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Original Article

VDR polymorphisms ApaI (rs7975232), TaqI (rs731236) and FokI (rs2228570) in Pakistani vitiligo patients and controls

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Abstract

Objective To understand the role of three single nucleotide polymorphisms (SNP) of vitamin D receptor (VDR) gene rs7975232, rs731236 and rs2228570 in Pakistani vitiligo patients and ethnically matched controls.

Methods 196 vitiligo patients and 262 controls were included in this study. Genomic DNA was extracted and polymerase chain reaction, restriction length polymorphism (PCR-RFLP) was done.

Results Genotype CA (rs7975232) was found to be associated with susceptibility (Odd ratio=1.46, 95% CI=1.01-2.13, *p*=0.046) and TT genotype (rs731236) with protection (Odd ratio=0.48, 95% CI=0.25-0.92, *p*=0.003) to vitiligo. TT genotype for rs731236 was significantly high in controls and absent in undetermined/unclassified group of patients. Vitiligo patients and controls were also compared on the basis of gender. Genotype CA (OR=2.12, 95% CI=1.18-3.79, *p*=0.013) and CC (OR =0.21 95% CI=0.06-0.73, p=0.007) for rs7975232 were significantly high in male patients and controls, respectively. For rs2228570 CC genotype was significantly high in controls (OR=0.52, 95% CI=0.2-0.97), *p*=0.04) while TC genotype showed significant difference between patient and controls (OR=1.93, 95% CI=1.02-3.6), *p*=0.05). In combined effect haplotype A-T-C was found significantly high in controls as compared with the patients (Odd ratio=0.57, 95% CI=0.34-0.97, *p*=0.04).

Conclusion VDR polymorphisms may be involved in etiology of vitiligo and future studies should be design to screen large sample size for more VDR polymorphism to get more precise picture.

Key words
Vitamin D receptor, vitiligo, polymorphism, polymerase chain reaction, restriction length polymorphism.

Introduction

Vitiligo is a depigmentation skin disorder caused by destruction of melanocytes.[1] The prevalence of the disease is around 1% in the United States and Europe, while in rest of the world its ranges from less than 0.1% to greater than 8%. Gujrat India tops the list in having prevalence of about 8.8%.[2] According to US Census Bureau, International Data Base 2004 estimated prevalence in Pakistan is 0.1%. The etiopathogenesis of vitiligo has not been fully elucidated and several theories have been proposed. Among those, the autoimmune hypothesis is currently the most accepted one. The presence of T cell-mediated melanocyte destruction and the association of vitiligo with autoimmune conditions, such as Hashimoto's and Addison's diseases, pernicious anemia, type 1 diabetes and systemic lupus erythematosus, are

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major evidences that supports the autoimmune hypothesis.[3-5]

Vitiligo is associated with low level of vitamin D.[6] There is increasing evidence that vitamin D may have an immune regulatory role in various autoimmune diseases.[7,8] It has multiple effects on innate and adaptive immune responses through its effects on T and B lymphocytes, macrophages, and dendritic cells (DC), all of which express the vitamin D receptor (VDR). The activation of VDR leads to suppression of activated T cells, so it can protect melanocytes against auto immune attack.[9,10]

The VDR gene is located on the long (q) arm of chromosome 12 with 11 exons.[11] Several polymorphisms have been identified in VDR gene that has functional significance. In general, the majority of polymorphisms in the VDR gene are found to be in regulatory areas such as the 5' promoter area and the 3' UTR region rather than in coding exons.[12] Three of these polymorphism Apa1 (rs7975232), Taq1 (rs731236) at the 3'UTR region and Fok1 (rs2228570) at the translational initiation site have gain a lot of attention in various association studies.[13-17]

Association studies of VDR polymorphism with several human disorders including skin cancers diabetes mellitus, sarcoidosis, metabolic syndrome etc. have also been studied.[13-17] It has been demonstrated that a length of the VDR, affected by the presence of the polymorphisms, could lead to a lower activation of target cells, since a VDR gene polymorphism, FokI, (rs10735810/ rs2228570) is reported to be in linkage disequilibrium with other VDR polymorphisms. A change in the sequence from C to T in the start codon translation site leads to generation of a polymorphic variant (TT) which is three amino acids longer and has decreased trans activation capacity as compared to the short CC allele and longer VDR protein appears to have a decreased transcriptional activity.[18] The aim of this study was to screen Pakistani vitiligo patients and controls for VDR polymorphisms to assess the role of VDR in etiology of vitiligo in our samples.

Methods

The research work was approved by ethical committee of Institute of Biomedical and Genetic Engineering (IBGE) and funding for this research work was also provided by the same institute.

Blood samples of 196 vitiligo patients (123 females and 73 males of mean age 25) and 262 ethnically-matched controls (137 females and 125 males of mean age 26) were collected from different hospitals of Rawalpindi (Punjab) and Islamabad along with complete clinical detail **(Table 1)** and informed consent. DNA was extracted from blood samples using standard DNA extraction protocol.[19] The three VDR polymorphisms were analyzed by polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) method and agarose gel electrophoresis was done on 2.5% for rs7975232 and rs731236 and 3.5% for rs2228570.[20]

Patients were classified into three classes segmental (SV), non-segmental (NSV) and undetermined/unclassified vitiligo (UDV), therefore on the bases of classification they were compared with normal controls for these three VDR SNPs.

Bands were counted to score different genotypes, odds ratios (OR) with 95% confidence intervals (CI) were estimated using the calculator for confidence intervals for OR (http://www.hutchon.net/ConfidOR.htm) while haplotype frequencies were calculated by statistical program Arlequin version 3.1

softwarehttp://cmpg.unibe.ch/software/arlequin. Significance level was set $p<0.05$.

Results

The associations of allele, genotypes and haplotypes for all three SNPs (rs7975232, rs731236, rs2228570) are presented in **Tables 2** to **5**. We observed statistically significant association of CA genotype (rs7975232) with susceptibility (OR=1.46, 95% CI=1.01-2.13, p=0.046) and TT genotype (rs731236) with protection (OR=0.48, 95% CI=0.25-0.92, p=0.003) to vitiligo. But the alleles for these SNPs showed no significance. Genotypes and their alleles for rs2228570 were also statistically non-significant **(Table 2)**.

When different types of vitiligo were compared with normal controls for these three VDR SNPs, no significance was observed in case of non-segmental (n=133) and segmental (n=33) samples in comparison with normal controls. However, for undetermined/unclassified (n=30) TT genotype for rs731236 was significantly high in controls and absent in undetermined/unclassified (n=30) group of patients **(Table3)**.

Vitiligo patients and controls were also compared on the basis of gender **(Table 4)**. Genotype CA (OR =2.12, 95% CI=1.18-3.79, p=0.013) and CC (OR =0.21 95% CI=0.06-0.73, p=0.007) for rs7975232 were significantly high in male patients and controls, respectively. Therefore we could say that genotype CA is risk genotype for vitiligo and genotype CC showed protective effect in this comparison. For rs2228570 CC genotype was significantly high in controls (OR=0.52, 95% CI=0.2-0.97, p=0.04) while TC genotype showed significant difference between patients and controls (OR=1.93, 95% CI=1.02-3.6), p=0.05). No association with VDR polymorphism was seen in female patients/controls **(Table 4)**.

To gain knowledge about the combined effect of these three polymorphisms, three locus haplotype of wild type and mutant allelic frequencies between patients and controls were compared **(Table 5)**. Haplotype A-T-C was found significantly high in controls as compared with the patients (OR=0.57, 95% CI=0.34-0.97, p=0.04).

Table 1 clinical detail of vitiligo patients and controls

Clinical details	*Vitiligo Patients*	*Controls*
Male	73	137 (105 for rs2228570)
Female	123	125 (90 for rs2228570)
Total	196	262 (195 for rs2228570)
Mean age (years)	25	26
Types		
Nonsegmental	133	
Generalized	61	
Acrofacial	24	
Mixed	35	
Universalis	10	
Rare variants	3	
Segmental	33	
Unisegmental	17	
Bisegmental	10	
Plurisegmental	5	
Segmental follicular	1	
Undetermined	30	
Focal	25	
Mucosal	4	

Table 2 VDR SNP's rs7975232, rs731236and rs2228570 polymorphisms in vitiligo patients and controls

rs7975232 Genotype	*Patients n=196*	*Controls n=262*	*Chi-square*	*OR(95%CI)*	*p-value*
AA	62 (31%)	102 (38%)	2.29	0.72 (0.4-1.07)	0.115
CA	101 (51%)	110 (41%)	3.74	1.46 (1.01-2.13)	0.046
CC	33 (16%)	50 (19%)	0.25	0.85 (0.52-1.39)	0.54
rs 7975232 Alleles					
A	225 (58%)	314 (60%)	0.49	1.10 (0.85-1.44)	0.48
C	167 (42%)	210 (40%)			
rs 731236 Genotype	Patients n=196	Controls n=262	Chi-square	OR (95% CI)	p-value
CC	96 (48%)	124 (47%)	0.07	1.06 (0.73-1.54)	0.77
TC	86 (43%)	102 (38%)	0.94	1.22 (0.84-1.78)	0.29
TT	14 (7%)	36 (13%)	4.36	0.48 (0.25-0.92)	0.03
rs 731236 Alleles					
C	278 (71%)	350(67%)	1.58	0.82 (0.62-1.09)	0.19
T	114 (29%)	174(33%)			
rs2228570 Genotype	Patients n=196	Controls n=195	Chi-square	OR(95%CI)	p-value
CC	103(50%)	113(60%)	0.94	0.8(0.5-1.19)	0.309
TC	77(40%)	66(30%)	1.02	1.26(0.8-1.91)	0.294
TT	16(10%)	16(10%)	0.03	0.99(0.48-2.04)	1
rs2228570 Alleles					
C	283(72%)	292(75%)	35.06	2.88(2.03-4.10)	2.2
T	109(28%)	98(25%)			

Table 3 VDR SNPs in non segmental (NSV), segmental (SV) and undetermined/ unclassified (UDV) vitiligo patients and healthy controls

SNP	*Genotypes*	*Patient*	*Control*	*Chi-square*	*OR(95%CI)*	*p-value*
rs7975232		NSV (133)	n=262			
(APAI)	AA	41(30%)	102(38%)	2.17	0.69(0.4-1.08)	0.12
	CA	69(51%)	110(41%)	3.1	1.48(0.97-2.26)	0.06
	CC	23(17%)	50(19%)	0.09	0.88(0.51-1.52)	0.68
		SV(33)				
	AA	12(36%)	102(38%)	0.01	0.89(0.4-1.9)	0.85
	CA	15(45%)	110(41%)	0.04	1.15(0.55-2.3)	0.71
	CC	6(18%)	50(19%)	0.01	0.94(0.36-2.40)	1
		UDV(30)				
	AA	9(30%)	102(38%)	0.57	0.67(0.2-1.5)	0.42
	CA	17(56%)	110(41%)	1.8	1.8(0.8-3.87)	0.17
	CC	4(13%)	50(19%)	0.27	0.65(0.21-1.95)	0.47
rs731236		NSV (133)	n=262			
(TAQI)	CC	67(50%)	124(47%)	0.22	1.12(0.74-1.71)	0.59
	TC	54(40%)	102(38%)	0.04	1.07(0.70-1.64)	0.82
	TT	12(9%)	36(13%)	1.42	0.62(0.31-1.24)	0.19
		SV(33)				
	CC	16(48%)	124(47%)	0	1.04(0.50-2.16)	1
	TC	15(45%)	102(38%)	0.28	1.30(0.63-2.7)	0.57
	TT	2(6%)	36(13%)		0.4(0.09-1.76)	0.27
		UDV(30)				

	CC	14 (47%)	124 (47%)	0.02	0.97 (0.45-2.07)	1
	TC	16 (53%)	102 (38%)	1.76	1.79 (0.83-3.8)	0.16
	TT	0 (0%)	36 (13%)	-	0 (0)	0.03
rs2228570		NSV (133)	n= 195			
(FOKI)	CC	71 (53%)	113 (60%)	0.5	0.83 (0.53-1.29)	0.42
	TC	48 (36%)	66 (30%)	0.09	1.1 (0.69-1.75)	0.72
	TT	14 (11%)	16 (10%)	0.27	1.31 (0.61-2.79)	0.55
		SV (33)				
	CC	16 (48%)	113 (60%)	0.68	0.68 (0.32-1.43)	0.34
	TC	16 (48%)	66 (30%)	2.13	1.83 (0.87-3.8)	0.11
	TT	1 (4%)	16 (10%)		0.34 (0.04-2.72)	0.47
		UDV (30)				
	CC	16 (53%)	113 (60%)	0.08	0.82 (0.38-1.79)	0.69
	TC	13 (43%)	66 (30%)	0.65	1.49 (0.68-3.26)	0.41
	TT	1 (3%)	16 (10%)	-	0.38 (0.04-3.02)	0.48

Table 4 VDR SNP's rs7975232, rs731236 and rs2228570 polymorphisms in Male (M) and Female (F) vitiligo patients and controls.

SNP	*Genotypes*	*Patients Male M) & Female (F)*	*Control*	*Chi-square*	*OR (95%CI)*	*P value*
rs7975232		M (73)	n=137			
(APAI)	AA	25 (34%)	55 (40%)	0.47	0.77 (0.4-1.40)	0.45
	CA	45 (61%)	59 (43%)	5.85	2.12 (1.18-3.79)	0.013
	CC	3 (4%)	23 (16%)	5.94	0.21 (0.06-0.73)	0.007
		F (123)	n=125			
	AA	37 (30%)	47 (37%)	1.25	0.71 (0.4-1.2)	0.22
	CA	56 (45%)	51 (40%)	0.39	1.21 (0.73-2.00)	0.52
	CC	30 (24%)	27 (21%)	0.14	1.17 (0.64-2.11)	0.65
rs731236		M (73)	n= 137			
(TAQI)	CC	31 (42%)	60 (43%)	0	0.94 (0.53-1.68)	0.88
	TC	37 (50%)	58 (42%)	1.02	1.39 (0.79-2.47)	0.30
	TT	5 (6%)	19 (13%)	1.68	0.45 (0.16-1.27)	0.17
		F (123)	n= 125			
	CC	65 (52%)	64 (51%)	0.02	1.06 (0.64-1.75)	0.8
	TC	49 (39%)	44 (35%)	0.39	1.21 (0.72-2.03)	0.51
	TT	9 (7%)	17 (13%)	1.98	0.50 (0.21-1.17)	0.14
rs2228570		M (73)	n= 105			
(FOKI)	CC	36 (49%)	68 (64%)	3.62	0.52 (0.2-0.97)	0.04
	TC	31 (42%)	29 (27%)	3.61	1.93 (01.02-3.6)	0.05
	TT	6 (8%)	8 (7%)	0.02	1.08 (0.36-3.27)	1
		F (123)	n= 90			
	CC	67 (54%)	45 (50%)	0.26	1.19 (0.6-2.06)	0.57
	TC	46 (37%)	37 (41%)	0.17	0.85 (0.49-1.49)	0.66
	TT	10 (8%)	8 (6%)	0	0.90 (0.34-2.39)	1

Table 5 Frequencies of the 3-locus (ApaI allele-TaqI allele-FokI allele) haplotypes among patients and healthy controls.

Haplotypes	*Patient n=196*	*Controls n=262*	*Chi-square*	*OR(95%CI)*	*p-value*
C-T-T	25(13%)	20(8%)	-	1.03(0.55-1.94)	1
C-T-C	30(15%)	43(16%)	0.04	0.92(0.55-1.52)	0.79
C-C-T	31 (16%)	27(10%)	2.6	1.63(0.94-2.84)	0.08
C-C-C	43(22%)	71(27%)	1.33	0.75(0.48-1.16)	0.23
A-T-T	19(10%)	22(8%)	0.1	1.17(0.61-2.22)	0.74
A-T-C	24(12%)	51(19%)	3.76	0.57(0.34-0.97)	0.04
A-C-T	10(5%)	12(5%)	-	1.12(0.47-0.97)	0.82
A-C-C	14(7%)	16(7%)	0.06	1.18(0.56-2.48)	0.80

Table 6 Results of VDR polymorphisms studies on different populations

Studies	*Country*	*Case/Controls*	*Results*
Birlea *et al.* 2006 [30]	Romania	33/31	Apa1 mutant homozygous genotype associated with vitiligo risk
Aydingoz *et al.* 2012 [33]	Turkey	98/216	Homozygous Taq1variant CC genotype associated with vitiligo
Li *et al.* 2012 [31]	China	749/763	Variant of bsma1 with protection
Li *et al.* 2015 [32]	China Turkey Romania	Meta analysis of 6 studies	Apa1and Bsma 1 variant associated with risk of vitiligo
Present study 2015	Pakistan	196/262	Apa1 variant genotype associated with susceptibility, Taq1 and fok1 variants associated with protection

Discussion

Vitamin D is a lipophilic hormone that functions through the nuclear vitamin D receptor (VDR). The biological activation of vitamin D involves the conversion of vitamin D into 25-hydroxyvitamin D [25(OH)D], followed by 1∞-hydroxylation to yield 1,25-dihydroxyvitamin D3 [1,25(OH)2D3].[12] It is widely accepted that 25(OH)D is the most appropriate indicator of overall vitamin D status, accurately reflecting vitamin D levels in humans. Therefore, it could be said that VDR mediates the effects of vitamin D, as a member of the nuclear receptor superfamily of ligand-inducible transcriptional factors, they are involved in many pathological processes.[21] Several studies have been done on vitiligo patients in whom levels of 25(OH)D are measured and compared with its levels in control group. Plasma levels of 25(OH)D were significantly lower in vitiligo patients.[22-29] Details regarding the genetic basis for this are still require high level of research. Our study was meant to study involvement of VDR polymorphisms in the etiology of vitiligo.

In our results, we found CA genotype (for rs7975232) as risk genotype and TT (for rs731236) as protective genotype when vitiligo samples were compared with ethnically matched controls. On the basis of classification no significance association was found for segmental and nonsegmental vitiligo patients and controls for VDR polymorphisms however for

unclassified/undetermined patients TT genotype for rs731236 was again significantly high in controls. Gender comparison provided us with data that in male vitiligo patients and normal controls CC genotype for rs7975232 and CC genotype for rs2228570 were significantly high in controls while TC and TT were significantly high in patients. Three locus based haplotypes comparison showed that haplotype A-T-C was found significantly high in patients. Unfortunately only few association studies regarding VDR polymorphisms studies for vitiligo patients are available. One study from Romania showed that mutant CC homozygous for APa1 (rs7975232) was significantly associated with susceptibility to vitiligo which contradicts our results.[30] In another study on Chinese population variants for rs7975232 and rs731236 and rs2228570 were associated with decreased risk of vitiligo.[31] Meta analysis done by Lie *et al.*[24] 2015 using data of 6 studies showed that Apa1 and Bsma1 variants are associated with the risk of vitiligo. A study by Aydıngöz *et al*.[33] on Turkish population demonstrated that TaqI polymorphism had a 2.23-fold increased risk of developing vitiligo.[32] Furthermore, a haplotype analysis showed that BsmI/ApaI/TaqI/FokI/Cdx2 GCCCG was significantly over represented in the vitiligo patients. This study showed that VDR TaqI SNP and the haplotype BsmI/ApaI/ TaqI/FokI/Cdx2 GCCCG may be considered as risk factors in vitiligo.[32] Meta-analysis done by Lie et al 2015 using data of 6 studies showed that Apa1 and Bsma1 variants are associated with the risk of vitiligo.[33] All the above mentioned studies result are compared to our research work for the three VDR SNP and are also represented in **(Table 6)**. The difference in association results of our study from other could be due to different geographical distribution, ethnicity, sample size etc. Another important difference could be due to consanguineous marriage practices in Pakistan. To our knowledge this is the only study from Pakistan regarding the VDR SNP polymorphisms and vitiligo. Therefore, it provides valuable information regarding the role of VDR polymorphisms. A future study should be designed with large sample size, wide range of VDR polymorphisms along with clinical manifestation of the genetic changes.

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