

Study of Vitamin D Receptor Gene Polymorphism (FokI, TaqI and ApaI) Among Prostate Cancer Patients in North India

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ABSTRACT

Introduction: Incidence of prostate cancer is rising worldwide. Multiple factors have been suggested for the aetiology of prostate cancer including ethnic, genetic and diet. Vitamin D (calcitriol) has been shown to have role in cell growth and differentiation and its deficiency is implicated as one of the aetiological factors in prostate cancer. Prostatic epithelial cells express Vitamin D Receptor (VDR) as well as 1α -hydroxylase enzyme that are required for the synthesis of calcitriol and its action. Polymorphism in VDR gene has been associated with prostate cancer in some epidemiological studies; but, there is paucity of information in the Indian context.

Aim: The present study was aimed to explore the association of VDR gene polymorphism with the development of prostate cancer.

Materials and Methods: Three Single Nucleotide Polymorphisms (SNP) sites viz., FokI, TaqI and ApaI were analysed in 120 cases of prostate cancer which were compared with their 120 healthy first degree relatives and 120 non-related controls in the Department of Biochemistry in collaboration with the Department of Urology.

Results: Analysis showed significantly decreased incidence of Tt and Aa genotype in prostate cancer patients as compared to healthy non-relative controls ($p=0.016$ and 0.043 respectively). As compared to first degree relatives, incidence of Tt genotype is significantly lower in cases ($p=0.005$). No significant association was found with FokI polymorphism.

Conclusion: This study suggests the protective role of heterozygous genotypes of TaqI and ApaI polymorphism against the development of prostate cancer.

Keywords: Polymerase chain reaction, Restriction fragment length polymorphism, Single nucleotide polymorphisms

INTRODUCTION

Prostate cancer is the most commonly diagnosed non-cutaneous cancer [1] and its incidence is rising rapidly in most countries including India [2]. As prostate cancer mostly affects older people, its incidence is likely to increase in near future as average life expectancy is on a rise. Men over 50 years have about 40% chance of having cancer in the prostate, regardless of nationality, race or ethnicity [3]. The aetiology of prostate cancer is unclear, although current evidence suggests that it is the result of multiple factors such as ethnic, environmental, genetic, hormonal and diet [4]. A significant hereditary element in the susceptibility of development of prostate cancer has been recognized by various genetic studies. These studies have revealed two-fold to three-fold increased risk of developing prostate cancer in men who have a first-degree relative (father, brother, son) with prostate cancer as compared to men with no family history [5]. Numerous genes' polymorphic sites have been studied to establish an association with prostate cancer to predict its risk of development which yielded inconsistent results. These genes include genes encoding the Androgen Receptor (AR), CYP17, and 5α -reductase type 2 (SRD5A2), CYP3A, Prostate Specific Antigen (PSA), Insulin-like Growth Factor (IGF)-1, and IGF-binding protein 3 [6,7]. But, prostate cancer lacks an established genetic marker for predicting susceptibility and progression.

Besides its role in maintaining calcium homeostasis, vitamin D is now known to affect cell growth and differentiation, immune function and can protect against cardiovascular disease, infections, cancer and autoimmune diseases such as multiple sclerosis [8-12]. By expressing 1α -hydroxylase, prostatic epithelial cells synthesize active form of vitamin D i.e., $1,25$ -dihydroxyvitamin D_3 which plays a

growth regulating role in prostate [13]. In addition, calcitriol exhibits anti-proliferative and pro-differentiating activities in malignant prostate cell lines and in some in vivo models of prostate cancer [14-17]. Mechanism of action of $1,25(OH)_2D_3$ is mediated by its binding to VDR. VDR functions as a heterodimer, generally with the retinoid X receptor for regulation of vitamin D target genes. The gene encoding the VDR is located on chromosome 12q13.11, contains 14 exons and spans approximately 75 kilobases of genomic DNA [18-20]. Various polymorphisms have been identified in the VDR gene such as *ApaI*, *BsmI*, *TaqI*, *FokI*, *Tru9I*, *cdx2* and *EcoRV*. FokI (rs 2228570) polymorphism, present in exon 2, produce a shorter VDR protein which is more effective in transactivation of the $1,25(OH)_2D_3$ signal [20,21]. TaqI (exon 9) and ApaI (intron 9) polymorphisms, however, do not alter the amino acid of the VDR protein; but, they may influence gene transcription and mRNA stability [22].

There is paucity of knowledge about VDR gene polymorphism and its association with prostate cancer in Indian population. Therefore, we conducted this study to investigate possible association of three VDR gene polymorphisms (FokI, TaqI and ApaI) with prostate cancer.

MATERIALS AND METHODS

This case-control study was conducted in the Department of Biochemistry, Vardhman Mahavir Medical College and Safdarjung Hospital, New Delhi, India, in association with the Department of Urology, at a tertiary care hospital. Clearance from Institutional Ethical Committee was obtained preceding the study. Cases included 120 newly diagnosed patients of prostate cancer which were histologically confirmed. All cases were in advance stage of

the disease. Advanced prostate cancer was defined according to Surveillance Epidemiology and End Results (SEER) 1995 pathologic and clinical extent of disease codes 41–85 [23]. Control groups consisted of group I which included 120 normal age and sex matched healthy controls selected from the volunteers with Prostate Specific Antigen (PSA) levels <4.0ng/ml and no history of prostate cancer among their first degree relatives and group II consisted of 120 first degree relatives of prostate cancer patients. First degree relatives include prostate cancer patients' sons or their brothers. All normal healthy controls as well as first degree relatives were screened for PSA level (normal <4.0 ng/ml). Selected controls did not have any history of cancer and/or prostate surgery. Written informed consent was taken from all subjects. Blood samples were collected in vials containing EDTA K2 anticoagulant and stored at -80°C till further analysis. Total genomic DNA was isolated from whole blood using the method described by Daly AK et al., [24]. The required region of VDR gene from the genomic DNA was amplified by PCR in MJ Research PTC-100™ (Peltier Thermal Cycler) using the primers as follows:

Primers for FokI: (Harris SS et al., [25])

Forward: 5'- AGC TGG CCC TGG CAC TGA CTC TGC TCT-3'

Reverse: 5'- ATG GAA ACA CCT TGC TTC TTC TCC CTC-3'

Primers for TaqI and Apal: (Riggs BL et al., [26])

Forward: 5'- CAG AGC ATG GAC AGG GAG CAA-3

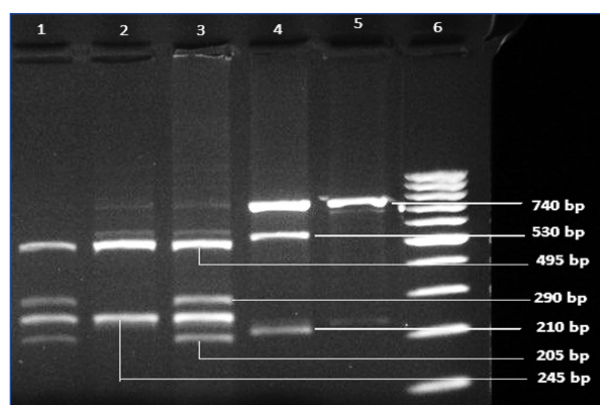
Reverse: 5-GCA ACT CCT CAT GGC TGA GGT CTC-3

For FokI, PCR product of 265 bp was obtained and verified using a 2% agarose gel. The PCR product was digested with Fok-I restriction enzyme obtained from New England Biolabs (NEB). The FF genotype lacked a Fok-I site and showed only one band of 265 bp. The ff genotype generated two fragments of 196 and 69 bp. The heterozygote displayed three fragments of 265, 196 and 69 bp, designated as Ff.

[Table/Fig-1] shows Agarose gel picture of electrophoresis pattern of restricted enzyme digested PCR products showing Heterozygous (Tt) in lane 1 and 3, Homozygous (TT) in lane 2, Heterozygous (Aa) in lane 4, Homozygous (AA) in lane 5 and 100 bp marker in lane 6.

In case of TaqI polymorphism, the PCR product of 740 bp was obtained and digested with Taq-I restriction enzyme from NEB. Taq-I digestion revealed one obligatory restriction site, the homozygous TT (absence of the specific Taq-I restriction site) yielded bands of 245 bp and 495 bp. The homozygous tt exhibited 205, 245, 290 bp and the heterozygous Tt provided 495, 205, 245, 290 bp fragments.

For Apal polymorphism, PCR product of 740 bp was digested with Apa-I restriction enzyme. The lack of Apal site showed only one band at 740 bp and designated as AA, whereas presence of restriction site showed two fragments at 210 and 530 bp and designated as



[Table/Fig-1]: Agarose gel picture of electrophoresis pattern of restricted enzyme digested PCR products showing Heterozygous (Tt) in lane 1 and 3, Homozygous (TT) in lane 2, Heterozygous (Aa) in lane 4, Homozygous (AA) in lane 5 and 100 bp marker in lane 6.

aa. The heterozygote displayed three fragments of 740, 210 and 530 designated as Aa [Table/Fig-1].

STATISTICAL ANALYSIS

Data was analysed using Graph Pad Prism 5.0 version. Group difference between ages was evaluated by unpaired t-test. Conformity towards Hardy-Weinberg equilibrium was calculated by the Chi-square test. Association between groups for genotypes and alleles were determined by contingency table analysed by calculating Odd's ratio. A p-value <0.05 was considered as statistically significant.

RESULTS

Age group analysis showed that 80% of the patients were above 60 years of age and 20% between the ages 51-60 years [Table/Fig-2].

[Table/Fig-3] and [Table/Fig-4] shows the distribution of VDR gene polymorphism (TaqI, Apal and FokI) in the study groups. Frequencies of single allele are shown in [Table/Fig-5]. Difference between single allelic frequencies was not significant. Distribution of genotypes by Chi-square test was carried out to find whether these genotypes are in accordance with Hardy-Weinberg Equilibrium. Comparison of VDR gene polymorphism between prostate cancer and control Group I (healthy non-related controls) showed that incidence of Tt and Aa genotype was more in controls as compared to cases and this was statistically significant [Table/Fig-3]. No significant association was found in FokI polymorphism. Single alleles were also compared between each group and no significant difference was found. Comparison of VDR gene polymorphism between prostate cancer and control Group II (first degree relatives of cases) was made [Table/Fig-4]. It shows higher incidence of Tt genotype in control Group II as compared to cases. None of the Apal and FokI genotype was found to be statistically significant. Proportion of each single allele was also compared and no significant difference was found.

Thus, it was observed that Tt and Aa genotype was significantly low in prostate cancer patients when compared to healthy controls.

Characteristics	Cases (N=120)	Control Group I (N=120)	Control Group II (N=120)
Mean age in years	68.3	65.8	40.4
Alcohol drinking			
Yes	52 (43.3%)	44 (36.7%)	48 (40%)
No	68 (56.7%)	76 (63.3%)	72 (60%)
Smoking			
Yes	63 (52.5%)	58 (48.3%)	54 (45%)
No	57 (47.5%)	62 (51.7%)	66 (55%)

[Table/Fig-2]: Demographic characteristics of study subjects.

Polymorphism	Genotype and allele	Cases n (%)	Control Group I n (%)	Odds Ratio	95% CI	p-value
TaqI	TT	68 (56.7)	40 (33.3)	1 (ref)		
	Tt	36 (30)	74 (61.7)	0.286	0.108–0.758	0.016*
	tt	16 (13.3)	6 (5)	1.569	0.307–8.014	0.692
Apal	AA	56 (46.6)	40 (33.4)	1 (ref)		
	Aa	32 (26.7)	70 (58.3)	0.336	0.120–0.941	0.043*
FokI	FF	64 (53.3)	86 (71.7)	1 (ref)		
	Ff	52 (43.3)	30 (25)	2.329	0.911–5.956	0.091
	ff	4 (3.3)	4 (3.3)	1.344	0.114–15.87	1.00

[Table/Fig-3]: Comparison of VDR gene polymorphism between cases v/s control group I (healthy non-related controls). *p<0.05

Polymorphism	Genotype	Cases n (%)	Control Group II n (%)	Odds Ratio	95% CI	p-value
TaqI	TT	68 (56.6)	28 (23.3)	1 (ref)		
	Tt	36 (30)	84 (70)	0.177	0.054-0.573	0.05*
	Tt	16 (13.4)	8 (6.7)	0.824	0.121-5.575	1.00
Apal	AA	56 (46.6)	44 (36.7)	1 (ref)		
	Aa	32 (26.7)	60 (50)	0.419	0.131-1.345	0.160
	Aa	32 (26.7)	16 (13.3)	1.571	0.373-6.613	0.723
FokI	FF	64 (53.3)	84 (70)	1 (ref)		
	Ff	52 (43.3)	32 (26.7)	2.133	0.713-6.376	0.274
	Ff	4 (3.3)	4 (3.3)	1.313	0.0761-22.64	1.00

[Table/Fig-4]: Comparison of VDR gene polymorphism between cases v/s control Group II (first degree relatives of cases).
p* < 0.01

Polymorphism	Allele	Cases n (%)	Control Group I n (%)	Control Group II n (%)
TaqI	T	172 (71.7)	154 (64.2)	140 (58.3)
	t	68 (28.3)	86 (35.8)	100 (41.7)
Apal	A	144 (60)	148 (61.7)	148 (61.7)
	a	96 (40)	92 (38.3)	92 (38.3)
FokI	F	180 (75)	202 (84.2)	200 (83.3)
	f	60 (25)	38 (15.8)	40 (16.7)

[Table/Fig-5]: Distribution of allelic frequency among cases and controls.

Sr. No.	Study	Country	Polymorphism	Association
1.	Chokkalingam et al., [36]	China	FokI, BsmI	NS
2.	Taylor JA et al., [30]	USA	TaqI	'tt' protective
3.	Correa-Cerro L et al., [29]	Germany	TaqI	'Tt' protective
4.	Medeiros R et al., [28]	Portugal	TaqI	T causative
5.	Luscombe CJ et al., [37]	UK	FokI, TaqI	NS
6.	Mishra DK et al., [31]	India	FokI	'FF' causative
7.	Torkko KC et al., [38]	USA	FokI	NS
8.	Bai Y et al., [39]	China	FokI, TaqI, Apal	NS
9.	Jingwi EY et al., [40]	USA	TaqI, Apal, BsmI	TT and AA causative
10.	Nunes HB et al. [41]	Brazil	FokI, TaqI, Apal, BsmI	NS
11.	Present study	India	FokI, TaqI, Apal	'Tt' and 'Aa' protective

[Table/Fig-6]: Worldwide studies on genetic variants of VDR and association with risk of prostate cancer [28-31,36-41].
NS: Not significant

Frequency of *Tt* genotype was also low in prostate cancer patients as compared to relative control group. No significant association was found with FokI polymorphism.

DISCUSSION

In the present study, we investigated the association of three VDR gene polymorphisms (FokI, TaqI and Apal) with the development of prostate cancer. Our study revealed that persons having heterozygous allele, *Tt* and *Aa* have protection against the development of prostate cancer. However, we did not find association between prostate cancer and FokI polymorphism.

There is a large variation in the incidence rates of prostate cancer among racial ethnic groups worldwide. Incidence is high in Western world as compared to Asia [27]. The epidemiological data supports a major genetic component to prostate cancer risk but the studies

associating VDR gene polymorphisms with the risk of development of prostate cancer has shown conflicting results.

Medeiros R et al., conducted a study in Portugal and found that both *TT* and *Tt* genotypes are over represented in prostate cancer patients [28]. In a study consisting of French and German men; the *t* allele was found to be the risk allele and linked with increase prostate cancer rates. In this case however, only the *Tt* genotype was found to be statistically significant in the risk of prostate cancer [29]. Taylor JA et al., claimed that men with the homozygous *t* allele had a one-third risk for developing prostate cancer compared with men who were heterozygotes or homozygotes for *T* allele [30]. In our study, we found that heterozygous *Tt* allele has protective role against the development of prostate cancer.

The study conducted in India by Mishra DK et al., found a higher incidence of FF genotype in patients as compared to controls (60.9 vs. 42.2 %). The frequency of *ff* genotype was significantly lower in cases (3.9%) while our study observed *ff* genotype in 3.3% of patients. They also opined that the *f* allele could be protective in nature and hence less aggressive [31].

Several authors have conducted meta-analysis of the published studies so far. A meta-analysis of 36 published studies carried out by Yin M et al., suggested that TaqI *t* allele was associated with reduced prostate cancer risk in overall population, whereas Apal *a* allele was associated with reduced prostate cancer risk only in Asian population. In contrast, FokI *f* allele was associated with a trend of increased prostate cancer risk only in Caucasian population [32]. Zhang Q et al., performed a meta-analysis of 40 studies associating VDR gene polymorphism and prostate cancer and concluded that FF genotype had protective effect on prostate cancer in the Caucasian population. Conversely, TT genotype was associated with increased risk of prostate cancer whereas no significant association was found between Apal gene polymorphism and prostate cancer risk [33]. A recent meta-analysis done by Wang K et al., demonstrated a non-significant association Apal polymorphism with prostate cancer risk [34].

These conflicting reports may be due to the differences in the ethnicity, as it has been observed that polymorphisms having positive association in Asian population have little or no effect on prostate cancer risk in Caucasians and vice versa [35]. Based on the observations of the present study, it can be suggested that '*Tt*' and '*Aa*' genotypes might have a protective role against the development of prostate cancer. [Table/Fig-6] depicts different reports regarding VDR gene polymorphism and prostate cancer risk [28-31,36-41]. Although, TaqI and Apal polymorphism does not seem to affect VDR amino acid sequence, their distribution may be relevant with VDR mRNA stability and gene transcription [42].

This would result in alteration in the ability of VDR protein to bind $1,25(\text{OH})_2\text{D}_3$ or activate VDRE gene and results in changes in the expression of regulatory genes, such as CDK, which control prostatic cell division. The protective effect of *Tt* and *Aa* suggests that these genotypes may be less responsive to cell proliferation. But, further research is required including large cohort population of different race and ethnicity using more polymorphic sites to apply this finding for clinical application. This study can also be useful in near future for genetic screening of prostate cancer susceptibility.

LIMITATION

The limitation of the study is small sample size. A study on larger sample size is needed to further explore association of VDR gene polymorphism with development of prostate cancer.

CONCLUSION

This study indicated protective role of '*Tt*' and '*Aa*' genotypes against the development of prostate cancer in North Indian population while FokI polymorphism did not reveal any association.

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