RESEARCH ARTICLE

Association of Vitamin D Receptor Gene Polymorphisms with Prostate Cancer Risk in the Pakistani Population

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Abstract

<u>Background</u>: Vitamin D receptor (VDR) gene has been a subject of extensive pharmacogenetic research recently. Association studies between different types of cancers including prostate cancer (PCa) and VDR gene polymorphism have also been conducted. The objective of this study was to find possible associations between PCa and VDR gene polymorphisms in the Pakistani population. <u>Materials and Methods</u>: A total of 162 subjects, including prostate cancer patients and controls, were genotyped for *Apa I, Taq I* and *Fok I* polymorphisms in the VDR gene using allele specific PCR, PCR-RFLP and direct DNA sequencing. Allelic frequencies were tested for Hardy-Weinberg equilibrium and associations between the genetic markers and PCa were calculated using logistic regression. <u>Results</u>: *Apa I* CC genotype was found to have strongest association with PCa risk, and "A" genotype was found to have protective effect. *Fok I* and *Taq I* did not have appreciable levels of association with PCa, although *Taq I* "TC" heterozygotes seemed to have some protective effect. Similarly the "C" allele of *Fok I* also seemed to have protective effect. <u>Conclusions</u>: To our knowledge, this is the first report showing association between VDR gene polymorphisms and PCa in Pakistan. Our findings may be somewhat skewed because of small sample size and tendency of consanguineous marriages in Pakistani society; nevertheless, it shows the trend of association and protective effects of certain VDR gene polymorphisms against PCa.

Keywords: Prostate cancer - VDR gene - poklymorphisms - Pakistan

Introduction

Prostate Cancer (PCa) is one of the most common cancer in men above 60 years of age (Stangelberger et al., 2008). The National Cancer Institute predicts 233,000 new cases in year 2014 alone. In Pakistan, PCa is reported as the most common cancer in elderly males (Arshad and Ahmad, 2013). Another recently study conducted, places prostate cancer at the third position of all the most commonly encountered malignancies among all Pakistani males (Jamal et al., 2014). Pathology of PCa is mainly chalked up to imbalance between cell growth and apoptosis in prostate tissue. Other cancers, PCa is multi-step and multi-factorial disease. Age, genetics and ethnicity are the factors most commonly implicated in the pathogenesis of PCa (Sporn and Suh, 2002; Nelson et al., 2003). Low serum levels of vitamin D are found to be associated with risk of different types of cancers (Giovannucci, 2009). The link between calcitriol and the inhibition of PCa is already well-established (Krishnan et al., 2003; Beer and Myrthue, 2004). Antiproliferative effect of calcitriol has been ascertained on many PCa cell lines (Peehl et al., 1994; Miller et al., 1995). Similarly, reports have been unanimous in hailing anticancer effect of calcitriol on prostate cancer mice models (Feldman et al., 1995; Trump et al., 2004). The actions of calcitriol require intact signaling pathways, which are all initiated by binding of active vitamin D to vitamin D receptor (VDR) (Vanoirbeek et al., 2011). *VDR* belongs to a steroid nuclear receptor super-family, which is a ligand-activated transcription factors (Haussler et al., 1995). Vitamin D- Vitamin D receptor complex bind to retinoid X receptor to form a heterodimer, which is responsible for expression/repression of genes involved in cell cycle arrest, apoptosis and differentiation (Jensen et al., 2001).Vitamin D receptors are present in bone, intestine,

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kidney and the parathyroid gland, but more importantly, high levels of *VDR* are taken as a positive indicators of prostate cancer regression (Krishnan and Feldman, 2010; Hendrickson et al., 2011). This discovery led to the idea that variation in *VDR* gene might have some effect in the risk &/or prognosis of PCa. *VDR* gene is more than 100 kb in size and is located on q arm of chromosome 12 (Uitterlinden et al., 2004). Four most commonly studied *VDR* polymorphisms are *Fok* I, *Taq* I, *Apa* I and *Bsm* I. The *Fok I* polymorphism lies in the exonic region and is associated with change in the reading frame of *VDR* gene (Gross et al., 1998). On the other hand, *Bsm* I, *Apa* I and *Taq I* polymorphisms are located in the 3'-UTR region and have no direct effect on the protein sequence. It is are however, reported that 3'-UTR region may change the mRNA stability (Whitfield et al., 2001).

Association between *VDR* gene polymorphisms and prostate cancer risk and/or prognosis has been a matter of debate. Several studies have shown correlation between prostate cancer and *VDR* polymorphisms are (Mishra et al., 2005; Onen et al., 2008; Bai et al., 2009; Raimondi et al., 2009; Oh et al., 2014; Xu et al., 2014). However, there are other studies that could not find any significant association (Guo et al., 2013). One of the reason for these conflicting reports may be differences in the population as it has been observed that SNPs bearing positive association in Asian population have little or no effect on prostate cancer risk in Caucasians and vice versa (Dianat et al., 2009). Association between *VDR* gene polymorphisms and prostate cancer risk has not been established in Pakistani population. In view of these facts, we designed this study to identify any possible association of *VDR* gene polymorphisms with prostate cancer risk in Pakistani population.

Materials and Methods

Study design and population

We designed our research as a case control study and non-probability purposive sampling was employed to obtain the samples. The study was approved by institutional ethical committee and was in concordance to Helsinki declaration. In total 162 samples of Pathan descent, including 114 normal healthy controls (age:

50 years±10) and 48 prostate cancer patients (age: 55 years±4.5) were collected. Informed written consent was obtained from all the participants. The patients were collected from Institute of Radiotherapy and Nuclear Medicine (IRNUM), Peshawar. All patients had confirmed prostate cancer following histopathological examination. They were at advanced stage of the disease and were under radiation therapy. Prostate-specific antigen levels of the patients was also obtained.

DNA extraction and SNP genotyping

Genomic DNA was extracted from the venous blood samples of selected patients using standard DNA extraction kit (Vivantis, CA, USA). Allele specific primers were designed for SNPs in *VDR* gene region i.e. rs731236 (*Taq* I), rs2228570 (*Fok* I) and rs7975232 (*Apa* I) using Primer 3 online tool (Koressaar and Remm, 2007; Untergasser et al., 2012). Standard polymerase chain reaction (PCR) of 50μl was carried out using 50ng of genomic DNA and 2 units of Taq DNA polymerase. Primer annealing temperatures were different for each SNP and are mentioned in Table 1. The PCR product was run on 2% agarose gel prestained with ethidium bromide and alleles were assigned based on the presence or absence of allele specific band. Alleles that could not be characterized through allele specific PCR were confirmed through PCR-RFLP using respective restriction enzymes. Finally, the results were confirmed through direct DNA sequencing of the regions of *VDR* gene flanking the polymorphism.

Statistical analysis

We used OEGE online tool to test the distribution of different alleles (Rodriguez et al., 2009) Web-based tool (http://www.socscistatistics.com/tests/chisquare/Default2.aspx) was used for calculation of Chi-square statistics and the odds ratio was calculated using Medcalc easy-to-use software (http://www.medcalc.org/calc/odds_ratio.php). A p-value of less than 0.05 was considered as statistically significant.

Results

The genotype distribution in control samples of three SNP (rs7975232, rs731236, rs2228570) were not

Table 1. Sequences of Primers Used for Genotyping by Allele Specific PCR

#	Primer ID	Sequence	Annealing Temperature (ºC)	Exp. Product size
rs731236 (Taq I)	1. VDR3-36-F	GCAGAGCAGAGTTCCAAGCAGAG	61	592
	2. VDR3-36T-R	CGGTCCTGAATGGCCACA		
	3. VDR3-36C-F	CAGGACGACGCGCTGTTC	60	396
	4. VDR3-36-R	GCCAAACACTTCGAGCACAAG		231
rs7975232 (Apa I)	5. VDR3-32-F	CCAAACACTTCGAGCACAAGG	60	589
	6. VDR3-32-R	AGAGCAGAGTTCCAAGCAGAGG		
	7. VDR3-32T-R	GGTGGGATTGAGCAGTGAAGT	58	313
	8. VDR3-32G-R	GTGGGATTGAGCAGTGATGG		312
rs2228570 (Fok I)	9. VDR3-70-F	AACACCTCTTCCAATGCAGACA	57	519
	10. VDR3-70T-R	TGGACGCCATTGCCTA6A		
	11. VDR3-70C-F	CCTGCTTGCTGTTCTTACAGGTAC	58	217
	12. VDR3-70-R	CTTCTTCTCCCTCCCTTTCCAC		
	13. VDR3-70G-F	CCTGCTTGCTGTTCTTACAGGTAG	58	
	14. VDR3-70-R	CTTCTTCTCCCTCCCTTTCCAC		
	15. VDR3-70A-F	CCTGCTTGCTGTTCTTACAGGTAA	57	
	16. VDR3-70-R	CTTCTTCTCCCTCCCTTTCCAC		343

in HWE (p≤0.00001). The associations of allele and genotypes for all the three SNPs are presented in Tables 2-4. We observed statistically significant association between rs7975232 genotype 'CC' and prostate cancer (p=0.041). The 'CC' genotype exhibit a protective role for the disease (OR=0.383; 95% CI=0.149-0.984). These results are further strengthened from the 'C' allele carriers (OR=0.536; 95% CI=0.305-0.943).

For rs731236, a decrease in risk for prostate cancer was found for 'CC' genotype (OR=0.352; 95% CI=0.114-1.087) and for the 'C' allele carriers (OR=0.681; 95% CI=0.389-1.194). However, these results did not reach a statistical significance.

Analysis for the rs2228570 revealed that this SNP also plays a protective role for homozygous form 'TT' (OR=0.375; 95% CI=0.081-1.739). However, the effect was not statistical significant. We could not detect any heterozygous individual in control or patient group for this SNP.

As, all the three SNPs have protective effective therefore we also performed diplotype analysis to estimate the combination effect of these SNPs (Table 5). The diplotypes 'AA/CC/CC' is associated with the disease (OR=3.4; 95% CI=0.889-13.00) and 'CA/TC/CC' have shown protective role (OR=0.12; 95% CI = 0.013-1.098); however, the effect could not reach the significance level.

Table 2. *Apa I* Polymorphism Frequencies and Effect Estimation by Age in Prostate Cancer Cases and Controls

rs7975232		Cases % (n=47)	Controls % (n=134)	OR (95% CI)	P-Value
Genotypes	CC	6 (12.76)	41 (30.6)	2.61 (1.01-6.70)	0.04
	CA	7 (14.89)	4 (2.99)	0.218 (0.06-79)	0.02
	AA	34 (72.34)	89 (66.42)	-	
Alleles	C	19 (20.21)	86 (32.09)	-	
	A	75 (79.78)	182 (67.91)	053 (0.3-0.94)	0.03
Age<50	CC	0	38 (35.51)	-	
	CA	1 (20)	3 (2.8)	0.18 (0.01-2.16)	0.17
	AA	4 (80)	66 (61.68)	-	
Age >50	CC	6 (14.28)	5 (18.51)	-	
	CA	6 (14.28)	1 (3.7)	0.2 (0.01-2.26)	0.19
	AA	30 (71.42)	21 (77.77)	0.84 (0.22-3.11)	0.79

Table 3. *Taq I* Polymorphism Frequencies and Effect Estimation by Age in Prostate Cancer Cases and Controls

rs7975236		Cases % (n=44)	Controls % (n=119)	OR (95% CI)	P-Value
Genotypes	TT	27 (61.36)	76 (63.86)	-	
	TC	13 (29.54)	11 (9.24)	0.3 (0.12-0.75)	0.01
	CC	4 (9.09)	32 (26.89)	2.84 0.91-8.78)	0.06
Alleles	T	67 (76.13)	163 (68.49)	-	
	C	21 (23.86	75 (31.51)	1.46 (0.83-2.57)	0.18
Age<50	TT	1 (20)	63 (64.94)	-	
	TC	3 (60)	9 (9.27)	0.04 (0.0004-0.5)	0.01
	CC	1 (20)	25 (25.77)	0.39 (0.02-6.59)	0.51
Age >50	TT	26 (66.66)	14 (60.86)	-	
	TC	10 (25.64)	28.69)	0.37 (0.07-1.93)	0.23
	CC	3 (7.69)	7 (30.43)	4.33 (0.96-19.43)	0.05

Table 4. *Fok I* Polymorphism Frequencies and Effect Estimation by Age in Prostate Cancer Cases and Controls

rs2228570		Cases % (n=41)	Controls % (n=108)	OR (95% CI)	P-Value
Genotypes	TT	2 (4.87)	13 (12.03)	2.66 (0.57-12.38)	0.21
	TC	0	0		
	CC	39 95.12)	95 (87.96)		
Alleles	T	4 (4.87)	26 (12.03)		
	C	78 (95.12)	190 (87.96)	037 (0.12-1.10)	0.07
Age<50	TT	0	10 (12.03)		
	TC	0	0		
	CC	5 (100)	76 (88.37)		
Age >50	TT	2 (5.55)	3 (13.04)		
	TC	0	0		
	CC	34 (94.44)	20 (86.95)	0.39 (0.06-2.55)	0.32

Table 5. Apa 1, Taq 1 and Fok 1 Haplotype Frequencies and Risk Estimates in Prostate Cancer

No	Apa I/Taq I/ Fok I	Controls	Cases	OR	95% CI	P value
1	AA/TC/CC	35	21	-	-	-
2	AA/TC/CC	6	6	0.6	0.171-2.103	0.424
3	AA/CC/CC	17	3	3.4	0.889-13.00	0.0737
4	CA/TC/CC	1	5	0.12	0.013-1.098	0.06
5	CA/TC/TT	0	1	-	-	-
6	CC/CC/CC	5	1	3	0.327-27.461	0.33
7	CC/TT/TT	12	2	3.6	0.733-17.684	0.115
8	AA/TT/TT	4	1	2.4	0.2512-22.93	0.447
9	CC/TC/CC	5	0	-	-	-
10	AA/CC/TT	3	0	-	-	-
11	AA/CC/TT	1	0	-	-	-
12	CC/CC/AA	1	0	-	-	-
	Total	90	40	-	-	-

*Diplotype analysis of VDR polymorphisms *Apa I*, *Taq I* and *Fok I* revealed twelve combinations. The diplotype AA/TT/CC was considered as reference for statistical analysis. The diplotype 'AA/TC/CC' is associated with prostate cancer and 'CA/TC/TT' was found to have a protective role; however, the differences were not statistically different. The combinations with zero values in cases or controls were not included in the calculations

Discussion

There are various factors that contribute to the basic pathology of prostate cancer. Clinical diagnosis of the disease is helped by PSA and biopsy, but none of these methods provide definitive diagnosis and/or credible account of progression of the disease (Scher and Heller, 2000; Harnden et al., 2008). Lately, research has been tilting towards genetics to provide a better account of disease risk and progression. Several report have shown positive association between the genetic polymorphisms and prostate cancer risk, including those on androgen receptor (AR) gene (Sun et al., 2010), angiotensin I-converting enzyme gene (Wang et al., 2012) and *VDR* gene. The reason why *VDR* gene polymorphism has attracted so much attention is because of the overall anticancer effect of the vitamin D itself (Lou et al., 2004). In this study, we have analyzed the role of *VDR* gene polymorphisms with prostate cancer, and found significant association between them. As it has been previously reported T allele of *Taq* I is associated with higher incidence of prostate cancer (Medeiros et al., 2002), we couldn't find that much significant association between the genotypes and alleles with the risk of prostate cancer. This matter can perhaps be addressed by increasing the number of the cases. However, low level of association has been reported by others in meta-analysis (Chen et

al., 2009). Contrarily, we found *Apa* I CC genotypes to increase the prostate cancer risk while A allele to be having protective effect. *Apa* I polymorphism has not been reported to have such strong correlation with PCa to the best of our knowledge. We also couldn't find any significant association between *Fok* I TT genotype and PCa which confirms meta-analyses (Yin et al., 2009; Zhang and Shan, 2013). However, protective effect of "C" allele seemed to be something unique in this study.

This study is by far, the first attempt to establish possible relationship between genetic markers and risk of PCa in Pakistani population. Different *VDR* gene polymorphisms seem to have association with the PCa but the findings are not all consistent with international literature. The difference of population may be one of the reasons. Although small sample size also seems to obscure pharmacogenetic findings, we believe that strong associations as those seen with *Apa* I genotype and *Fok* I allele is significant enough to warrant reporting. Future studies, with large enough sample size may be able to find a better correlation between *VDR* gene polymorphisms and PCa risk.

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