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[1] De**Interaction of NKX3.1 and SELENOP Genotype with Prostate Cancer Recurrence**

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Running Title: NKX3.1 and SELENOP genotypes affect prostate cancer

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ABSTRACT

NKX3.1 is a tumor suppressor frequently lost in prostate cancer. Previous studies by others indicated that the risks associated with reduced NKX3.1 levels can be enhanced by anti-oxidant supplementation. Selenium is an essential component of several proteins with anti-oxidant functions and lower levels of selenium have been associated with greater risk of prostate cancer. In contrast, participants of the SELECT prostate cancer prevention trial were at increased risk of prostate cancer when supplemented with selenium when their baseline selenium levels were high. In order to investigate whether there was an interaction between a functional polymorphism in *NKX3.1* that results in less protein and selenium status with prostate cancer grade or outcome, plasma selenium levels and the genotypes of *NKX3.1* and the selenium carrier protein *SELENOP* were determined from a cohort of men who underwent radical protatectomy. *NKX3.1* and *SELENOP* genotypes were associated with a more aggressive prostate tumor grade at the time of prostatectomy, but there were no significant interactions of *NKX3.1* genotype with either selenium status or *SELENOP* genotype. There was also a significant association between *NKX3.1* genotype and prostate cancer recurrence, however this association was modified by *SELENOP* genotype, but not with plasma selenium levels. These data indicate that the impact of selenium status on prostate cancer may be influenced by factors other than the amount of selenium in circulation.

1. INTRODUCTION

The use of dietary supplements for the prevention of prostate cancer has been evaluated in randomized clinical trials. One supplement that has received attention for this purpose due to encouraging *in vitro*, *in vivo* and clinical trials is vitamin E (reviewed in[1]). Data obtained from the Alpha-Tocopherol, Beta-Carotene Cancer Prevention trial (ATBC) indicated that supplementation with alpha-tocopherol reduced the incidence of prostate cancer with an RR of 0.88 (95% CI, 0.76-1.03)[2]. Selenium, an essential micronutrient previously considered to be an excellent candidate for prostate cancer chemoprevention, was evaluated in the Nutritional Prevention of Cancer Trial (NPCT)[3]. Using selenium-enriched yeast as a source of selenium, there was a significant reduction in prostate cancer incidence in the selenium group among participants with lower plasma selenium at baseline. Based on these data and supporting results from *in vitro* and preclinical studies, the larger multicenter Selenium and Vitamin E Cancer Prevention Trial (SELECT) was initiated to evaluate whether vitamin E and selenium, alone or in combination, were effective in preventing prostate cancer. Men who were cancer-free at baseline were randomized to one of four groups: 200µg/day of selenomethionine, 400 IU/day of vitamin E (a-tocopherol), both or placebo[4]. The trial was terminated early due to the lack of evidence that either supplement reduced prostate cancer risk and because participants in the Vitamin E arm exhibited increased prostate cancer incidence[5]. Subsequent analysis indicated that men assigned to the selenium arm who had higher baseline selenium levels were also at increased risk of high-grade prostate cancer[6]. Although selenium in its elemental form is not an anti-oxidant, it indirectly contributes to anti-oxidant defenses due to its role as an essential component of several anti-oxidant enzymes that contain selenocysteine[7]. Thus, two anti-oxidants, vitamin E and selenium, may in some circumstances contribute to the elevated risk of prostate cancer.

An unexpected effect of anti-oxidant supplementation was revealed when mice null for the *NKX3.1* tumor suppressor were used to investigate the impact of anti-oxidant supplementation on prostate pathology[8]. The diet of these mice was supplemented with the anti-oxidant N-acetylcysteine (NACS) and consequential effects on prostatic hyperplasia, dysplasia and prostatic intraepithelial neoplasia (PIN) were determined[8]. *NKX3.1* is encoded by an androgen-regulated homeobox gene expressed specifically in the prostate gland and located in the chromosome 8p21[9], a region frequently exhibiting loss of heterozygosity in human prostate cancers[10,11]. Although treatment of the *NKX3.1*-null mice with NACS reduced reactive oxygen species (ROS), there was increased proliferation of prostate epithelium proliferation in the NACS-treated mice[8]. Given these results, these same authors extended their animal data to investigate whether *NKX3.1* genotype was associated with the development of prostate cancer among men provided vitamin E or selenium in participants of SELECT[12]. DNAs were genotyped for a functional single nucleotide polymorphism (SNP) in the *NKX3.1* promoter (rs17881886) where the presence of the minor C allele results in its lower mRNA expression by altering the binding of the SP1 transcription factor to the 5'-untranslated region (UTR) of the gene[10]. While no effect of *NKX3.1* genotype on prostate cancer risk was found, these studies revealed a significant interaction between the minor C allele in *NKX3.1* with either selenium and vitamin E supplementation. In the selenium-supplemented group, the *NKX3.1* rare genotype was associated with a 67% increased risk for total prostate cancer and with an 81% increased risk for low-grade prostate cancer[12]. Based on these data, we examined whether there were associations between *NKX3.1* genotype and selenium status with cancer grade at presentation or recurrence of disease following prostatectomy in a Chicago area cohort.

2. MATERIALS AND METHODS

2.1 Study Population

The cohort used in this study was part of the Adiposity and Outcomes of Clinically Localized Prostate Study, a prospective cohort investigating the impact of obesity on biochemical failure and other adverse outcomes after radical prostatectomy for clinically localized prostate cancer. A total of 129 volunteers were selected in the Chicago area. Participants were mostly middle-aged (mean = 60 years) and Caucasian (67%). Before surgery, blood samples and buffy coat DNA were collected and clinical status, lifestyle and anthropometric measures were assessed. Tumor diagnoses were determined by a single pathologist and a second pathologist reviewed 10% of samples (see reference[13] for details). The protocol was approved by the UIC Office for the Protection of Research Subjects.

2.2 Biochemical follow-up

After prostatectomy, volunteers were instructed to repeat the serum PSA (prostate-specific antigen) test every 3 months for the first year, every 3-6 months for the second year and once a year thereafter. Serum PSA concentration was determined using the Tandem PSA monoclonal antibody assay (Hybritech, San Diego, CA). Tumor recurrence within two years, previously shown to be associated with increased risk of distant metastasis and prostate cancer-specific mortality[14,15], was defined as detectable ($\geq$ 0.1 ng/mL) and subsequently rising serum PSA.

2.3 Genotyping

Archived DNA were originally obtained from buffy coat samples and genotyping of the *NKX3.1* rs11781886 was performed by real-time PCR using the QuantStudio 6K Real-Time PCR System

with Taqman SNP Genotyping Assays (Applied Biosystems, Thermo Scientific, Foster City, CA, USA). Allelic discrimination was obtained by performing an endpoint read using the Taqman Genotyper software. Validation was done by randomly sequencing 10% of the samples which were in agreement with the PCR generated results. Genotyping for rs7579 and rs3877899 in *SELENOP* and plasma selenium concentrations were previously reported on the same sample set as was used in this study[13].

2.4 Statistical analysis

Bivariate associations were evaluated using Fisher's Exact Test (Supplemental Table S1). The two main outcomes variables for this study were Gleason Score and tumor recurrence. Patients were divided into groups based on their Gleason Score sums divided as: 2 to 6, 7(3+4), 7(4+3) and ≥8, and cancer recurrence status after surgery (yes and no). An ordinary logistic regression model was used for outcome Gleason Score with the lower category (2 to 6) set as the reference, whereas a logistic regression model was used for tumor recurrence. In both models, a hypothesis based model was tested with the main goal of investigating the associations of *NKX3.1* SNP with selenium status on tumor grade and recurrence. The predictors of primary interest were plasma selenium, *NKX3.1* rs11781886, *SELENOP* rs3877899, *SELENOP* rs7579, interaction of selenium status with *NKX3.1*, and of *NKX3.1* with both *SELENOP* SNPs. A backwards selection was performed for demographic variables. The models were adjusted for age, body fat and race. Odds ratio, 95% confidence limits and *p* values are provided. All tests are two sided and with the significance level of 0.05. All statistical analyses were performed using R statistical software.

3. RESULTS

3.1 A polymorphism in *NKX3.1* is not associated with an increased chance of being diagnosed a more aggressive prostate cancer.

DNA obtained from men diagnosed with prostate cancer and participating in the Adiposity and Outcomes of Clinically Localized Prostate Study were genotyped for the functional polymorphism in the *NKX3.1* promoter (rs11781886). Overall, the genotypes frequencies for rs11781886 were 56% TT, 40% TC and 4% CC. Mean plasma selenium concentration (134.2 ± 24.8 ng/ml) and serum PSA (7.5 ± 6.3 ng/ml) were previously reported[13]. Biomarkers concentrations and the frequencies of Gleason score groups and recurrence status were not different between *NKX3.1* genotypes. The allele frequencies did not differ between African American and Caucasian men, as well as patient groups with different Gleason scores or tumor recurrence after prostatectomy (Supplemental Table S1).

Using ordinary multivariate logistic regression model, the odds of a higher Gleason sum increased with plasma selenium levels and NKX3.1 C genotype (Table 1). However, there was no interaction between *NKX3.1* genotype and selenium status with Gleason grade. Selenoprotein P (SELENOP) is the most abundant selenium-containing protein in plasma and the main selenium transporter in plasma[16,17]. Two functional SNPs in *SELENOP*, rs3877899 resulting in either an alanine or threonine in the coding region and rs7579 resulting in a G or A in the 3-untranslated region of the *SELENOP* gene, have been shown to impact plasma selenium levels and SELENOP concentrations as well as the levels of selenoproteins in plasma, erythrocytes and lymphocytes[18,19]. Associations between these polymorphisms and Gleason sum scores were assessed by combining the low frequency alleles (*SELENOP* rs3877899^{GA+AA} and *SELENOP* rs7579^{GA+AA}, respectively). There was a 3-fold higher risk (p = 0.019) of aggressive cancer in

men with *SELENOP* rs7579^{GA+AA} genotype compared to *SELENOP* rs7579^{GG} genotype ((OR [95% CI] = 3.02 [1.21 – 7.76]) while no association with the *SELENOP* rs3877899 polymorphism was observed (Table 1). Examining the data for an interaction between polymorphisms in *NKX3.1* and *SELENOP*, there was no interaction of the *NKX3.1* polymorphism with either *SELENOP* rs3877899 or *SELENOP* rs7579 (Table 1).

3.2 *NKX3.1* and *SELENOP* genotype are associated with an increased risk of prostate cancer recurrence following prostatectomy.

We previously reported the association of the *SELENOP* rs3877899^{AA} genotype status with prostate cancer recurrence in the same population as assessed here[13]. This observation was extended by testing for associations with tumor recurrence, defined as detectable (≥ 0.01 ng/mL) and rising serum PSA, with SNPs in *NKX3.1* and *SELENOP* genes as well as selenium levels (Table 2). Multivariate logistic regression model with backwards selection was used to assess such associations. There was significant interaction effect between NKX3.1 and SELENOP rs3877899. When present with the SELENOP rs3877899^{GG} genotype, NKX3.1^{TC+CC} is significantly more protective against cancer recurrence (NKX3.1^{TC+CC} vs. NKX3.1^{TT} OR = 0.19, or NKX3.1^{TT} versus NKX3.1^{TC+CC} OR = 5.3, p-value = 0.0183). This protective effect of NKX3.1^{TC+CC} was not found when *SELENOP* rs3877899^{GA+AA}. In contrast, *SELENOP* rs3877899^{GA+AA} was associated with increased risk of recurrence co-expressed with NKX3.1^{TC+CC} (OR = 4.85, p-value = 0.0415). No significant association between *SELENOP* rs3877899^{GA+AA} and NKX3.1^{TT} was found with prostate cancer recurrence.

DISCUSSION

The benefits of dietary anti-oxidant supplements remain uncertain due to conflicting results from epidemiological data, involving dietary intake and disease incidence, compared to supplementation trials, comprised of selected groups at elevated risk. Baseline status of an individual and the effects of an individual's genetic background may influence the consequences of consuming anti-oxidant supplements for cancer prevention. Previously published work indicated that the impact of the allelic identity of the *NKX3.1* gene on prostate cancer might be influenced by anti-oxidant intake or status[8,12]. These data were expanded here by examining whether serum selenium levels together with *NKX3.1* genotype were associated with either the aggressiveness of the tumor at the time of diagnosis or cancer recurrence following prostatectomy. No interaction between selenium status and *NKX3.1* genotype on either of these endpoints were found for the cohort of men who had undergone prostatectomy examined in this study.

Selenium status in a particular tissue may not reflect the levels determined from serum or plasma. For example, we previously reported a lack of association between serum selenium levels and the selenium concentration from prostate tissue[20]. Prostate tissue was not available for the men from this cohort examined, but we instead examined the genotype of the major selenium carrier protein, *SELENOP*, determining the allelic identity of SNPs previously shown to be functional and influence the levels of *SELENOP*[18,19]. The rare allele A of rs7579 in the *SELENOP* 3'-untranslated region, was associated with an increased risk of aggressive cancer, but not when co-expressed with *NKX3.1* rs11781886 minor allele C (Table 1). Moreover, the rare allele A for *SELENOP* rs3877899 was associated with an increased risk of recurrence of cancer only when co-expressed with the minor allele C for *NKX3.1* SNP (Table 2).

SELENOP, which contains 10 selenium atoms in the form of selenocysteine, is generated in the liver, enters the circulation and can gain entry into tissues by megalin, a carrier protein with many ligands, where SELENOP is hydrolyzed, releasing selenium for eventual use in the synthesis of prostatic selenoproteins[17,21]. Polymorphisms in *SELENOP* have been shown to be associated with prostate cancer risk[22,23] and interact with polymorphisms in the MnSOD anti-oxidant gene to further increase prostate cancer risk[24]. The lack of association between circulating and tissue selenium levels[20] and the association between *NKX3.1* genotype and *SELENOP* polymorphisms but not between *NKX3.1* genotype and plasma selenium levels indicate that the efficiency or regulation of selenium transport may be relevant to understanding the effects of selenium on prostate cancer. Although megalin can transport many ligands in addition to selenium, it is interesting to note that polymorphisms in the megalin gene were significantly associated with both prostate cancer recurrence and mortality[25].

NKX3.1 is a transcription factor that has been implicated in prostate cancer due to its frequent deletion of its gene in the disease and its loss being associated with more aggressive disease[9,26,27]. It regulates several anti-oxidant enzymes and the loss of *NKX3.1* results in increased oxidative damage[28]. Here, we have shown that the SNPs in *NKX3.1* and *SELENOP,* that are likely to result in reduced levels of each of those proteins, interact to increase the risk of prostate cancer recurrence (Table 2). It is therefore possible that the reduction of both NKX3.1 and SELENOP results in elevated oxidative stress due to diminished expression of NKX3.1-regulated anti-oxidant genes, as well as reduced levels of anti-oxidant selenoproteins, resulting in growth promoting reactive oxygen signaling and accelerated accumulation of mutations that contribute to progression.

In summary, both *NKX3.1* and *SELENOP* genotypes were associated with a more aggressive prostate tumor grade at the time of prostatectomy, but there were no significant interactions of *NKX3.1* genotype with either selenium status or *SELENOP* genotype. There was also a significant association between *NKX3.1* genotype and prostate cancer recurrence although this association was modified by *SELENOP* genotype, but not with plasma selenium levels. Weaknesses of the study include the small sample size and the relatively short period of follow up. In addition, the study was not originally designed to investigate the particular endpoints reported herein, raising the possibility that there were confounding factors that were not taken into account in the analyses. While additional studies with larger cohorts and longer follow up are warranted, these data indicate that the impact of selenium on prostate cancer risk and outcome needs to consider the levels of selenium in prostatic tissue, which may be influenced by selenium transport and metabolism.

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AUTHORS' CONTRIBUTIONS

JLSD was responsible for genotyping, data analysis, biostatistics and manuscript writing, LL for biostatistics, VLF for access to patient dataset and DNA samples and data analysis, DNE, GB and AMD contributed data analysis and writing the manuscript.

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TABLE 1 Associations of higher tumor Gleason grade group[a] with selenium, *NKX3.1* and *SELENOP* genotypes.

Selenium, NKX3.1 and SELENOP genotypes	COR[b] (95% CI)	P value
Selenium	1.01 (1.00-1.03)	0.046
NKX3.1 rs11781886^{TC+CC}	2.12 (0.86-5.33)	0.010
SELENOP rs3877899^{GA+AA}	-	-
SELENOP rs7579^{GA+AA}	3.02 (1.21-7.76)	0.019
Selenium * rs11781886^{TC+CC}	-	-
NKX3.1 * *SELENOP* rs3877899	-	-
NKX3.1 * *SELENOP* rs7579	0.31 (0.07-1.21)	0.094

COR: cumulative odds ratio; CI: confidence interval.

[a]: Gleason sums were categorized in to four Gleason grade groups (2 to 6, 7 [3+4], 7 [4+3] and ≥8) based on the 8th Edition of the American Joint Commission on Cancer tumor-node-metastasis staging for prostate cancer (Amin MB, Edge SB, Greene FL, et al. AJCC Cancer Staging Manual. 8th ed. American Joint Committee on Cancer. Switzerland: Springer; 2017).

[b]: CORs were derived using ordinal multivariate logistic regression with backwards selection of selenium, NKX3, and SELENOP genotypes and interaction terms followed by further adjustment for age, race and body fat mass. After backwards selection, the model was: Gleason grade group (1 to 4) = β_1NKX3 + β_2SELENOP rs7579 + β_3NKX3*SELENOP rs7579 + ε.

TABLE 2. Interaction of *NKX3.1* and *SELENOP* genotypes with biochemical recurrence.

Tumor Recurrence [a]	Estimates (SE)	*p*-value
NKX3.1^{TC+CC}	-1.62 (0.68)	***0.018***
SELENOP rs3877899^{GA+AA}	-0.80 (0.59)	0.173
NKX3.1 * selenium	-	-
NKX3,1 * *SELENOP* rs7579	-	-
NKX3.1 $^{TC+CC}$ * *SELENOP* rs3877899^{GA+AA}	2.38 (0.97)	***0.041***
	OR (95% CI)	*p*-value
NKX3.1^{TC+CC} (at SELENOP rs3877^{GG})	0.19 (0.04-0.68)	**0.0183**
NKX3.1^{TC+CC} (at SELENOP rs3877^{GA+AA})	2.14 (0.55-8.31)	0.2704
SELENOP rs3877899^{GA+AA} (at NKX3.1^{TT})	0.45 (0.14-1.43)	0.1734
SELENOP rs3877899^{GA+AA} (at NKX3.1^{TC+CC})	4.85 (1.06-22.19)	***0.0415***

OR: odds ratio; CI: confidence interval.
Tumor recurrence was defined as detectable (0.1ng/L) and rising serum PSA levels or additional therapy.
[a]: Multivariate Logistic Model; after backwards selection: Recurrence (yes x no) = β_1NKX3 + β_2SELENOP rs3877899 + β_3NKX3*SELENOP rs3877899.
[b]: OR for individuals with *NKX3.1* minor allele C to have tumor recurrence if also carrying rare allele A for SELENOP rs3877899
[c]: OR for individuals with *SELENOP* rs3877899 minor allele A to have tumor recurrence if also carrying rare allele C for NKX3.1
Significant p values are highlighted in bold and italic

Supplemental Table S1

Distribution of *NKX3.1* genotype according to race and prostate cancer biomarkers

	rs11781886 (NKX3)		p value
	TC+CC (N= 57)	TT (N= 72)	
Race			
African American	16 (55.2%)	13 (44.8%)	0.252
Caucasian	35 (39.3%)	54 (60.7%)	
Other	6 (54.5%)	5 (45.5%)	
Gleason score			
8 or higher	2 (3.5%)	5 (6.9%)	0.425
7 (4+3)	6 (10.5%)	6 (8.3%)	
7 (3+4)	29 (50.9%)	28 (38.9%)	
2 to 6	20 (35.1%)	33 (45.8%)	
Recurrence[a]			
yes	9 (15.8%)	18 (25%)	0.289
no	48 (84.2%)	54 (75%)	
PSA (ng/mL)	7.7 ± 5.1	7.3 ± 7.1	0.149
Plasma Se (µg/L)	132.1 ± 26.9	135.8 ± 23.0	0.407

Values are N (%) for categorical variables
[a]: Detectable (≥ 0.01 ng/ml) and rising serum PSA[24]