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Replication of Prostate Cancer Risk Variants in a Danish Case-Control Association Study

Diem Nguyen Bentzon[1*], Mette Nyegaard[2], Anders Børglum[2], Torben Ørntoft[3], Michael Borre[1], Karina Dalsgaard Sørensen[3]

[1]Department of Urology, Aarhus University Hospital Skejby, Aarhus, Denmark
[2]Department of Human Genetics, Aarhus University, Aarhus, Denmark
[3]Department of Molecular Medicine, Aarhus University Hospital Skejby, Aarhus, Denmark
Email: [*]diem.bentzon@dadlnet.dk

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ABSTRACT

Background: Prostate cancer is one of the main causes for cancer morbidity and mortality in Western countries. Recently, several single nucleotide polymorphisms (SNPs) associated with prostate cancer have been identified in genome-wide association studies and multiple variant models have been developed to predict prostate cancer risk. The association between genetic markers and clinico-pathological tumor variables has, however, been inconsistent. **Methods and Materials:** A total of 32 previously identified prostate cancer-associated risk SNPs were genotyped in 648 prostate cancer cases and 526 age-matched controls. Family history was obtained by questionnaire. Age at diagnosis, clinical tumor variables including pre- and postoperative PSA, Gleason score, and T stage were obtained from prospectively collected clinical data (Aarhus Prostate Cancer Study). The SNPs were genotyped using Sequenom and Taqman assays and associations between SNPs, prostate cancer risk, and clinico-pathological variables were assessed. **Results:** Seventeen SNPs were successfully replicated in our case-control study and the association estimates were consistent with previous reports. Four markers were excluded from further analysis due to linkage disequilibrium. The cumulative effect of having the disease-associated genotype at five SNPs (rs4430796, rs6983267, rs1859962, rs1447295 and rs16901979) increased the prostate cancer risk with odds ratio 6.02 (95% CI: 2.21 - 16.38; $P = 1.0 \times 10^{-4}$) in patients with any combination of ≥4 markers compared with patients without any of the five SNPs (P for trend = 1.0×10^{-4}). Six markers were significantly associated with clinico-pathological variables: SNP rs2735839 (GG) at locus 19q13, which is in the *KLK*3 gene encoding PSA, was associated with high preoperative PSA ($P = 0.04$), low Gleason score ($P = 0.01$) and low T stage ($P = 0.02$). Variants rs5759167 (GG/GT) (22q13) and rs7679673 (CC/CA) (4q24) were correlated with low risk for biochemical relapse ($P = 0.015$ and $P = 0.009$, respectively), whereas rs6983267 (GG) (8q24) was significantly associated with biochemical recurrence ($P = 0.045$). In addition, variants rs6983267 (GG) and rs5759167 (GG/GT) were significantly associated with negative family history ($P = 0.04$ and $P = 0.02$, respectively). **Conclusion:** We replicated 17 previously identified prostate cancer-associated risk SNPs in a Danish case-control study and found a cumulative and significant association between five SNPs and prostate cancer. Overall, we noted significant associations between several prostate cancer-associated risk genotypes and less aggressive tumor variables, high level of PSA, and low risk for biochemical reccurrence.

Keywords: SNP; Family History; Cumulative Effect; Biochemical Relapse; Prostate Cancer Risk

1. Introduction

Prostate cancer is the most common cause of non-skin cancer morbidity and mortality in Western countries [1,2]. Family history is one of the strongest risk factors for prostate cancer in addition to age and ethnicity [3,4]. Based on large epidemiological cohort studies, familial prostate cancer accounts for 10% - 20% of all prostate cancer cases and hereditary prostate cancer for 5% - 10%. Except for early age at onset, no clinical patterns have been consistently associated with hereditary prostate cancer [5-13].

Since the introduction of PSA screening, more prostate cancer cases are diagnosed at a treatable stage. However, since not all cases are likely to progress it is important to distinguish the indolent from the aggressive form of prostate cancer.

Recently, more than 40 single nucleotide polymerphisms (SNP) associated with prostate cancer have been identified in genome-wide association studies [14-16]. Individually, these variants contribute only modestly to the risk; combined, they have cumulative risk [17-19]. However, findings on associations between genetic mark-

[*]Corresponding author.

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ers and clinico-pathological traits have been inconsistent [19-21].

In the present study, we evaluated 32 previously reported prostate cancer-associated risk variants in a case-control study comprising 526 control subjects and 648 cases diagnosed with prostate cancer.

2. Methods and Materials

2.1. Patients

Between 1997 and 2009, 648 patients with prostate cancer were recruited from the Central Denmark Region through the Department of Urology, Aarhus University Hospital, for the Aarhus Prostate Cancer Study. Prostate cancer patients between the ages of 36 and 77 were enrolled within few weeks of diagnosis. Of the 648 prostate cancer patients, 535 underwent retropubic or computer-assisted laparoscopic radical prostatectomy for clinically localized prostate cancer (stage T1c-T2c).

In the current study, all 648 patients had DNA extracted from whole blood and provided information about prostate cancer in first-degree relatives. Demographic information and clinical characteristics including age at diagnosis, preoperative PSA, postoperative Gleason score and TNM (Tumor Node Metastasis) stage were obtained from the Aarhus Prostate Cancer Study database (**Table 1**). Based on preoperative PSA, preoperative T stage, and preoperative Gleason score the patients were grouped as low, intermediate, and high-risk according to D'Amico *et al*. [22]. Biochemical relapse was defined by a postoperative increase in PSA after radical prostatectomy based on a single measure of PSA ≥ 0.2 ng/ml.

Control individuals (n = 526) were patients either treated with percutaneous transluminal coronary angioplasty or enrolled in the DANish trial on Acute Myocardial Infarction-2 (DANAMI-2) [23]. All control subjects were age-matched and information about their disease status was requested from the Danish Cancer Registry and the Cause of Death Registry; men diagnosed with prostate cancer were excluded from the control group. Written informed consent was obtained from both case patients and control patients and the Central Denmark Committee on Biochemical and Research Ethics and the Danish Data Protection Agency approved the study.

2.2. Selection of SNPs

Genotyping of eligible genetic variants included 32 SNPs with previously reported prostate cancer association (**Table 2**). The search strategy for the 32 prostate cancer-associated SNPs was as follows: genome-wide association studies on prostate cancer in the Pub Med database were selected. Only major association studies including cases diagnosed with prostate cancer and prostate cancer-free controls were included. SNPs reported as being signifycantly associated with prostate cancer risk and validated on at least one independent case-control study were included (genome-wide statistical significance level of 10^{-6}) [24-39].

Table 1. Clinico-pathological characteristics of the prostate cancer cases.

	Case Group	
Number of cases	648	
Age—yr		
Mean age (95% CI)	64.0	(63.5 - 64.6)
Age at diagnosis—no. (%)		
<63	288	(44.4)
≥63	360	(55. 6)
First-degree relatives (%)		
No	438	(67.6)
Yes	98	(15.1)
Missing	112	(17.3)
PSA level—no. (%)		
<10 ng/ml	215	(33.2)
≥10 ng/ml	433	(66.8)
Gleason score[a]—no. (%)		
≤6	354	(54.6)
7	248	(38.3)
≥8	46	(7.1)
Tumor stage[a]— no. (%)		
≤pT2a	90	(13.8)
pT2b	23	(3.5)
≥pT2c	535	(82.6)
Nodal stage—no. (%)		
N_0	459	(70.8)
N_1	29	(4.5)
N_x	160	(24.7)
Metastatis stage—no. (%)		
M_0	576	(88.9)
M_1	27	(4.2)
M_x	45	(6.9)

[a]In 83% of cases, patients underwent radical prostatectomy due to clinically localized prostate cancer. In these cases, tumor (T) stage and Gleason score were based on the pathological evaluation of the prostatectomy specimen. For the remaining 17% of patients that were diagnosed with localized advanced or metastasized prostate cancer, clinical T stage and biopsy Gleason score were used.

To optimize the genotyping regarding to cost, all candidate SNPs were combined into Sequenom assays. The two largest multiplex assays (*i.e*. with the highest numbers of SNPs incorporated) were analysed using the Sequenom platform. The remaining SNPs were genotyped using Taqman assays.

2.3. SNP Genotyping

Genotyping of 18 SNPs was performed using the Sequenom MassARRAY Genotyping system (Sequenom, San Diego, CA) (**Table 2**). Primers for PCR and extension probes were designed using the MassARRAY Assay Design 3.1 software (Sequenom). Multiplex PCR was performed in 5 μl reactions containing 10 ng of genomic DNA, 1.25 × PCR buffer (Qiagen, Valencia, CA), 0.5 mM dNTP (Roche, Geneva, Switzerland), 100 nM of

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Table 2. List of 32 SNPs preselected from published literature.

							Frequency			
SNP[a]	**Locus**	**Gene**	**Platform**	**BP-build37**	**Allele 1**	**Allele 2**	**Cases**	**Controls**	**OR (95% CI)**	**P Value**
rs721048	2p15	*EHBP*1	Taqman	63,131,731	A	G	0.19	0.18	1.09 (0.88 - 1.35)	0.44
rs1465618	2p21	*THADA*	Taqman	43,553,949	A	G	0.23	0.22	1.03 (0.84 - 1.25)	0.79
rs12621278	2q31	*ITGA*6	Taqman	173,311,553	G	A	0.94	0.93	1.17 (0.83 - 1.63)	0.35
rs2660753	3p12		Sequenom	87,110,674	T	C	0.92	0.90	1.22 (0.90 - 1.65)	0.18
rs12500426	4q22	*PDLIM*5	Taqman	95,514,609	A	C	0.54	0.54	1.01 (0.86 - 1.20)	0.87
rs17021918	4q22	*PDLIM*5	Taqman	95,562,877	T	C	0.36	0.35	1.02 (0.86 - 1.22)	0.79
rs7679673	4q24		Taqman	106,061,534	C	A	0.65	0.60	1.20 (1.01 - 1.43)	**0.03**
rs9364554	6q25	*SLC*22*A*3	Sequenom	160,753,654	T	C	0.35	0.34	1.05 (0.88 - 1.25)	0.58
rs10486567	7p15	*JAZF*1	Sequenom	27,976,563	A	G	0.80	0.76	1.22 (0.99 - 1.49)	0.051
rs6465657	7q21	*LMTK*2	Sequenom	97,816,327	C	T	0.48	0.47	1.06 (0.90 - 1.25)	0.47
rs2928679	8p21		Taqman	23,438,975	T	C	0.59	0.55	1.18 (1.00 - 1.40)	0.050
rs1512268	8p21		Taqman	23,526,463	A	G	0.44	0.41	1.13 (0.95 - 1.34)	0.15
rs1447295	8q24		Sequenom	128,485,038	A	C	0.18	0.14	1.35 (1.07 - 1.72)	$\mathbf{9.9 \times 10^{-3}}$
rs1016343	8q24		Sequenom	128,093,297	T	C	0.25	0.21	1.25 (1.03 - 1.53)	**0.02**
rs16901979	8q24		Sequenom	128,124,916	A	C	0.05	0.03	1.62 (1.03 - 2.59)	**0.03**
rs6983267	8q24	*POU5F1P*1	Taqman	128,413,305	T	G	0.56	0.52	1.20 (1.02 - 1.42)	**0.03**
*rs*4242384	8*q*24		*Sequenom*	128,518,554	*C*	*A*	0.17	0.14	1.35 (1.07 - 1.71)	**0.01**
*rs*7812894	8*q*24		*Taqman*	128520479	*A*	*T*	0.18	0.14	1.31 (1.04 - 1.65)	**0.02**
rs1571801	9q33	*DAB2IP*	Taqman	124,427,373	A	C	0.70	0.70	1.01 (0.84 - 1.21)	0.95
rs10993994	10q11	*MSMB*	Sequenom	51,549,496	T	C	0.41	0.36	1.21 (1.02 - 1.44)	**0.02**
rs4962416	10q26	*CTBP*2	Sequenom	126,696,872	C	T	0.73	0.73	1.00 (0.83 - 1.21)	0.99
*rs*7931342	11*q*12		*Sequenom*	68,994,497	*T*	*G*	0.53	0.48	1.24 (1.05 - 1.47)	**0.01**
rs7127900	11q13		Taqman	2,233,574	A	G	0.26	0.22	1.22 (1.00 - 1.49)	**0.04**
rs10896449	11q13		Sequenom	68,994,667	A	G	0.52	0.47	1.25 (1.06 - 1.48)	**0.01**
rs4054823	17p12		Sequenom	13,625,024	C	T	0.54	0.55	1.00 (0.85 - 1.19)	0.96
rs11649743	17q12	*HNF*1*B*	Sequenom	36,074,979	A	G	0.80	0.79	1.09 (0.89 - 1.35)	0.39
rs4430796	17q12	*HNF*1*B*	Taqman	36,098,040	G	A	0.58	0.53	1.23 (1.04 - 1.45)	**0.01**
rs1859962	17q23		Sequenom	69,108,753	T	G	0.56	0.49	1.32 (1.12 - 1.56)	$\mathbf{8.0 \times 10^{-4}}$
rs2735839	19q13	*KLK*3	Sequenom	51,364,623	A	G	0.89	0.84	1.48 (1.15 - 1.89)	**0.001**
rs5759167	22q13		Tagman	43,500,212	G	T	0.54	0.49	1.21 (1.02 - 1.43)	**0.02**
*rs*5945619	*Xp*11	*NUDT*11	*Sequenom*	51,241,672	*C*	*T*	0.68	0.61	1.34 (1.04 - 1.72)	**0.02**
rs5945572	Xp11	*NUDT*11	Sequenom	51,246,423	A	G	0.67	0.61	1.32 (1.03 - 1.70)	**0.02**

[a]SNPs in linkage disequilibrium are indicated in italics.

each primer (Metabion, Martinsried, Germany) and 0.5U Taq polymerase (Qiagen), using the standard cycling conditions described by Sequenom. The PCR products were treated with arctic shrimp alkaline phosphatase (SAP) and the probe extension reaction (iPLEX) was carried out in accordance with the standard protocol (Sequenom). The iPLEX reactions were desalted using resin and spotted on a SpectroCHIP (Sequenom) using a nanodispenser. The samples were analyzed using a Bruker matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer (Sequenom) and the genotypes were determined using the MassARRAY Type 4.0 software (Sequenom). The primer sequences used can be obtained on request.

Fourteen SNPs were genotyped using commercial TaqMan genotyping assays from Applied Biosystems (**Table 2**). All genotyping reactions (5 μL total volume; 10 ng gDNA per reaction) were performed in 386-well plates on the Applied Biosystems 7900HT Fast Real-Time PCR System using TaqMan Universal PCR Master Mix according to the manufacturer's protocol (Applied Biosystems).

2.4. Statistical Analysis

Quality Control (QC) of the genotyping results was performed per individual and per marker. QC was conducted in PLiNK [40]. The individual DNA threshold call rate was set at > 0.8. For both cases and control, we used Fisher's

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exact test to assess Hardy-Weinberg equilibrium for each SNP. Tests for pairwise linkage disequilibrium among variants in the control group were calculated by the Stata command pwld (David Clayton for Stata).

Assuming a multiplicative model, per-allele odds ratio (OR) and 95% confidence interval (CI) were calculated and allele frequency differences between 648 cases and 526 controls were tested using the Chi-square test df = 1-2. Bonferroni correction for multiple testing was performed. Variants with significantly ($P < 0.05$) different allele frequencies between cases and controls were included in the genotype testing under the assumption of a dominant or recessive model by logistic regression controlled for age (continuous variable). ORs and 95% CIs for prostate cancer risk were calculated for individuals with risk genotypes compared with those with non-risk genotypes for each genetic model. The best-fitting model of each of the SNPs was conferred highest likelihood. Missing data were managed by omitting individuals with missing data in genotype counts.

The five variants presenting the best fitting genetic model with the highest likelihood were included in the cumulative effect model by assessing the presence of the prostate cancer-associated genotypes in each study subject. The five variants were rs16901979, rs6983267, rs4430796, rs1859962 and rs1447295 also previously tested by Zheng *et al*. [41]: one (1) was assigned for the presence of prostate cancer-associated genotypes of each of the five variants in each individual and zero (0) for non-presence. The cumulative effects of the five SNPs were tested by adding up the points for each SNP in each individual. Using logistic regression, the combinations of prostate cancer-associated genotypes (1, 2, 3, 4 and 5 SNPs) in each individual was estimated.

Associations between SNPs associated with prostate cancer risk and the clinico-pathological parameters were conducted on prostate cancer cases undergoing prostatectomy (n = 535).

The chi-square test was used in 2 × C contingency analysis for association between the prostate cancer-associated variants and preoperative PSA (<10 ng/ml; ≥10 ng/ml), postoperative T stage, postoperative Gleason score, D'Amico risk score, and family history (familial prostate cancer vs. sporadic prostate cancer). Postoperative T stage and Gleason score were stratified into two levels (≤T2c vs. >T2c and ≤6 vs. >6, respectively). The disease-free survival of the study subjects was shown in Kaplan-Meier plots. Time at risk was estimated from the date of surgery to the date of biochemical recurrence or prostate cancer-specific death, and case subjects without the events were censored at their last blood test. Log rank test (Mantel-Cox test) was used to compare the recurrence distribution of the genotypes. All Fischer's exact tests and Chi-square tests in association analysis, logistic regression, Chi-square tests and Cochrane-Armitage test for trend were performed using intercooled Stata 10.0 (StataCorp LP, Texas, USA). The *P*-value significance level was set at <0.05.

3. Results

Thirty-two genetic variants were selected from published literature and genotyped for evaluation in our study population comprising 648 men diagnosed with prostate cancer and 526 heart patients as the control group. All 32 markers were polymorphic and in Hardy-Weinberg equilibrium. Thirty samples from the control group were genotyped in duplicates and the genotype concordance rate was above 99.5%.

Demographic information and clinical characteristics are listed in **Table 1**. Radical prostatectomy for clinically localized prostate cancer (cT2) was performed in 83% (535/648) of cases. Information about family history of prostate cancer in first-degree relatives was obtained from 535 (82.7%), of which 442 (82.5%) reported that none of their relatives were affected, whereas 94 (17.5%) stated at least one affected relative in the family (**Table 1**) The overall mean age of the prostate cancer group was 64.0 (95% CI: 63.5 - 64.6) years and 63.2 (95% CI: 62.5 - 64.1) years for control subjects.

3.1. Association Analysis of Alleles

Table 2 shows the unadjusted allelic ORs for each of 32 genetic variants comparing prostate cancer cases and control subjects. Four of the 32 markers were excluded due to linkage disequilebrium: SNPs rs78122894 and rs4242384 at 8q24 were filtered due to strong linkage with rs1447295 ($r^2 = 0.94$; and $r^2 = 0.96$, respectively); at chromosome 11, rs7931342 was excluded due to strong linkage with rs10896449 ($r^2 = 0.95$). Finally, variants rs5945619 at Xq11 was in strong linkage with rs5945572 ($r^2 = 0.94$) and therefore not included for further analysis. Variant rs5945572 at Xq11 was excluded from genotypic analysis due to only one allele. Thirteen SNPs exhibited significant ORs ranging from 1.20 to 1.62 ($P = 8.0 \times 10^{-4}$ - 0.04) including one marker at 4q24, 10q11, 11q13, 17q12, 17q23, 19q13, 22q13 and four at 8q24. After correction for multiple testing, only marker rs1859962 at 17q23 survived.

Variant rs16901979 was statistically significantly associated with prostate cancer risk presenting the highest OR at 8q24 and overall (1.62; 95% CI: 1.03 - 2.59; $P = 0.03$), whereas the OR of rs1447295 was the most statistically significant (1.35; 95% CI: 1.07 - 1.72; $P = 9.9 \times 10^{-3}$). The remaining markers at 8q24, rs6983267 and rs1016343 were significantly associated with prostate cancer risk (OR: 1.20; 95% CI: 1.02 - 1.42; $P = 0.03$ and OR: 1.25; 95% CI: 1.03 - 1.53; $P = 0.02$). Four SNPs each located

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at 4q24, 10q11, 19q13 and 22q13 showed a significant association with prostate cancer (rs7679673, rs10993994, rs2735839 and rs5759167), of which the rs2735839 that was previously reported to be associated with PSA exhibited the statistically most significant OR (1.45; 95% CI: 1.15 - 1.89; $P = 0.001$). At 11q23 (rs10896449 and rs7127900), 17q (rs4430796 and rs1859962) and Xq11 (rs5945572), five markers were significantly associated with prostate cancer risk with ORs ranging from 1.22 to 1.34 and *P*-values between 8.0×10^{-4} and 0.04.

Overall, 17 markers including four in linkage disequilibrium, were successfully replicated in the present study.

3.2. Best-Fitting Models

Thirteen markers were tested for the best-fitting genetic model (recessive or dominant); the model with the highest likelihood was considered the best fit for the particular marker. Ten out of 13 markers remained significant. The dominant model was the best-fitting model for the seven markers at 4q24, 10q11, 11q13, 17q23 and 22q23 (rs7679673, rs1447295, rs16901979, rs10993994, rs10896449, rs1859962 and rs5759167) (**Table 3**). The best-fitting model for rs6983267 (8q24), rs4430796 (17q12) and rs2735839 (19q13) was the recessive.

All 10 variants with significantly best-fitting genetic models were subjected to further analysis for correlation with clinico-pathological variables and for cumulative effects.

3.3. Cumulative Effect Model

Zheng *et al*. found a cumulative association between five of the SNPs genotyped in the present study and prostate cancer [41]. We tested this association on the basis of the best-fitting genetic models of the present study controlled for age. The five markers were rs4430796 (17q12), rs6983267 (8q24), rs1859962 (17q23), rs1447295 and rs16901979 (both at 8q24). Marker rs1859962 showed best fit under the dominant model in the current study in contrast to the recessive model in the study by Zheng *et al*. (dominant mode $P = 9.6 \times 10^{-4}$ versus recessive mode $P = 0.025$). The models were otherwise similar. The estimated ORs (95% CI) for men carrying any combination of 1, 2, 3 or ≥4 of the disease-associated genotypes at these five SNPs were 2.11 (1.26 - 3.52), 2.58 (1.55 - 4.31), 3.97 (2.22 - 7.12) and 6.02 (2.21 - 16.38), respectively, for prostate cancer compared to those without any risk variants (**Table 4**). The test for trend was statistically significant ($p = 1.0 \times 10^{-4}$).

Table 3. Ten SNPs associated with prostate cancer were tested significant for the best fitting genetic model.

			Allele Frequency				Best Fitting Model[a]				
SNP ID	**Reference Allele**	**Associated Allele**	*Cases*	*Controls*	**OR (95% CI)**	***P*-Value[b]**	**Model**	**Associated Genotype**	**OR**	**95% CI**	***P*-Value[c]**
rs7679673	A	C	0.65	0.60	1.20(1.01 - 1.43)	0.03	Dom	CA + CC	1.43	(1.04 - 1.99)	0.03
rs1447295	C	A	0.18	0.14	1.35(1.07 - 1.72)	9.9×10^{-3}	Dom	AC + AA	1.37	(1.05 - 1.78)	0.02
rs16901979	C	A	0.05	0.03	1.62 (1.03 - 2.59)	0.03	Dom	AC & CC	1.59	(1.02 - 2.49)	0.04
rs10993994	C	T	0.41	0.36	1.21 (1.02 - 1.44)	0.02	Dom	TC & TT	1.31	(1.03 - 1.65)	0.03
rs10896449	A	G	0.53	0.46	1.25 (1.05 - 1.48)	8×10^{-3}	Dom	GA & GG	1.44	(1.11 - 1.88)	6.6×10^{-3}
rs1859962	T	G	0.56	0.49	1.32 (1.12 - 1.56)	8×10^{-4}	Dom	TG & GG	1.60	(1.21 - 2.12)	8.5×10^{-4}
rs5759167	T	G	0.54	0.49	1.21(1.02 - 1.43)	0.02	Dom	GT & CC	1.41	(1.07 - 1.85)	0.01
rs6983267	T	G	0.56	0.52	1.20 (1.02 - 1.42)	0.03	Rec	GG	1.37	(1.06 - 1.78)	0.02
rs4430796	G	A	0.58	0.53	1.23 (1.04 - 1.45)	0.01	Rec	AA	1.45	(1.13 - 1.87)	3.6×10^{-3}
rs2735839	A	G	0.89	0.84	1.48 (1.15 - 1.89)	1.4×10^{-3}	Rec	GG	1.56	(1.19 - 2.04)	1.2×10^{-3}

[a]Model testing for best fit: dominant or recessive model is shown with corresponding genotypes; [b]Allele frequency between cases and controls was tested using Chi-square test; [c]Genotype frequency was assessed by logistic regression adjusted for age. Best-fitting genetic model was chosen based on the most significant best fit model (*P*-value).

Table 4. The Cumulative effect of five SNPs associated with prostate cancer risk.

Genotypes at five SNPs	Cases	Controls	OR (95% CI)	*P* Value	*P* Value for Trend[b]
Number of associated genotypes[a]	**Number of cases (%)**				
0	27 (4.2%)	52 (8.0%)	1		
1	198 (30.6%)	181 (27.9%)	2.11 (1.26 - 3.52)	3.5×10^{-4}	
2	240 (37.0%)	179 (27.6%)	2.58 (1.55 - 4.31)	2.0×10^{-4}	
3	130 (20.1%)	63 (9.7%)	3.97 (2.22 - 7.12)	1.0×10^{-4}	
4	25 (3.9%)	8 (1.2%)	6.02 (2.21 - 16.38)	1.0×10^{-4}	
					1.0×10^{-4}

[a]Number of PC-associated genotypes at the five SNPs for 648 cases and 526 controls; [b]Trend test by Cochrane-Armitage test for trend.

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3.4. Clinico-Pathological Variables

The association between the 10 SNPs and clinico-pathological variables under the best fitting genetic model was assessed for prostatectomized cases. Variables tested were age at diagnosis, family history, PSA, postoperative Gleason score and T stage, and biochemical recurrence. Five markers with significant associations are summarized in **Table 5**. SNPs rs6983267 (GG) and rs5759167 (GG/GT) were significantly associated with negative family history ($P = 0.04$ and $P = 0.02$, respectively). SNP rs2735839(GG) and rs10896449(GG/GA) were significantly associated with high level of PSA (>10 ng/ml) ($P = 0.04$). Marker rs2735839 (GG) demonstrated a significant association with lower Gleason score (<7) ($P = 0.0096$). Lower T stage (≤T2c) was significantly associated with rs2735839 (GG) and rs4430796 (AA) ($P = 0.02$ and $P = 0.03$, respectively). Markers rs5759167 (GG/GT) and rs7679673 (CC/CA) demonstrated significant association with lower risk for biochemical recurrence ($P = 0.015$ and $P = 0.009$, respectively), whereas rs6983267 (GG) was significantly associated with biochemical relapse ($P = 0.045$) (**Figure 1**). None of the SNPs were associated with D'Amico score.

Table 5. Assocaitions between five SNPs and clinico-pathological parameters.

	rs6983267		rs4430796		rs2735839		rs10896449		rs5759167	
	Reference	*Associated*	*Reference*	*Associated*	*Reference*	*Associated*	*Reference*	*Associated*	*Reference*	*Associated*
	GT & TT	*GG*	*GA & GG*	*AA*	*AG & AA*	*GG*	*AA*	*GA & GG*	*TT*	*GT & GG*
Number of genotypes	353	176	344	186	110	425	117	414	107	426
Age at diagnosis										
Mean	62.9	63.4	63.3	62.5	63.4	63	63	63.1	62.8	63.1
P-value[b]		0.43		0.15		0.54		0.91		0.64
Family history[a]—no. (%)										
No	283 (80.2)	154 (87.5)	278 (80.8)	159 (85.5)	92 (83.6)	350 (82.4)	98 (83.8)	340 (82.1)	80 (74.8)	359 (84.3)
Yes	70 (19.8)	22 (12.5)	66 (19.2)	27 (14.5)	19 (17.3)	75 (17.6)	19 (16.2)	75 (18.1)	27 (25.2)	67 (15.7)
P-value[c]		0.04		0.17		0.9		0.65		0.02
PSA—no. (%)										
≤10 ng/ml	136 (38.5)	59 (33.5)	127 (36.9)	68 (36.6)	50 (45.5)	148 (34.8)	53 (45.3)	144 (34.8)	33 (30.8)	165 (38.7)
>10 ng/ml	217 (61.5)	117 (66.5)	217 (63.1)	118 (63.4)	60 (54.5)	277 (65.2)	64 (54.7)	270 (65.1)	74 (69.2)	261 (61.3)
P-value[c]		0.26		0.94		0.04		0.04		0.13
D'Amico risk score—no. (%)										
Low	55 (15.6)	21 (11.9)	53 (15.4)	24 (12.9)	20 (18.2)	58 (13.6)	15 (12.8)	62 (15.0)	13 (12.1)	65 (15.3)
Intermediate	131 (37.1)	65 (36.9)	128 (37.2)	67 (36.0)	43 (39.1)	154 (36.2)	50 (42.7)	147 (35.5)	36 (33.6)	161 (37.8)
High	167 (47.3)	90 (51.1)	163 (47.4)	95 (51.1)	47 (42.7)	213 (50.1)	52 (44.4)	205 (49.5)	58 (54.2)	200 (46.9)
P-value[c]		0.48		0.65		0.28		0.37		0.38
Gleason score—no. (%)										
≤6	188 (53.3)	78 (44.3)	172 (50.0)	93 (50.0)	43 (39.1)	225 (52.9)	63 (53.8)	205 (49.5)	52 (48.6)	216 (50.7)
>6	165 (46.7)	98 (55.7)	172 (50.0)	93 (50.0)	67 (60.9)	200 (47.1)	54 (46.2)	209 (50.5)	55 (51.4)	210 (49.3)
P-value[c]		0.053		1.00		0.0096		0.41		0.7
T stage—no. (%)										
≤pT2c	251 (71.1)	132 (75.0)	238 (69.2)	145 (78.0)	70 (63.6)	317 (74.6)	86 (73.5)	299 (72.2)	72 (67.3)	314 (73.7)
>pT2c	102 (28.9)	44 (25.0)	106 (30.8)	41 (22.0)	40 (36.4)	108 (25.4)	31 (26.5)	115 (27.8)	35 (32.7)	112 (26.3)
P-value[c]		0.35		**0.03**		**0.02**		0.78		0.18

[a]Family history included at least one first-degree relative; [b]Mean age at diagnosis was tested with two-sample t-test; [c]Associations between five SNPs and clinico-pathological tumor variables tested by Chi-square test.

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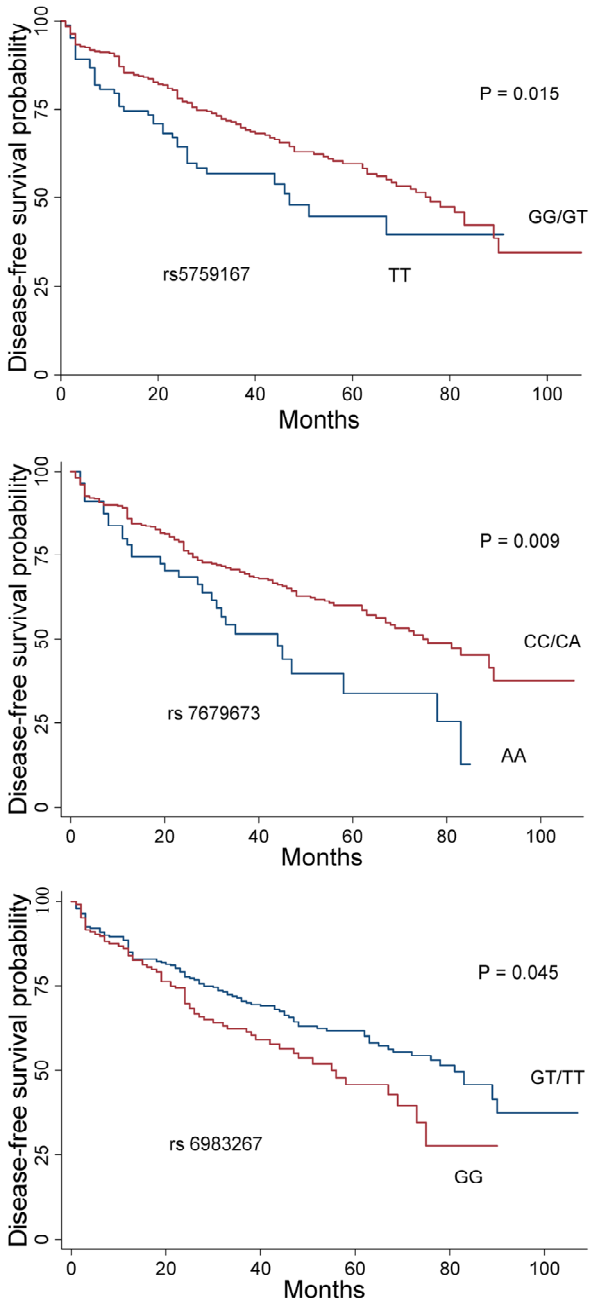

Figure 1. Kaplan-Meier plot of disease-free survival related to the genotype at SNPs rs6983267, rs7679673, and rs-5759167. Red curves represent prostate cancer-associated genotypes under the dominant mode (rs7679673 and rs-5759167, respectively), or recessive model (rs6983267). Blue curves are reference genotypes.

4. Discussion

In recent years, numerous association studies have provided information about the genetic foundation of prostate cancer and more than 40 genetic variants associated with the disease have been identified [16]. However, only a few have demonstrated a significant correlation between SNPs and clinico-pathological variables [25,41-43]. In the present study, we genotyped 32 genetic variants in a Danish study population comprising 648 prostate cancer cases and 526 control subjects. We demonstrated that 17 genetic variants were associated with prostate cancer risk in our cohort, and that the association estimates (ORs) were similar to those previously reported [14-16]. In addition to confirming previous studies, we also observed several interesting and significant associations between clinico-pathological parameters and certain variants under either recessive or dominant models.

Considering the modest effect of each SNP on the heterogeneous and common prostate cancer disease, several thousands of subjects are typically required to detect such genetic variants. We were able to replicate 17 genetic markers in nine different chromosomal regions in our study population that was only 1/3 the size of larger association studies [41,43,44]. Although the ORs were similar with those estimated in larger studies, the width of the confidence intervals reflected the comparably modest size of our study population.

We evaluated the cumulative effects of five markers also previously tested by Zheng *et al*. [41]. Zheng *et al*. demonstrated that, individually, these five markers might only contribute a modest proportion of risk, but collectively, they have considerable composite risk. Our analysis of the cumulative effects of the five SNPs was conducted without including family history as a sixth risk factor, in contrast to Zheng *et al*. [41], because family history was not obtained from control subjects in the present study. We were, nevertheless, able to reproduce the cumulative association of the five genetic variants with prostate cancer risk [41]. In the work of Salina *et al*., it was claimed that the clinical relevance of cumulative effects of five genetic variants could not be established to predict more aggressive prostate cancer, as the SNPs were not correlated with the clinical and pathological tumor variables including age at diagnosis, preoperative PSA, tumor grade and stage [44]. In our study, we observed that two of the SNPs included in the cumulative model were correlated with clinico-pathological variables. The variant rs4430796(AA) was significantly associated with lower T stage (≤T2c). Furthermore, we found that rs6983267 (GG) was associated with negative family history implying a potentially protective effect of the risk genotype (GG) against familial/hereditary prostate cancer, the marker was, however, significantly correlated with biochemical relapse (**Figure 1**). In contrast, Freedman *et al.* observed a significant association between rs6983267 (GG) and a positive family history, advanced stage, and younger age, whereas Zheng and Sun found no association between this marker and family history, tumor grade and stage, age and PSA [24,31,45].

More interestingly, we found rs2735839 (GG) in the

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KLK3 gene encoding PSA to be associated with increased levels of PSA (>10 ng/ml), lower Gleason score (<7) and lower T stage (≤T2c). In men undergoing radical prostatectomy, Xu *et al*. reported that rs2735839 (GG) was significantly associated with less aggressive prostate cancer and low T stage similar to our findings; in healthy individuals, Xu *et al*. also found an association with an elevated pre-diagnostic PSA [46]. It is not yet elucidated how genetic variation in rs2735839 influence PSA levels, but our findings suggest the following scenario: triggered by increased PSA (>10 ng/ml), carriers of the described risk genotype were more likely to be diagnosed with an indolent type of prostate cancer that was less aggressive in terms of lower Gleason score (≤6) and lower T stage (≤T2c). It may be speculated that the increased PSA levels in these patients make them prone to diagnostic examinations like needle biopsy [43,47]. Consequently, prostate cancer screening can detect indolent prostate cancer in carriers of rs2735839 (GG). Having said that, Pomerantz *et al*. found no association between rs2735839 and PSA, while Penney *et al*. observed that rs2569735 in linkage disequilibrium with rs2735839 was associated with lower PSA levels [43,47]. Such conflicting discoveries with generally opposite SNP effects on phenotypes has been explained by differentially structured linkage disequilibrium across diverse populations when the interrogated genetic variant is closely related; this occurrence is called the flip-flop phenomenon [48].

Variant rs5759167 (GG/GT) was significantly associated with a negative family history of prostate cancer, similar to rs6983267, but with a low risk for biochemical recurrence. Like rs2735839, rs10896449 (GG/GA) was significantly associated with higher level of PSA (>10 ng/ml). Pomerantz *et al*. reported a statistically significant association between rs7679673 (CC/CA) and prostate cancer specific death, but in our study, the marker was associated with a significantly lower risk for biochemical recurrence [43]. Like Pomerantz *et al*., the associations between the prostate cancer-associated SNPs and D'Amico risk score were non-significant suggesting that the risk (associated) genotypes or non-risk (reference) genotypes may have poor value in predicting aggressiveness of prostate cancer (**Table 5**).

We acknowledge that there are several potential limitations of our study. As previously mentioned, our sample size was relatively modest, and particular variants that presented borderline significance or insignificance might reflect the limitation of our sample size. However, we were able to replicate several previously reported risk SNPs. In the current study, we did not adjust for possible ancestral admixture in the study population, as information about the origins of the patients was not provided. The effect of ancestral admixture would probably not influence the association estimates significantly because we have replicated 17 prostate cancer-associated risk SNPs reported in European case subjects [16]. However, we were not able to reproduce the remaining previously identified prostate cancer-associated risk SNPs. There may be following reasons: 1) the study was not powered to estimate the modest effect measure; 2) the SNPs might confer risk in one population but not the other; and 3) the SNP effect might vary from population to population due to gene-gene or gene-environment interactions. Only one SNP survived Bonferroni correction; we chose, however, to carry on with the 10 SNPs that were statistically significant under either the recessive or the dominant model, because the allele frequencies and ORs were consistent with previous reports. Our main concern has been whether the patients with myocardial infarction were appropriate control subjects for the prostate cancer cases. A recently published meta-analysis of 14 genome-wide association studies of coronary artery diseases has, however, at least temporarily, closed the discussion whether acute myocardial infarction patients as controls were appropriate [49]. None of the reviewed variants associated with coronary heart disease were correlated with prostate cancer.

5. Conclusion

We replicated 17 previously identified prostate cancer-associated risk SNPs in a Danish case-control study and found a cumulative and significant association between five SNPs and prostate cancer. Furthermore, we noted significant associations between several prostate cancer-associated risk genotypes and less aggressive tumor variables, high level of PSA, and low risk for biochemical recurrence.

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