Association of Genetic Polymorphisms in Vitamin D Receptor Gene and Susceptibility to Sporadic Prostate Cancer

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Genetic and environmental factors are involved in prostate cancer (PCa) etiology. Single nucleotide polymorphisms (SNPs) may contribute to the PCa pathogenesis. The goal of this study is to determine the role of vitamin D receptor (*VDR*) gene polymorphisms and haplotypes in the development and progression of sporadic PCa. One hundred and thirty-three PCa patients and 157 age-matched healthy controls were genotyped for the *Apa*I (rs7975232), *Bsm*I (rs1544410) and *Taq*I (rs731236) polymorphisms in *VDR* gene by using polymerase chain reaction-restriction fragment length polymorphism. An association was observed between the *Apa*I polymorphism and PCa predisposition (P = 0.03). When compared with AA genotype, there was a highly notable difference in the frequencies of the Aa (P = 0.02), aa (P = 0.026) and *Apa*I "a" allele carriers (Aa + aa) (P = 0.009) genotypes. Furthermore, we found a statistical difference in the allele frequencies of the *Apa*I polymorphism between the sporadic PCa patients and control subjects (P = 0.013). The genotype distribution for the *Bsm*I and *Taq*I polymorphisms were similar between cases and controls ($P > 0.05$). No clinically significant relationship was found between the three-locus haplotypes and development of sporadic PCa. The genotype frequencies for the three polymorphisms of the *VDR* gene within subgroups of PCa (defined by tumor stage, Gleason score, PSA levels) were also analyzed, but no statistically noteworthy difference was observed ($P > 0.05$). As far as we know, this is the first study which investigates the relationship between *VDR* genotypes and sporadic PCa in the Turkish population. Our findings suggest that the *VDR Apa*I (rs7975232) polymorphism may play a role in the development of sporadic PCa. Exp Biol Med 233:1608–1614, 2008

Key words: *VDR* gene; polymorphism; SNP; prostate cancer

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Introduction

Prostate cancer (PCa) is one of the most commonly diagnosed forms of cancer among men in industrialized countries (1, 2). It is believed that many factors such as age, ethnicity, family history, diet, environment and genetic predisposition contribute to the etiology of this disorder (3–5). The risk of development of PCa varies among countries and ethnic groups. PCa incidence and mortality rates in Asian countries are much lower than those observed in western populations (5, 6). However, in general PCa incidence rates are rising rapidly in most countries, including low-risk populations (5, 7). The reasons for the ethnic and geographic variations in the incidence of, and mortality from, prostate cancer could be polymorphisms of genes associated with androgen secretion and metabolism (8, 9).

Vitamin D plays a prominent role in control of bone and calcium metabolism (10). Moreover, it is involved in a variety of biological processes including immune response metastasis, angiogenesis and apoptosis (11). In target cells, the biological action of 1 alpha, 25 dihydroxyvitamin D3 [$1,25(OH)_2D_3$]—active form of the vitamin D—is mostly mediated through the interaction of its receptor (*VDR*) (12). Then, this complex binds the retinoid X receptor to form a heterodimer. The heterodimer is responsible for the regulation of the other transcriptions of genes which result

Table 1. Clinical Characteristics of Prostate Cancer Patients and Controls

Characteristics	Cases ($N = 133$)	Controls ($N = 157$)
Age (mean ± SD)	68 ± 02	69 ± 01
Age range (years)	45–89	50–78
Clinical staging (cTNM)[a]		
Group I (cT1a-cT1c)	22	-
Group II (cT2a-cT2c)	57	-
Group III (cT3a-cT4)	52	-
TX	2	-
Lymph nodes		
N0	133	-
Metastasis		
M0	133	-
Pathological grade (GS)[b]		
≤6	83	-
7	23	-
8–10	26	-
GX	1	-
PSA[c] **level (ng/ mL)**		
<10	64	-
10–20	36	-
>20	33	-

[a] Tumor-lymph nodes-metastasis. According to bone scan at the time of diagnosis. TX, primary tumor that cannot be assessed; N0, no regional lymph node metastasis; M0, no distant metastasis.
[b] Gleason score. GX, the grade can not be assessed.
[c] Total serum prostate-specific antigen (PSA).

in cell cycle arrest in G1/S, apoptosis and differentiation (13). Chemopreventive effects of 1,25$(OH)_2D_3$ were shown in animal models of colon (14), gastrointestinal (15) and skin (16) cancer. It was also shown, *in vitro,* that 1,25$(OH)_2D_3$ has notable antiproliferative effects on malignant cells of prostate (17), breast (18), and colon (19) cell lines. Furthermore, Corder *et al.* (20) revealed a correlation between low 1,25$(OH)_2D_3$ blood levels and prostate cancer predisposition.

The vitamin D receptor gene is a member of the nuclear receptor family and expressed in over 30 different cell types and located on chromosome 12q12–14 (21, 22). *VDR* gene is highly polymorphic and allele frequencies are highly variable among different races and ethnic groups (23). To date, several polymorphisms at the 3′ end of the *VDR* gene have been described using different restriction enzymes such as *ApaI* (24), *Bsm*I (25), *Taq*I (26), *Tru*9I (27), and *EcoR*V (25). *Bsm*I (rs1544410) and *Apa*I (rs7975232) (both in intron 8), and *Taq*I (rs731236) (in exon 9) are the most widely studied polymorphisms in linkage disequilibrium. *Taq*I polymorphism is located at codon 352, and T→C alteration (AT$\underline{\text{T}}$ to AT$\underline{\text{C}}$) does not result in amino acid sequence change (26).

Although it is not known how these polymorphisms affect the *VDR* protein levels and functions, it is thought that 3′ untranslated (UTR) sequence variances may affect the mRNA stability and protein translation efficiency (23).

The aim of this study is to investigate the association between the *VDR* polymorphisms and haplotypes and the development and progression of sporadic PCa in a specific Caucasian (Turkish) population.

Materials and Methods

Study Population. The study group consisted of 157 controls and 133 newly diagnosed sporadic PCa cases, recruited from the Departments of Urology of the Gazi University and Abant Izzet Baysal University between 2003 and 2006. The study populations were Caucasian. Cases were classified according to the 2002 tumor-lymph nodes-metastasis (TNM) system of the American Joint Committee on Cancer (AJCC) (28) and pathological grades (29). Clinical characteristics of the cases such as prostate specific antigen (PSA) at the time of diagnosis, tumour node metastasis (TNM) stage, tumour grade, as well as age at diagnosis and family history were obtained from medical records. All patients reported that there was no history of PCa in their first and second degree relatives. This information was recorded in form of a pedigree. The control group underwent clinical urologic examination which included digital rectal examination (DRE), transrectal ultrasound of the prostate (TRUS), residual urine volume, measurement of serum PSA and physical check-up. Any samples with abnormal DRE, suspicious lesion detected by TRUS, elevated serum levels of PSA ≥ 4 ng/ml were excluded from the control group. In addition, control subjects who had family history of cancer or a previous diagnosis of cancer were excluded from the study. Written informed consent was obtained from all cases and controls. The study was approved by the Ethical Committee of the Faculty of Medicine, Gazi University (Ankara, Turkey). Pathologic grades were determined according to the Gleason pattern and classified into (6 or lower), (7), or (8 or higher). Serum PSA levels were categorized into three groups: <10, 10–20 and >20 ng/ml. The clinical stages at the time of diagnosis were classified into Group I (cT1a–cT1c), Group II (cT2a–cT2c) and Group III (cT3a–cT4). Clinical profiles of the subjects are given in Table 1.

Genotyping. Genomic DNA was extracted from peripheral blood lymphocytes using a commercially available DNA extraction kit (Heliosis®, Metis Biotechnology). Polymorphic sites in *VDR* (*Bsm*I A/G, rs1544410), (*Apa*I A/C rs7975232), (*Taq*I C/T, rs731236), were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. Primer sequences used for the amplification of a 191 bp fragment containing the *Bsm*I polymorphic site in intron 8 and PCR conditions were the same as described in Sosa *et al.* (30). The PCR products were digested by *Bsm*I (Roche Diagnostics GmbH, Mannheim, Germany) restriction enzyme at 65°C overnight. Digested PCR products with B allele remained uncut (191 bp) whereas those with b allele generated two fragments (115 bp and 76 bp).

The *Apa*I and *Taq*I polymorphisms were identified as reported by Ozkaya *et al*. (31). The 490-bp fragment generated by PCR was digested with the restriction endonucleases *Taq*I (MBI Fermentas, Lithuania) and *Apa*I (MBI Fermentas, Lithuania) at 65°C and 37°C respectively. Amplicons were digested with the restriction enzyme *Taq*I to yield a 490 bp fragment for the T allele, 290 and 200 bp for the t alleles. After digestion with *Apa*I, same PCR product was cut into 280 and 210 bp fragments in the presence of a allele, whereas A allele was undigested (490 bp). The PCR products and the restriction fragments were separated in 2% agarose gel stained with ethidium bromide, and were visualized by Logic 100 gel image system (Kodak, USA).

Analyzed *VDR* polymorphisms used to detect the base changes are shown in Table 2 (Methodical Nomenclature Recommended by Human Genome Variation Society, www.hgvs.org).

Statistical Analysis. The observed genotype frequencies were used to test for Hardy-Weinberg equilibrium (HWE) and the $\chi 2$ test. The association between VDR genotypes defined by *Bsm*I, *Apa*I, *Taq*I polymorphisms and prostate cancer was determined by analysis of Pearson chi square (χ^2). The relationship between the genotype distribution and the clinicopathological parameters such as PSA levels, GS or tumor stage was analyzed using Pearson chi square (χ^2) and Fisher's exact probability tests. Crude odds ratios (ORs) were reported with 95% confidence intervals (CI). Differences between groups were considered statistically significant if $P < 0.05$. Statistical analysis was performed using SPSS version 15.0. The Linkage Disequilibrium (LD) values for the three pairs of SNPs have been calculated using Haploview Version 4.0 (Website: http://www.broad.mit.edu/mpg/haploview). One haplotype block was identified (32). Two different software, both of which were based on the expectation maximisation (EM) algorithm, were used for the estimation of the haplotype frequencies: Arlequin (version 3.1) (33) and SNPStats (34). Odds ratios (ORs) were calculated by χ^2 test in comparison with the most common homozygote genotype as well as haplotype observed in studied population. The statistical power was calculated using the software QUANTO 1.2 (Website: http://hydra.usc.edu/gxe) (35). For the less frequent alleles (39.5% for *Bsm*I, 39.8% for *Taq*I and 37.9% for *Apa*I) with $P = 0.05$, the study had a power >80 for all polymorphisms (OR = 2.0; mode of inheritance: log-additive).

Results

The observed control genotype frequencies of the two *VDR* polymorphisms, with the exception of the *Bsm*I (rs1544410) polymorphism ($\chi^2 < 4.06$, $P = 0.04$), did not differ from those expected from Hardy-Weinberg equilibrium ($\chi^2 < 0.14$, $P = 0.71$ for the *Apa*I (rs7975232) and $\chi^2 < 0.01$, $P = 0.99$ for the *Taq*I (rs731236)). The genotype distribution in the cases was in Hardy-Weinberg equilibrium ($\chi^2 < 0.26$, $P = 0.61$ for the *Bsm*I and $\chi^2 < 0.05$, $P = 0.82$ for the *Apa*I, $\chi^2 < 0.01$, $P = 0.91$ for the *Taq*I). Genotype distributions and allele frequencies of three *VDR* polymorphisms are displayed in Table 3. There was a statistical difference in the genotype distribution of the *Apa*I polymorphism among cases and controls ($P = 0.03$). Furthermore, compared with the AA genotype, the ORs for the Aa, aa and Aa + aa increased 1.88 (95% Cl, 1.10–3.20), 2.15 (95% Cl, 1.09–4.24) and 1.95 (95% Cl, 1.18–3.23) times, respectively. Therefore, a statistical difference was found in genotypic frequencies of the *Apa*I polymorphism between the sporadic PCa patients with Aa, aa, Aa + aa genotypes and those with AA genotype ($P < 0.05$) (Table 3). In addition, we found a statistical difference in the allele frequencies of the *Apa*I polymorphism between the sporadic PCa patients and control subjects ($P = 0.013$). When we compared the genotype distribution of the *Taq*I between PCa patients and controls, we found that the TT genotype was more frequent in cases (46.6%) than in controls (36.3%), although this difference was not statistically significant. The *Bsm*I genotype distribution was similar in patients and healty controls (Table 3). We also analyzed the relation of the *Apa*I (rs7975232), *Taq*I (rs731236) and *Bsm*I (rs1544410) genotypes to the clinicopathological parameters including PSA level, GS and tumor stage but observed no statistical differences. No significant association was found between allele frequencies of *Taq*I and *Bsm*I of the *VDR* polymorphisms and prostate cancer. Pairwise LD coefficients (D′ values) for three SNPs based on genotypes of 290 individuals of the case-control study were calculated and plotted (Fig. 1). Since we observed that all three SNPs were in LD, we continued with our haplotype study. We analyzed the *Bsm*I, *Apa*I and *Taq*I separately as SNPs and also observed these three linked polymorphisms as haplotypes. The distributions of VDR haplotypes with estimation of ORs in PCa patients and controls are presented in Table 4.

Table 2. Polymorphisms in the *VDR* Gene and the Methods of Their Genotyping

	Analyzed polymorphisms		
Gene	Common nomenclature used in paper (alleles)	Methodical nomenclature	db SNP
VDR	*Apa*I (a A)	1025 - 49 G > T	rs7975232
	*Taq*I (T t)	c.1056 T > C	rs731236
	*Bsm*I (b B)	1024 + 283 G > A	rs1544410

Discussion

PCa is a heterogeneous disease (36). The prevalence of PCa varies dramatically among different geographic loca-

Table 3. Genotype and Allele Distribution of *Apa*I (rs7975232), *Taq*I (rs731236) and *Bsm*I (rs1544410) Genotypes of *VDR* Gene in the Study Subjects

	Controls		Cases		*P* values	OR (95% CI)	*P* values
Genotypes	*N*	(%)	*N*	(%)			
*Apa*I (rs7975232)					**0.03**		
AA	63	40.1	34	25.6		1[a]	
Aa	69	44	70	52.6		1.88 (1.10–3.20)	**0.020**
aa	25	15.9	29	21.8		2.15 (1.09–4.24)	**0.026**
Aa + aa[b]	94	59.9	99	74.4		1.95 (1.18–3.23)	**0.009**
AA + Aa[c]	132	84.1	104	78.2		0.68 (0.38–1.23)	0.200
Alleles							
A	195	62.1	138	51.9		1[a]	
a	119	37.9	128	48.1		1.52 (1.09–2.12)	**0.013**
Genotypes	*N*	(%)	*N*	(%)			
*Taq*I (rs731236)					0.173		
TT	57	36.3	62	46.6		1[a]	
Tt	75	47.8	56	42.1		0.69 (0.42–1.13)	0.139
tt	25	15.9	15	11.3		0.55 (0.26–1.15)	0.110
Tt + tt[d]	100	63.7	71	53.4		0.65 (0.41–1.05)	0.075
TT + Tt[e]	132	84.1	118	88.7		1.49 (0.75–2.96)	0.253
Alleles							
T	189	60.2	180	67.7		1[a]	
t	125	39.8	86	32.3		0.72 (0.51–1.02)	0.062
Genotypes	*N*	(%)	*N*	(%)			
*Bsm*I (rs1544410)					0.350		
bb	50	31.9	53	39.9		1[a]	
Bb	90	57.3	66	49.6		0.69 (0.42–1.14)	0.148
BB	17	10.8	14	10.5		0.78 (0.35–1.74)	0.539
Bb + BB[f]	107	68.1	80	60.1		0.71 (0.44–1.14)	0.156
Bb + bb[g]	140	89.2	119	89.5		1.03 (0.49–2.18)	0.934
Alleles							
b	190	60.5	172	64.7		1[a]	
B	124	39.5	94	35.3		0.84 (0.60–1.17)	0.304

OR, odds ratio; CI, confidence interval. *P* values $<$0.05 are shown in **bold**.
[a] Reference genotype/allele.
[b] Comparing of subjects with Aa + aa genotypes versus AA genotype.
[c] Comparing of subjects with AA + Aa genotypes versus aa genotype.
[d] Comparing of subjects with Tt + tt genotypes versus TT genotype.
[e] Comparing of subjects with TT + Tt genotypes versus tt genotype.
[f] Comparing of subjects with Bb + BB genotypes versus bb genotype.
[g] Comparing of subjects with Bb + bb genotypes versus BB genotype.

tions. Single nucleotide polymorphisms involved in steroid metabolism might be the cause of this difference (8, 9).

It is possible that various genes act together, in connection with other factors of the individual or the individual's environment, to induce PCa development, prognosis and metastasis (37). Owing to the biological importance of Vitamin D function, polymorphisms of the *VDR* gene are the most widely studied (11, 26). Although the effects of the *Bsm*I and *Apa*I polymorphisms on any splicing or transcription factor binding site are not presently known (38), it is possible that these polymorphisms might be linked to another genetic variation in the *VDR* gene itself or nearby polymorphic gene.

A study on the *Apa*I *VDR* polymorphism in Asian population did not observe an association between the frequency of any other particular genotypes and PCa (39). Two family-based association studies investigated the relationship between the *VDR* gene polymorphisms and PCa predisposition in Caucasian (40), African-American (40) and Asian (41) populations but no differences were found among cases and controls. Habuchi *et al.* (5) observed a significant difference between PCa and female controls. In a recent family-based study, Cicek *et al.* (42) found an

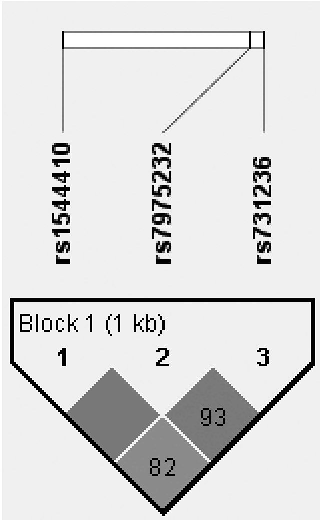

Figure 1. Locations and the linkage disequilibrium plot (obtained using Haploview) showing pairwise D′ values (as percentage) between the three polymorphisms on *VDR* gene. LD blocks are framed in black and were classified according to the "solid spine" option (32). Each square plots the level of D′ values between a pair of SNPs. The markers 1, 2, 3 are *Bsm*I (rs1544410) A/G, *Apa*I (rs7975232) A/C, *Taq*I (rs731236) C/T, respectively.

association between the *Apa*I polymorphism and PCa. As far as we are concerned, the present study is the first one that demonstrates a relationship between the Aa, aa, *Apa*I "a" allele carriers (Aa + aa) genotypes of the *Apa*I (rs7975232) polymorphism and sporadic PCa in Caucasians. In our population-based case control study, we found that the PCa risk, in comparison to individuals with the AA genotype, increased in all individuals with either the Aa genotype ($P = 0.02$) or the aa genotype ($P = 0.026$) or *Apa*I "a" allele carriers ($P = 0.009$). These results show that, *Apa*I "a" allele of the *VDR* gene may be a risk factor for sporadic PCa. Furthermore, compared to the findings of Cicek *et al.* (42), we observed a significant increase in the distribution of the AA genotype frequency in our control group. Suzuki *et al.* (41) did not observe a relationship between clinicopathologic parameters and genotype distributions of the *Apa*I (rs7975232) polymorphism. Our results are similar to the findings of Suzuki *et al.* (41).

In this research, the genotype distributions and allele frequencies of the *Taq*I (rs731236) polymorphism were consistent with the findings of Cicek *et al.* (42). However, previous population-based case-control studies have been inconclusive regarding the association between the *Taq*I (rs731236) polymorphism and the PCa predisposition. Taylor *et al.* (2) indicated a positive association between the T alleles and PCa development. Correa-Cerro *et al.* (43) also observed that men with the Tt genotype had half the risk of PCa compared with those with the TT genotype. However, a meta-analysis which included 14 studies (44), and an additional five studies (39, 40, 45–47) observed no relationship between this polymorphism and PCa. Our findings did not support the previously reported association of T allele for the *VDR* gene with an increased risk of PCa (2, 43). Besides, Ma *et al.* (48) displayed the reduced risk observed for the *tt* genotype among men with lower $1,25(OH)_2D_3$. Moreover, two researchers reported a noteworthy association between the TT genotype and advanced stage (3, 45) of PCa. However, these findings were not confirmed by Ma *et al.* (48), Blazer *et al.* (49) and Gsur *et al.* (50). Our results displayed no correlation between the TT genotype and the clinical variables as well.

The *Bsm*I (rs1544410) genotype distribution indicated a narrow departure from Hardy-Weinberg equilibrium due to excess heterozygotes and low frequency of the BB genotype in controls. Furthermore, deviation from Hardy-Weinberg equilibrium was also found in control groups in three previous case-control studies (5, 51, 52). According to a meta-analysis, B allele frequency of the *VDR* was 41% in Caucasian and 14% in Asian control groups (44). Our genotype distributions are similar with those in the Caucasians studies by Ntais *et al.* (44). While a family-based study (40) found no difference between *Bsm*I genotype and PCa, Suzuki *et al.* (41) showed a weak association between the *Bsm*I genotype and PCa in subjects less than 70 years of age. Population-based case-control studies on PCa did not reveal a relation between any

Table 4. Distribution of *VDR* Haplotypes in Controls and Prostate Cancer Cases

Haplotypes	Controls ($2N = 314$) (%)	Cases ($2N = 266$) (%)	OR (95% CI)	P^a value
baT	119 (37.9)	125 (46.85)	1[b]	
BAt	111 (35.45)	75 (28.28)	0.64 (0.44–0.95)	**0.025**
bAT	57 (18.25)	38 (14.15)	0.64 (0.39–1.03)	0.070
BAT	13 (4.04)	17 (6.58)	1.25 (0.58–2.67)	0.699
bAt	14 (4.36)	8 (2.86)	0.54 (0.22–1.34)	0.265
bat	—	2 (0.80)	NC[c]	
Bat	—	1 (0.48)	NC[d]	

OR, odds ratio; CI, confidence interval. P values <0.05 are shown in **bold**.
[a] Haplotype frequencies between controls and prostate cancer cases were calculated by χ^2 test.
[b] Reference haplotype.
[c] NC, not calculated. There are no controls having bat haplotype.
[d] NC, not calculated. There are no controls having Bat haplotype.

particular genotype of the *Bsm*I and the risk of developing PCa (4, 5, 39, 51–53). Clinicopathological parameters were also examined in relation to *Bsm*I genotype but no correlation was found between them (4, 51, 53) except in Huang *et al*. (39). Furthermore, Ma *et al*. (48) revealed that serum 1,25(OH)$_2$D$_3$ levels were significantly higher in men with BB genotypes.

A meta-analysis found no evidence indicating that any of the three alleles alone, was associated with PCa (44). Our findings did not differ from the mentioned study.

BAt haplotype was defined as protective for Caucasian populations in two investigations (2, 26). Moreover, Morrison *et al*. (26) showed that BAt haplotype had 140% more excessive receptor activity than baT haplotype. Cicek *et al*. (42) also showed that the distribution of a baT haplotype was slightly higher in patients with PCa than in the controls. In accordance with the findings of Cicek *et al*. (42), our study confirms that the most common haplotype in Caucasians is baT. Although, a statistical difference was observed haplotype frequency of BAt (OR: 0.64; 95% CI, 0.44–0.95; $P = 0.025$), this result was not clinically significant due to its negligible OR.

Consequently, our findings suggest that *Apa*I (rs7975232) polymorphism in the intron 8 of the *VDR* gene may confer susceptibility to sporadic PCa which may have important implications for understanding the pathogenesis of this cancer. With the support of our findings by successive research studies, *Apa*I (rs7975232) polymorphism may help detect individuals with higher risk of PCa. A limited number of patients were enrolled in our study which led us to work with a relatively small sample size. Therefore, further large-scale case-control studies on PCa, like Thomas *et al*. (54) and Eeeles *et al*. (55), are needed to better understand susceptibility to PCa. Confirmation of the importance of *Apa*I polymorphism in the development and progression of sporadic PCa and selection of the best treatment strategies in this respect could be possible through further studies based on larger sample sizes.

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