the association between individual SNP genotypes and prostate cancer risk. Potential confounding factors, including age at reference date, PC screening history and first-degree family history of PC, were examined to see if such factors changed the risk estimates by ≥ 10%. After these analyses, only age at reference date was included in the final models. Polytomous regression models adjusted for age were used to test a log-additive genetic model of SNP genotypes for cases stratified by Gleason score [≤7 (3+4) *vs.* ≥ 7 (4+3)], tumor stage (local *vs.* regional/distant), and diagnostic PSA level [low (<10ng/mL) *vs.* high (≥10ng/mL)] compared to controls. To test for significant differences in risk estimates, a Wald chi-square test was used ($p_{heterogeneity}$). The primary endpoint for the survival analyses was time to death from PC. Survival time, i.e., time elapsed from diagnosis until death, was the time dependent variable used. For deceased cases, a death certificate was obtained to confirm the underlying cause of death (PC or other). Cases who died of other causes were censored at time of death, and living cases were censored as of November 30th, 2011. The association between survival and SNP genotype was evaluated using the Cox's proportional hazard model [Cox, 1972] to estimate hazard ratios (HR) and 95% confidence intervals (CIs). Final survival analysis models were adjusted for age at diagnosis, Gleason score, stage, primary treatment and diagnostic PSA value.

Differences in the distributions of cell type, clinical features of disease, and frequencies of genotypes of SNPs between PSP94 and NCOA4 protein expression categories (no/weak, moderate or intense) were evaluated using the Fisher's Exact test as some categorical

distributions had an expected frequency of five or less. SAS version 9.1.3 was used for all statistical analyses.

RESULTS

MSMB and NCOA4 Genetic Associations

The *MSMB* SNP, rs10993994:C>T, and seven *NCOA4* tagSNPs were genotyped in our population-based dataset of 1,323 cases and 1,268 controls (Table 1). The *MSMB* SNP, rs10993994:C>T, was previously shown to be associated with an increased risk of PC (OR=1.31) and to be correlated with lower Gleason scores [FitzGerald et al., 2009]. Of the remaining seven SNPs, the *NCOA4* tagSNP, rs7350420:T>C, was also found to be significantly associated with disease risk (OR=0.85; 95% CI 0.75 0.95; $p=5.6\times10^{-3}$; Table 2), after multiple testing was taken into account. In addition, the minor allele of rs7350420:T>C was associated with a reduced risk of lower Gleason grade disease (OR=0.84; 95% CI 0.74 – 0.95; $p=6.0\times10^{-3}$) but not higher Gleason grade disease (OR=0.87; 95% CI 0.69 – 1.10; p=0.24); however this difference was not significant ($p_{heterogeneity}=0.77$). The minor allele of rs10993994:C>T was associated with both lower (OR=1.25; 95% CI 1.10 – 1.42; $p=5.4\times10^{-4}$) and higher (OR=1.56; 95% CI 1.30 – 1.88; $p=1.99\times10^{-6}$) diagnostic PSA levels, but the association with higher PSA levels was stronger ($p_{heterogeneity}=0.02$). The rs7350420:T>C minor allele was also shown to be more strongly associated with higher (OR=0.77; 95% CI 0.63 – 0.94; p=0.009) than with lower PSA levels (OR=0.88; 95% CI 0.77 – 1.01; p=0.06), however the difference between the two groups was not significant ($p_{heterogeneity}=0.19$). No associations were seen with either SNP and disease stage or prostate cancer-specific mortality (data not shown).

We next tested for independence of effects by including both rs10993994:C>T, *MSMB*, and rs7350420:T>C, *NCOA4*, in the risk model, despite little evidence for linkage disequilibrium between the two SNPs in the 1000 Genomes CEU population ($r^2=0.02$; www.1000genomes.org/) or in our own dataset ($r^2=0.07$). The association between the variant rs7350420:T>C allele and PC risk was not significant (OR=0.91; 95% CI 0.80 1.03; p=0.13) after adjustment for rs10993994:C>T, which remained significantly associated with risk (OR=1.29; 95% CI 1.15 – 1.45; $p=3.0\times10^{-5}$). This was also the case when adjusting for rs10993994:C>T in the analyses of clinicopathological covariates; rs7350420:T>C no longer remained significantly associated with low Gleason grade (p=0.17) or higher PSA levels (p=0.21), while the associations between rs10993994:C>T and these clinical features remained significant, albeit slightly diminished.

PSP94 and NCOA4 Protein Expression

From the original 519 TMA samples, successful PSP94 and NCOA4 protein staining was available for 475 and 468 cases, respectively. Data for PSP94 and NCOA4 protein staining of normal prostate tissue was also available for 395 and 369 cases, respectively. A significant difference was observed in PSP94 protein expression between tumor and normal tissue ($p<0.0001$), where expression was predominantly absent or weak in tumor tissue, but over 60% of normal tissue samples showed moderate to intense staining (Table 3; Figure 1). NCOA4 expression levels did not differ substantially between tumor and normal tissues. In fact, intense staining was observed in the majority of both tumor and normal prostate tissue samples (p=0.14; Figure 1). There were no significant differences in either PSP94 and NCOA4 protein expression in tumor or normal tissue when cases were stratified by Gleason score or stage of disease (Table 3). There were also no significant differences in PSP94 expression when stratified by diagnostic PSA levels, however significant differences were seen in NCOA4 protein expression. In those cases with higher versus lower PSA levels,

there was a significantly greater proportion of cases that had no or weak NCOA4 staining in both tumor (p=0.01) and normal tissue (p=0.007).

Associations Between MSMB and NCOA4 SNP Genotypes and Protein Expression

There were 398 cases with both genotyping and tumor tissue PSP94 protein expression data, and 331 cases with both genotyping and normal tissue PSP94 protein expression data. Three SNPs, rs10993994:C>T (*MSMB*), rs10761581:T>G and rs7350420:T>C (both in *NCOA4*), were significantly associated with PSP94 protein expression (Table 4). The rs10993994:C>T minor allele was associated with no to weak staining in both tumor ($p=1.32\times10^{-5}$) and normal tissue (p=0.002). The rs10761581:T>G minor allele was also associated with no to weak PSP94 staining but only in tumor tissue (p=0.04). Conversely, the rs7350420:T>C minor allele was associated with an increase in intense PSP94 staining in normal tissue only (p=0.03). NCOA4 genotyping and protein expression data were available for 391 cases with tumor tissue and 308 cases with normal tissue. Two *NCOA4* SNPs, rs10761618:T>C and rs7085433:G>A, were significantly associated with NCOA4 protein expression (Table 5). The rs10761618:T>C minor allele was associated with a reduction in NCOA4 staining from intense to moderate (p=0.02) in normal tissue. Finally, an association was observed in tumor tissue where all men homozygous for the rs7085433:G>A minor allele had intense NCOA4 protein expression (p=0.04).

DISCUSSION

Through recent GWAS, resequencing and functional studies, the 10q11 locus has been shown to contain at least one gene, *MSMB* [Chang et al., 2009; Eeles et al., 2008; Lou et al., 2009; Pomerantz et al., 2010; Thomas et al., 2008], associated with PC risk. One of these studies provided additional evidence suggesting that the 10q11 risk allele is also associated with NCOA4 mRNA expression levels in normal prostate tissue [Pomerantz et al., 2010]. Here we explore this locus further, incorporating the *NCOA4* gene into our genetic analyses of 1,323 PC cases and 1,268 controls, and protein expression analyses of a TMA series of 519 patients. We have previously shown that the rs10993994:C>T variant allele is associated with PC risk in our study population [FitzGerald et al., 2009], but had not investigated the relationship between *NCOA4* variants and disease risk. A previous study presented evidence that the *NCOA4* SNP, rs10761581:T>G, was associated with PC, however the association was not consistent across replication studies [Chang et al., 2009] and we found no evidence for an association in our data. However, we did find an association between the *NCOA4* 3′ SNP, rs7350420:T>C, and PC risk, where the variant allele is associated with a 15% decrease in the relative risk. When looking at clinicopathological features of disease, similar to rs10993994:C>T, the rs7350420:T>C variant allele was also more strongly associated with low Gleason grade disease and higher diagnostic PSA levels. While there is no evidence for linkage disequilibrium between these two SNPs from the 1000 Genomes CEU population or our own dataset and evidence for a recombination hotspot between these loci has been presented [Chang et al., 2009], the similarity of association results prompted us to test for independence of effects for the two SNPs. After adjusting for rs10993994:C>T, rs7350420:T>C no longer remained significantly associated with disease risk, low Gleason grade disease, or PSA levels. However, the odds ratios were not greatly changed and the confidence intervals only just encompassed one, suggesting lack of statistical power in the adjusted analyses. Thus, while the data presented here suggest that at the germline level, genetic risk and clinical features of PC related to the 10q11 locus is primarily due to the *MSMB* variant allele of rs10993994:C>T, rs7350420:T>C should be further investigated in larger PC datasets.

The decrease in PSP94 mRNA and protein expression in prostate tumor compared to benign tissue is well documented [Chan et al., 1999; Girvan et al., 2005; Imasato et al., 2000;

Pomerantz et al., 2010]. However, only recently has this decrease in expression been associated with the *MSMB* promoter SNP, rs10993994:C>T [Chang et al., 2009; Pomerantz et al., 2010]. Pomerantz and colleagues (2010) observed an association of the variant allele, and not the reference allele, with decreases in mRNA levels in both tumor and normal prostate tissue, although the association was not as strong in the tumor tissue. Chang and colleagues also observed that the *MSMB* promoter activity was greatly reduced (13%) when the variant allele was present in LNCaP cells [Chang et al., 2009]. We also observed a significant decrease in PSP94 protein expression in tumor compared to normal tissue and the decrease in expression was associated with the rs10993994:C>T variant allele. Unlike Pomerantz and colleagues, we found the decrease in PSP94 expression was more strongly associated with the variant allele in tumor tissue than in normal tissue. This difference in findings may be explained by the fact that the former study measured mRNA levels while we measured protein levels: post-translational modifications may alter the final amount and ratio of protein present in the two tissue types. The stronger decrease in PSP94 expression in tumor compared to normal tissue is also consistent with its suggested function. PSP94 has been shown to inhibit the activity of MMP-9 and CD44, both of which play crucial roles in cancer cell adhesion to the extracellular matrix in the primary tumor and at several stages in the metastatic pathway [Annabi et al., 2005]. Removing the inhibitory effect of PSP94 in the tumor tissue may increase the activity of both MMP-9 and CD44, and facilitate tumor progression and metastasis. It is interesting to note that we did not observe a difference in PSP94 expression levels in low versus high Gleason grade tumors or local compared to regional/distant stage disease. Two additional SNPs were also associated with PSP94 expression levels: rs10761581:T>G in tumour tissue and rs7350420:T>C in normal tissue. The association between genotype and staining was not as striking for these two SNPs, reflected by the less significant p-values and, therefore, suggests that the rs10993994:C>T promoter SNP primarily determines PSP94 levels in both normal prostate and tumor tissue.

The majority of early NCOA4 expression studies observed a decrease in mRNA transcript levels in prostate cancer compared to benign tissue [Li et al., 2002; Mestayer et al., 2003; Tekur et al., 2001], however, one study noted an increase in expression in tumor compared to benign tissue [Hu et al., 2004]. We found no difference in protein expression levels between normal and cancerous tissue: there was intense NCOA4 staining in both cell types. In 1999, Alen and colleagues discovered that two NCOA4 isoforms existed, the full length alpha isoform and the internally spliced beta isoform [Alen et al., 1999]. Peng and collegues looked specifically at the expression of these two different isoforms and found that while NCOA4 alpha expression is lower in PC, the beta isoform was expressed at higher levels in tumor compared to normal cells [Peng et al., 2008]. Our study, like most previous NCOA4 expression studies, used an antibody that binds amino acids ~505–615 and as the beta transcript is missing amino acids 239–566, there therefore may be preferential binding to the alpha isoform. However, as we found no difference in NCOA4 expression levels in tumor compared to normal cells, it may be that our antibody was, in fact, also binding the beta isoform [Hu et al., 2004]. The resulting observation of no change in NCOA4 expression in the tumor cells may therefore be due to a balancing effect of loss of one isoform and gain of the other. In another recent study, Pomerantz and colleagues (2010) looked at five NCOA4 isoforms and found all five mRNA transcripts were expressed at slightly lower levels in tumor compared to normal cells, including the beta isoform [Pomerantz et al., 2010]. Notable differences in our study and those of Peng *et al* (2008) and Pomerantz *et al* (2010) include the fact that we and Peng and colleagues were measuring protein expression while Pomerantz and colleagues measured mRNA transcript levels, which may undergo further post-translational modification and not reflect the final expression pattern or levels. Another major difference in these three studies is the number of patients studied, whereas we had 468 patients with protein expression data, Peng and colleagues studied 50 patients and Pomerantz and colleagues studied 61 patients. While antibodies to all NCOA4 isoforms

were not available at the time the current study was being conducted, an investigation of the individual alpha and beta NCOA4 isoforms in our larger dataset may provide greater insight into whether these isoforms are differentially expressed in normal compared to tumor cells.

When looking at NCOA4 expression and clinical features of disease, we observed an association between expression levels in both tumor and normal cells and diagnostic PSA levels. In cases with higher PSA levels (≥10ng/mL), there was a decrease in protein expression levels from intense to none/weak. NCOA4 is known to have a role in androgen receptor (AR) transactivation in hormone-refractory prostate cancer [Niu et al., 2008]. NCOA4 forms a complex with PSA and AR, which acts to increase AR transactivation and results in decreased apoptosis and increased cell growth. A positive feedback loop is also activated whereby AR transactivation results in an increase in PSA protein levels. The inverse association between PSA levels and NCOA4 expression observed here is a novel and unexpected finding. One simple explanation for this result is that we are using a measure of PSA in the serum and not the prostate tissue. While PSA levels may increase due to the positive feedback loop, this may not be reflected in serum levels due to PSA being bound to the AR-NCOA4 complex. To understand our novel finding better, the relationship between NCOA4 and PSA requires further study.

Finally, we investigated whether NCOA4 expression levels in normal and tumor cells were associated with *MSMB* and *NCOA4* genetic variants. In normal cells, a decrease in expression from intense to moderate levels was associated with the variant allele of rs10761618:T>C, while in tumor cells an increase in expression of NCOA4 was associated with the variant allele of rs7085433:G>A. After adjusting for multiple testing, neither of these associations remained significant. These results are similar to those of Ligr and colleagues (2010). Through sequence analysis of the NCOA4 alpha transcript, that study identified four previously recognized common SNPs and two novel variants that were only present in a small number of cases; they therefore concluded that these variants were unlikely to have a role in NCOA4 alpha expression levels [Ligr et al., 2010]. Nonetheless, the associations with two variants observed in our study should be investigated further with respect to the distinct alpha and beta isoforms.

In summary, our data demonstrate that the *MSMB* promoter variant, rs10993994:C>T, is significantly associated with PSP94 expression levels in both normal prostate and tumor tissue. However, unlike a previous study, this effect was greater in tumor compared to normal tissue, which may reflect the role PSP94 plays in tumor progression and metastasis. In terms of NCOA4 expression levels, there was no difference between normal and tumor prostate cells, nor any significant associations with selected *MSMB* and *NCOA4* genetic variants; although these results may be due to our inability to differentiate between the alpha and beta protein isoforms and thus warrant further investigation.

Acknowledgments

This work was partially supported by grants RO1 CA056678 (J.L.S), R01 CA092579 (J.L.S), R03 CA121871 (J.L.S), R03 CA137799 (J.L.S), and P50 CA097186 (J.L.S & L.M.F) from the National Cancer Institute, National Institutes of Health. Additional support was provided by the Fred Hutchinson Cancer Research Center and the Intramural Program of the National Human Genome Research Institute. We thank all the men who participated in this study for their time, effort and cooperation. Thank you also to the Experimental Histopathology Laboratory, Fred Hutchinson Cancer Research Center, for their immunohistochemistry help, and to Brian T. Cox for bioinformatics support and programming.

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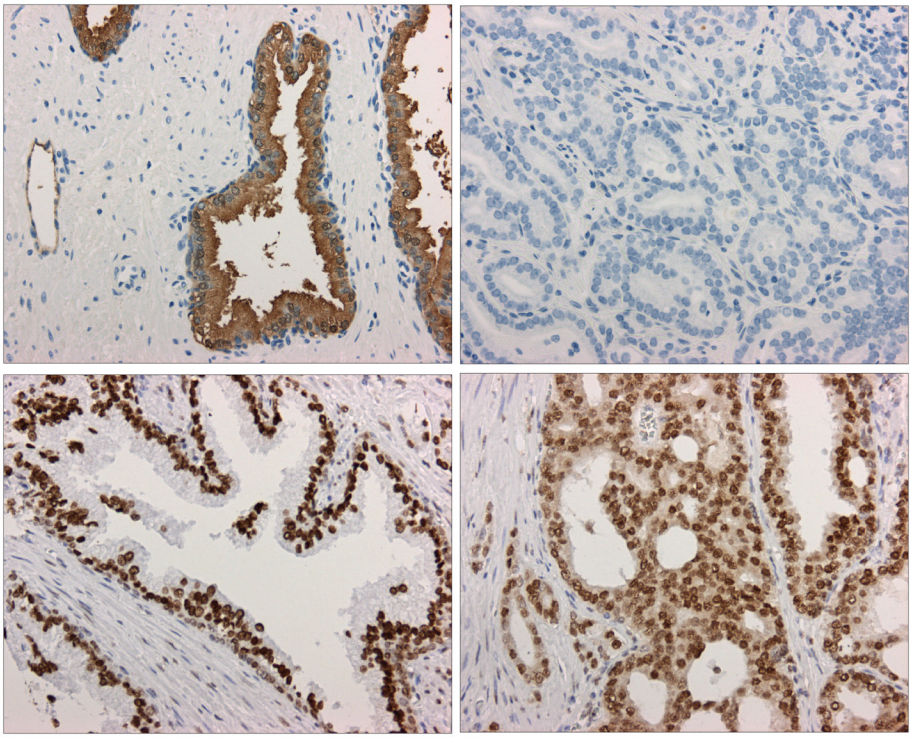

Figure 1.
PSP94 staining at 200x in normal prostate (top left) and tumor tissue (top right). NCOA4 staining at 200x in normal prostate (bottom left) and tumor tissue (bottom right).

Table 1

Characteristics of the study population

Characteristics	Cases	Controls	Cases on TMAs
	n = 1,323	**n = 1,268**	**n = 519**
Age at reference date			
Mean (±SD)	59.9 (7.0)	59.6 (7.2)	58.2 (7.0)
Range	35 – 74	40 – 74	35 – 74
Gleason score (%)			
2 – 7 (3+4)	1,115 (84.3)		440 (84.8)
7 (4+3) – 10	204 (15.4)		79 (15.2)
Missing	4 (0.3)		
Stage (%)			
Local	1,029 (77.8)		361 (69.6)
Regional/Distant	294 (22.2)		158 (30.4)[c]
PSA value at diagnosis (ng/ml) (%)			
0 – 9.9	907 (68.6)		385 (74.2)
≥ 10.0	312 (23.6)		102 (19.6)
Missing	104 (7.8)		32 (6.2)
Primary treatment			
Radical Prostatectomy	780 (59.0)		519 (100.0)
RAD[a] +/− ADT[b]	361 (27.3)		
ADT	62 (4.7)		
Other treatment	4 (0.3)		
Active surveillance	116 (8.7)		
Vital status (%)			
Alive	1,092 (82.5)		471 (90.7)
Deceased	231 (17.5)		48 (9.3)
Prostate cancer-specific death	81 (35.1)		12 (25.0)
Other cause of death	150 (64.9)		36 (75.0)

[a]Radiation;

[b]Androgen Deprivation Therapy;

[c]Regional stage only.

Table 2

Genotype distributions and relative risk estimates for prostate cancer according to *MSMB* and *NCOA4* SNP genotypes

		Case genotype counts (%) (N=1,323)			Control genotype counts (%)(N=1,268)						
SNP[a]	**Position[b]**	**Hom wt[c]**	**Het[d]**	**Hom var [e]**	**Hom wt [c]**	**Het[d]**	**Hom var [e]**	**MAF Ca/Co[f]**	**OR[g]**	**95% CI**	**p-value**
MSMB											
rs10993994:C>T	51,549,496	382 (30.4)	633 (50.4)	242 (19.2)	472 (37.7)	608 (48.5)	173 (13.8)	0.44/0.38	**1.31**	**1.17–1.47**	**3.5×10^{-6}**
NCOA4 tagSNPs											
rs17178655:G>A	51,561,799	718 (56.4)	473 (37.2)	81 (6.4)	746 (60.4)	428 (34.7)	60 (4.9)	0.25/0.22	1.17	1.02–1.33	0.02
rs10994675:G>A	51,563,993	384 (30.7)	609 (48.6)	260 (20.7)	415 (33.8)	579 (47.1)	235 (19.1)	0.45/0.43	1.10	0.98–1.22	0.10
rs10761581:T>G	51,568,378	422 (32.5)	651 (50.1)	227 (17.4)	390 (31.1)	640 (51.1)	223 (17.8)	0.43/0.43	0.96	0.86–1.08	0.51
rs10761618:T>C	51,574,606	614 (48.7)	537 (42.6)	109 (8.7)	610 (49.7)	511 (41.6)	107 (8.7)	0.30/0.30	1.02	0.90–1.15	0.75
rs11592181:G>A	51,582,686	977 (76.6)	275 (21.5)	24 (1.9)	921 (74.4)	289 (23.3)	28 (2.3)	0.13/0.14	0.89	0.76–1.05	0.17
rs7085433:G>A	51,593,354	969 (76.4)	280 (22.1)	19 (1.5)	971 (79.3)	236 (19.3)	18 (1.4)	0.13/0.11	1.15	0.96–1.36	0.12
rs7350420:T>C	51,594,462	606 (48.1)	533 (42.4)	120 (9.5)	531 (43.0)	555 (45.0)	148 (12.0)	0.31/0.34	**0.85**	**0.75–0.95**	**5.6×10^{-3}**

[a] Major/Minor allele;

[b] UCSC hg19 assembly, build GRCh37;

[c] Homozygous wildtype;

[d] Heterozygous;

[e] Homozygous variant;

[f] Case/control;

[g] Log-additive model adjusted for age; Number of samples in each analysis varies due to missing data.

Table 3

PSP94 and NCOA4 expression in tumor and normal prostate tissue from radical prostatectomy specimens

	Tumor Tissue Staining				Normal Tissue Staining			
	No/Weak	**Moderate**	**Intense**	**p-value**	**No/Weak**	**Moderate**	**Intense**	**p-value**
PSP94	388 (81.7)	55 (11.6)	32 (6.7)		144 (36.5)	83 (21.0)	168 (42.5)	**<0.0001**
Gleason score								
Low [≤7(3+4)]	325 (81.0)	48 (12.0)	28 (7.0)		120 (35.0)	73 (21.3)	150 (43.7)	
High [≥7(4+3)]	63 (85.1)	7 (9.5)	4 (5.4)	0.80	24 (46.2)	10 (19.2)	18 (34.6)	0.30
Stage								
Local	264 (81.5)	41 (12.6)	19 (5.9)		110 (37.6)	64 (21.8)	119 (40.6)	
Regional	124 (82.1)	14 (9.3)	13 (8.6)	0.34	34 (33.3)	19 (18.6)	49 (48.1)	0.44
PSA								
Low	286 (81.5)	43 (12.2)	22 (6.3)		113 (36.9)	65 (21.2)	128 (41.8)	
High	79 (83.2)	8 (8.4)	8 (8.4)	0.49	22 (35.5)	12 (19.3)	28 (45.2)	0.89
NCOA4	23 (4.9)	37 (7.9)	408 (87.2)		21 (5.7)	17 (4.6)	331 (89.7)	0.14
Gleason								
Low	20 (5.1)	32 (8.1)	342 (86.8)		18 (5.7)	14 (4.4)	286 (89.9)	
High	3 (4.1)	5 (6.8)	66 (89.2)	0.96	3 (5.9)	3 (5.9)	45 (88.2)	0.86
Stage								
Local	16 (5.0)	23 (7.2)	280 (87.8)		14 (5.0)	15 (5.4)	249 (89.6)	
Regional	7 (4.7)	14 (9.4)	128 (85.9)	0.69	7 (7.7)	2 (2.2)	82 (90.1)	0.33
PSA								
Low	10 (2.9)	26 (7.4)	313 (89.7)		8 (2.8)	13 (4.5)	267 (92.7)	
High	9 (9.8)	9 (9.8)	74 (80.4)	**0.01**	7 (12.5)	3 (5.4)	46 (82.1)	**0.007**

Number of samples in each analysis varies due to missing data.

Table 4

Associations between tumor and normal prostate PSP94 expression levels and *MSMB* and *NCOA4* SNP genotypes

	Tumor Tissue Staining				Normal Tissue Staining			
	No/Weak	**Moderate**	**Intense**	**p-value**	**No/Weak**	**Moderate**	**Intense**	**p-value**
MSMB								
rs10993994								
CC	88 (71.0)	25 (20.2)	11 (8.8)		31 (27.7)	21 (18.7)	60 (53.6)	
CT	149 (82.3)	20 (11.1)	12 (6.6)		62 (40.8)	27 (17.8)	63 (41.4)	
TT	80 (97.6)	2 (2.4)	0 (0.0)	**1.32×10^{-5}**	27 (47.4)	17 (29.8)	13 (22.8)	**0.002**
rs17178655								
GG	188 (83.9)	26 (11.6)	10 (4.5)		63 (34.8)	42 (23.2)	76 (42.0)	
GA	108 (80.0)	16 (11.8)	11 (8.2)		46 (39.0)	16 (13.5)	56 (47.5)	
AA	22 (75.9)	5 (17.2)	2 (6.9)	0.48	11 (44.0)	8 (32.0)	6 (24.0)	0.06
NCOA4								
rs10994675								
GG	105 (82.7)	16 (12.6)	6 (4.7)		35 (35.4)	19 (19.2)	45 (45.4)	
GA	145 (81.5)	20 (11.2)	13 (7.3)		55 (35.7)	28 (18.2)	71 (46.1)	
AA	67 (83.7)	10 (12.5)	3 (3.8)	0.83	30 (43.5)	18 (26.1)	21 (30.4)	0.23
rs10761581								
TT	104 (81.9)	17 (13.4)	6 (4.7)		38 (35.2)	16 (14.8)	54 (50.0)	
TG	161 (80.5)	21 (10.5)	18 (9.0)		64 (38.5)	36 (21.7)	66 (39.8)	
GG	61 (85.9)	10 (14.1)	0 (0.0)	**0.04**	18 (31.6)	15 (21.7)	24 (42.1)	0.28
rs10761618								
TT	149 (81.9)	24 (13.2)	9 (4.9)		58 (38.7)	25 (16.7)	67 (44.6)	
TC	137 (81.6)	17 (10.1)	14 (8.3)		49 (35.3)	32 (23.0)	58 (41.7)	
CC	32 (88.9)	4 (11.1)	0 (0.0)	0.32	13 (39.4)	9 (27.3)	11 (33.3)	0.48
rs11592181								
GG	250 (82.2)	35 (11.5)	19 (6.3)		98 (37.4)	51 (19.5)	113 (43.1)	
GA	60 (78.9)	12 (15.8)	4 (5.3)		20 (36.4)	13 (23.6)	22 (40.0)	
AA	7 (87.5)	1 (12.5)	0 (0.0)	0.84	3 (42.9)	1 (14.3)	3 (42.8)	0.96
rs7085433								
GG	255 (82.3)	38 (12.2)	17 (5.5)		89 (34.8)	55 (21.5)	112 (43.7)	

	Tumor Tissue Staining				Normal Tissue Staining			
	No/Weak	**Moderate**	**Intense**	**p-value**	**No/Weak**	**Moderate**	**Intense**	**p-value**
GA	59 (85.5)	5 (7.3)	5 (7.2)		30 (49.2)	8 (13.1)	23 (37.7)	
AA	5 (83.3)	1 (16.7)	0 (0.0)	0.61	0 (0.0)	2 (50.0)	2 (50.0)	0.06
rs7350420								
TT	160 (85.6)	17 (9.1)	10 (5.3)		61 (41.8)	35 (24.0)	50 (34.2)	
TC	124 (79.5)	21 (13.5)	11 (7.0)		52 (36.6)	24 (16.9)	66 (46.5)	
CC	34 (80.9)	7 (16.7)	1 (2.4)	0.40	7 (21.2)	6 (18.2)	20 (60.6)	**0.03**

Number of samples in each analysis varies due to missing data.

Table 5

Associations between tumor and normal prostate NCOA4 expression levels and *MSMB* and *NCOA4* SNP genotypes

	Tumor Tissue Staining				Normal Tissue Staining			
	No/Weak	**Moderate**	**Intense**	**p-value**	**No/Weak**	**Moderate**	**Intense**	**p-value**
MSMB								
rs10993994								
CC	7 (5.8)	8 (6.6)	106 (87.6)		7 (6.5)	7 (6.5)	94 (87.0)	
CT	8 (4.5)	16 (8.9)	155 (86.6)		8 (5.8)	7 (5.1)	123 (89.1)	
TT	3 (3.7)	6 (7.4)	72 (88.9)	0.92	2 (3.6)	1 (1.8)	53 (94.6)	0.72
rs17178655								
GG	10 (4.5)	14 (6.4)	196 (89.1)		8 (4.7)	6 (3.5)	156 (91.8)	
GA	7 (5.2)	16 (11.8)	112 (83.0)		7 (6.4)	9 (8.3)	93 (85.3)	
AA	2 (7.1)	1 (3.6)	25 (89.3)	0.32	1 (4.3)	1 (4.4)	21 (91.3)	0.43
NCOA4								
rs10994675								
GG	8 (6.4)	8 (6.5)	108 (87.1)		5 (5.4)	1 (1.1)	87 (93.5)	
GA	7 (3.9)	14 (7.9)	157 (88.2)		9 (6.3)	7 (4.9)	126 (88.7)	
AA	3 (3.9)	9 (11.5)	66 (84.6)	0.61	2 (3.1)	7 (10.8)	56 (86.2)	0.08
rs10761581								
TT	3 (2.4)	11 (8.9)	109 (88.6)		3 (3.0)	2 (2.0)	94 (95.0)	
TG	13 (6.6)	17 (8.6)	167 (84.8)		11 (7.0)	10 (6.4)	136 (86.6)	
GG	3 (4.2)	2 (2,8)	66 (93.0)	0.21	2 (3.8)	4 (7.7)	46 (88.5)	0.23
rs10761618								
TT	10 (5.6)	14 (7.9)	154 (86.5)		8 (5.8)	2 (1.5)	127 (92.7)	
TC	8 (4.8)	16 (9.6)	143 (85.6)		8 (6.0)	10 (7.5)	15 (86.5)	
CC	1 (2.8)	1 (2.8)	34 (94.4)	0.75	0 (0.0)	4 (13.3)	26 (86.7)	**0.02**
rs11592181								
GG	11 (3.7)	26 (8.7)	263 (87.7)		11 (4.5)	14 (5.7)	22- (89.8)	
GA	6 (7.9)	5 (6.6)	65 (85.5)		3 (5.9)	2 (3.9)	46 (90.2)	
AA	2 (25.0)	0 (0.0)	6 (75.0)	0.08	2 (28.6)	0 (0.0)	5 (71.4)	0.18
rs7085433								
GG	11 (3.6)	21 (6.9)	273 (89.5)		11 (4.6)	11 (4.6)	218 (90.8)	

	Tumor Tissue Staining				Normal Tissue Staining			
	No/Weak	**Moderate**	**Intense**	**p-value**	**No/Weak**	**Moderate**	**Intense**	**p-value**
GA	7 (10.0)	10 (14.3)	53 (75.7)		4 (7.1)	4 (7.2)	48 (85.7)	
AA	0 (0.0)	0 (0.0)	5 (100.0)	**0.04**	1 (25.0)	0 (0.0)	3 (75.0)	0.24
rs7350420								
TT	11 (5.9)	14 (7.5)	162 (86.6)		10 (7.3)	9 (6.5)	119 (86.2)	
TC	6 (4.0)	14 (9.2)	132 (86.8)		5 (3.9)	5 (3.9)	119 (92.2)	
CC	1 (2.4)	3 (7.3)	37 (90.2)	0.88	1 (3.1)	1 (3.1)	30 (93.8)	0.60

Number of samples in each analysis varies due to missing data.