

Association of Vitamin D and its Receptor (VDR) Gene Single Nucleotide Polymorphism (ApaI and TaqI) with Risk of Psoriasis

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ABSTRACT

Introduction: Psoriasis is one of the skin related inflammatory diseases that affects a low percentage of population around the globe. Vitamin D through Vitamin D Receptor (VDR) also regulates the function of white blood cells in psoriasis. Mutations in VDR gene have shown abnormalities in immune responses like psoriatic arthritis. To determine the possible association between Vitamin D Receptor (*ApaI* and *TaqI*) gene polymorphism and psoriasis, a case-control study was designed and conducted at the Institute of Basic Medical Sciences (IBMS), Khyber Medical University (KMU) Peshawar and health units of Peshawar.

Method: This multi-centre study included 220 samples (110 cases of psoriatic disease and 110 healthy controls). DNA was extracted using modified salting out protocol. VDR gene polymorphisms (*TaqI* and *ApaI*) were genotyped using amplification refractory mutation system using polymerase chain reaction (ARMS-PCR) method. Results were statistically analyzed.

Result: Our study showed significant association between VDR gene (*TaqI*, *ApaI*) polymorphisms and psoriasis with p-value of 0.009 (0.0019 and 0.0162) and odds ratios (95% confidence interval) for psoriasis of CC vs CT (*TaqI*) and AA vs AC (*ApaI*) were 2.963 (95% CI: 1.508-5.743) and 2.293 (95% CI: 1.22-4.246) respectively.

Conclusion: Findings indicate that VDR gene polymorphisms (*TaqI*, *ApaI*) are significantly associated with onset and progression of psoriasis, and mutations in these loci are risk factors for development of psoriasis.

Key words: Psoriasis, VDR polymorphism, *TaqI*, *ApaI*, South Asia, Pakistan

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INTRODUCTION

Psoriasis is a type of chronic skin inflammatory disorder which affects 1-3 % of total world population and adversely affects quality of life. It is an auto-immune type of disorder. Symptoms include rapid growth of epidermal cells accompanied by inflammation of underlying dermal layer¹. The word psoriasis was derived from Greek word "Psora" meaning "to be itchy"². It is one of the two familiar immune-mediated skin inflammatory diseases. The other one is atopic dermatitis³, characterized by skin inflammation and other symptoms including initial small and raised red

patches covered by silver-coloured dead skin cells appearing as scales. About half of the patients experience the onset of symptoms below 25 years of age and mostly the females tend to be affected earlier than males⁴. Environmental factors such as infections, chemical agents, alcoholism, smoking, drugs as stress may provoke early events of the disease especially in those who are genetically vulnerable to psoriasis. Recent studies have shown that the early appearance of symptoms as compared to late onset involve genetic influence⁵ which include mutations in different genes such as, TNFRSF9, IL-28RA, RUNX3, IL23R, LCE3B/LCE3C, REL, B3GNT2, IFIH1, ERAP1, TNIP1, IL12B, EXOC2, HLA-C, TRAF3IP2, TNFAIP3 and several others which are thought to be causing factors for initiation of early onset of disease⁶. Vitamin D is a steroidal hormone chemically known as 1, 25-dihydroxycholecalciferol⁷. The parent compound of vitamin D is cholecalciferol and found in vitamin supplements and dairy products. Inside our body, vitamin D exists in two forms i.e. 25 hydroxy VD (25-OH-VD) and 1, 25-dihydroxy VD (1, 25-[OH] 2 VD)⁸.

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Both forms of VD are found in the blood and carrier proteins. About 10 to 15% are bound to albumin and 85 to 90% are bound to VD binding proteins (VDBP)^{9,10}. Normal serum vitamin D level is around 20-50 ng/ml. Serum level less than 20 ng/ml is defined as vitamin D avitaminosis¹¹.

Vitamin D produces its action in body through its receptor (VDR). The receptor is present on long arm chromosome 12q13-12q14 near the centromere consisting of 11 exons that together with the associated introns makeup nearly 75kb of the DNA. The vitamin D receptor (VDR) mediates the effects of the physically active form of vitamin D 1,25-dihydroxy vitamin D3 (1,25(OH)2D3). Upon activation, VDR ligand/receptor complex regulates the transcription level of target genes which are involved in T helper (Th) cell improvement and Th cytokine profile change. The gene is involved in cellular growth, differentiation and activated inflammatory processes in keratinocytes. Vitamin D3 (calcitriol) through VDR gene regulates the functions of white blood cells like monocytes, T-cells, macrophages and dendritic cells. Based upon these mechanisms, the role of topical vitamin D formulations in curing psoriasis is still under investigation. These studies now give enough evidence for re-thinking vitamin D as possible candidate for the treatment of psoriasis¹². The studies have shown that vitamin D (VD) and its active metabolite 1, 25-dihydroxy VD has a role in the pathogenesis of psoriasis including genetic mutations in VDR. Most common Polymorphisms in VDR gene are Cdx2, Fok I, TaqI situated in exon 1, 2 and 9 respectively while the BsmI and ApaI are in intron region between exon 7 and exon 8^{13,14}. Therefore, in this study we analyzed and compared plasma levels of 25-(OH) D by ELISA in vitamin D deficient patients and normal healthy control samples along with VDR gene polymorphism (ApaI and TaqI) using allele specific polymerase chain reaction. This is the first report to show the role of VDR gene polymorphisms in the development of psoriasis in Pakistani population.

METHODOLOGY

The study was conducted on a total of 220 individuals. Out of these 220 individuals, 110 were the clinically diagnosed patients of psoriasis and 110 samples were taken from healthy control subjects. The cases included 48.18% females while in control group 49.10% were females. Similarly, the average ages of cases and controls were 57.36±6.25 years and 58.71±8.39 years, respectively.

Blood samples were taken from healthy individuals and psoriasis patients as per the inclusion criteria. Sampling was done at Khyber teaching Hospital (KTH) Peshawar, Lady Reading Hospital (LRH) Peshawar, Hayatabad Medical complex (HMC) Peshawar and experimental work was carried out in Institute of Basic Medical Sciences (IBMS), Khyber Medical University (KMU). Informed consents from the patients were taken. The study was approved from the Ethical Committee of the Khyber Medical University, Peshawar, Pakistan.

Clinically diagnosed psoriasis patients with complete medical history were included. There were no age and sex limitations. Patients with other skin diseases that mimic psoriasis were excluded from the study. Furthermore, patients not consenting and/or unwilling were also excluded from the study. Data of every sample was collected on a pre-designed pro forma. Apart from the demographic data, other information obtained from the patients included: name, age, gender, and ethnicity, duration of disease, family history of disease and history of other metabolic disorders.

Blood samples were collected in disposable syringes from every subject. A total of 3 ml of blood was withdrawn from the antecubital vein of every subject under sterile conditions. The blood was divided into two portions. Two ml of the blood was transferred to labeled plain vacuum tube and the remaining 1ml to labeled ethylene diamine tetra-acetic acid (EDTA) tube for separation of cell pellet. Within half an hour, the blood in plain tube was centrifuged at 3000 rpm for 10 minutes. A clear supernatant of serum was collected and transferred into a fresh Eppendorf tube and stored at -20°C for biochemical analysis. While the blood in EDTA tubes, was stored at 4°C for genotyping. ELISA Analysis: VD serum concentration was measured by an enzyme-linked immunosorbent assay (ELISA) kit (EQ 6411-9601 EUROIMMUN 25-OH VD ELISA kit manufactured by Medizinische labordiagnostika AG) Lot. No. E160916BH. The kit used solid phase competitive ELISA to assay the level of VD in human serum samples. The wells were pre-coated with monoclonal anti-25-OH VD antibodies. First the samples and calibrators/controls were diluted by adding 20 ul of each with 0.5 ml of working strength biotin. The solution was then incubated at room temperature for 10 minutes (+18°C to +25°C) and then pipetted into the microplate wells. 25-OH VD in the sample competed with biotin-labeled 25-OH VD enzyme conjugate for binding site. After washing completely, 3, 3', 5, 5'-tetramethylbenzidine (TMB) liquid substrate was added. The intensity of the colour produced was inversely proportional to the

concentration of 25-OH VD in the samples. The reaction was terminated by the addition of a stop solution (0.5 M Sulfuric acid) and the colour change was measured at a wavelength of 450 nm. All other steps of the assay were performed according to the manufacturer's instructions.

DNA Extraction: Before starting the DNA extraction procedure, all the plasticwares including tips, Eppendorf tubes, cell lysis buffer, nucleic lysis buffer and TE buffer were sterilized in auto clave. Chloroform and ethanol were pre-chilled at -20°C . Total 0.6 ml of blood was dissolved in 1.2 ml of cell lysis buffer in 2 ml pre-sterilized Eppendorf tube. The solution was mixed thoroughly and centrifuged at 7000 rpm for five minutes. The supernatant was discarded carefully, and the pellet was washed 2-3 times with cell lysis buffer up to clarity. The set speed was 5000 rpm after first washing. The Eppendorf tube was kept for 2-3 minutes until the pellet was dried. To the pellet, 0.5 ml nucleus lysis buffer was added and after dissolving the pellet by pipetting, 0.15 ml of saturated NaCl and 0.7 ml of pre-chilled chloroform was added. The Eppendorf tube was centrifuged at 7000 xg for three minutes. The two visible layers were achieved after centrifugation. The supernatant (0.5 ml) was carefully? 12000 xg for 2 minutes to precipitate the DNA. The supernatant was carefully discarded, and DNA was washed with 70% ethanol and centrifuged at maximum speed (14000xg) for 10 minutes. The supernatant was removed, and the tubes kept for 10 minutes at room temperature. The DNA was re-suspended in 0.1 ml TE buffer and was kept at -20°C .

PCR amplification: PCR assay was done for each DNA sample in a final reaction volume of 10 μl . Specific PCR primers were designed. The Control forward primers used for VDR-ApaI genotyping were 5'-CCA AAC ACT TCG AGC ACA AGG-3' and Control Reverse 5'-AGA GCA GAG TTC CAA GCAGAG G-3' with product size 592 bp. The allele specific reverse primer 5'-GGT GGG ATT GAG CAG TGA AGT-3' and reverse 5'-GGT GGG ATT GAG CAG TGA TGG-3' with product size 318 bp were respectively. Similarly, the control forward and reverse primers for VDR-TaqI genotyping were 5'-GCC AAA CAC TTC GGC AAG-3' and 5'-CGG TCC TGA ATG GCC ACA-3' respectively with product size of 592 bp. The allele specific forward and reverse primers were 5'-CAG AGC ATG GAC AGG GAG CAAG-3' and 5'-CAC ACTG CAG ACG TAC ATCC-3' respectively with PCR product size of 340 bp. The primers sequences are shown in Table 1 for allelic discrimination of both ApaI and TaqI polymorphisms in our study samples. The PCR product sizes are shown in Table 2 and the gel electrophoresis results are shown

in Fig. 1 and Fig. 2. For each sample, four PCR tubes were labeled i.e. two tubes for VDR-TaqI and two tubes for VDR-ApaI PCR assays. The tubes were labelled as 'C' for C allele, 'T' for T allele for VDR-TaqI PCR assay and 'A' for A allele, 'C' for C allele for VDR-ApaI. Final reaction volume was prepared by taking 7 μl of deionized water, 0.8 μl of 10x PCR buffer, 0.3 μl of MgCl_2 , 0.5 μl of 10mM dNTPs, 0.5 μl of template DNA, 0.3 μl of control primers, 0.3 μl of allele specific primers (C and T) in one PCR tube and 0.3 μl of allele specific primers (A and C) in the other PCR tube. The PCR tubes were vortexed for 5-10 seconds and then were run in a PCR thermal cycler (Multigene Optimax). The PCR conditions are mentioned in Table 2 and were as follows: denaturation at 94°C for 5 minutes, followed by 30 cycles of PCR: denaturation at 95°C for one minute, annealing at 64°C for 30 seconds, polymerization at 72°C for one minute and proof reading at 72°C for five minutes.

Stock Solutions Preparation: Total 46.1 gm of EDTA was dissolved in about 800 ml of distilled water and placed on magnetic stirrer. The pH was adjusted with sodium hydroxide (NaOH) to 7.5. After pH adjustment, sufficient quantity of distilled water was added to make up the volume 1000 ml; 121.14 gm of ultra-pure tris was dissolved in about 800 ml of distilled water and placed on magnetic stirrer. The pH was adjusted with hydrochloric acid (HCl) to 8.0. After pH adjustment, sufficient quantity of water was added to make up the volume 1000 ml. Then 12 mM Tris-HCl, 350 mM Sucrose, 6 mM MgCl_2 and 1% Triton X 100 were dissolved in 700 ml distilled water. After complete dissolution, sufficient distilled water was added to make up the volume 1000 ml.

Next, 12 mM Tris-HCL, 15 mM Ammonium Acetate 1.5 mM EDTA and 2% SDS solution were dissolved in 700 ml distilled water and after complete dissolution, enough water was added to make up the volume 1000 ml. NaCl 59 gm was dissolved in 150 ml of distilled water and after complete dissolution; sufficient water was added to make up the volume 200 ml; 5 ml 1M Tris-HCl solution and 2 ml 0.5M EDTA solutions were dissolved in 1000 ml of distilled water.

Statistical analysis: Statistical Package for the Social Sciences (SPSS) v23.00 software was used to determine the genetic association between psoriasis and VDR polymorphism. Chi-square test was performed to determine the association between alleles and genotypes while allele and genotype frequencies were assessed under Hardy-Weinberg Equilibrium (HWE). Odds Ratio (OR) was calculated with 95% Confidence Interval (CI).

RESULTS

The association study between VDR SNPs (apaI and TaqI) and psoriasis was conducted in 110 psoriasis cases and 110 healthy controls. The clinical demographic data of study samples is mentioned in Fig. 3. The average Body Mass Index (BMI) of both cases and controls was 25.79 ± 3.97 and 30.767 ± 6.59 , respectively. Similarly, initial serum vitamin D levels were calculated in both cases and healthy control samples were 13.5 ± 5.34 and 29.02 ± 17.65 , respectively. The results were statistically analyzed by using chi-square (χ^2) test as shown in Table 3, which showed significant association between the VDR SNPs (ApaI and TaqI) and psoriasis ($p = 0.0162$ and 0.0019 respectively). To check either this significant association is a risk factor for the onset of disease, we calculated the odds ratio between the cases and controls. The results are mentioned in Table 4 which showed that although our study SNPs were significantly associated with psoriasis, but they are not a risk factor for onset of psoriasis. The odds ratios also showed that these polymorphisms are risk factors for development of psoriasis (Odds ratios of 2.29 and 2.96). Similarly, the serum levels of vitamin D in both cases and controls were statistically analyzed through chi-square test. According to our results, the low levels of serum vitamin D in cases (13.5 ± 5.34 ng/ml) as compared to controls (29.02 ± 17.65 ng/ml) with p-value less than 0.005 (<0.0001) shows a strong association with psoriatic plaques. The results are summarized in Table 3. Finally, the levels of vitamin D in both cases and controls were statistically compared with VDR SNPs i.e. ApaI and TaqI. The results showed that these VDR mutations are risk factors for early onset of psoriasis symptoms in local population of Peshawar, KPK, Pakistan and nearby areas (Table 5).

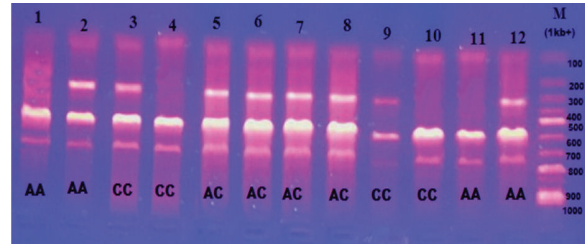


Fig. 1: PCR amplification ApaI

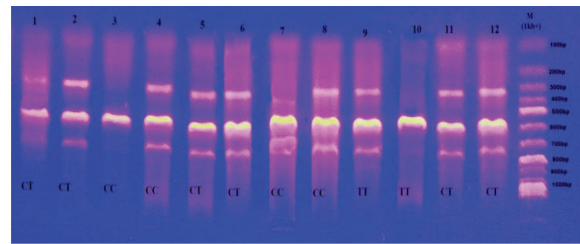


Fig. 2: PCR amplification TaqI

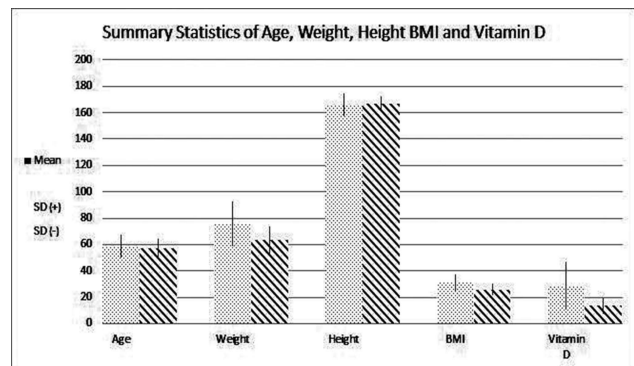


Fig. 3: Demographic Parameters of Controls and Cases

Table 1: ASPCR Primers with specific Sequences

Primers Type		Primers Sequence	PCR Product size
Apa I	Controls	Forward: 5° CCA AAC ACT TCG AGC ACA AGG 3° Reverse: 5° AGA GCA GAG TTC CAA GCAGAG G 3°	592 bp
	Allele Specific	Reverse: 5° GGT GGG ATT GAG CAG TGA AGT 3° Reverse: 5° GGT GGG ATT GAG CAG TGA TGG 3°	318 bp
Taq I	Controls	Forward: 5° GCC AAA CAC TTC GGC AAG 3° Reverse: 5° CGG TCC TGA ATG GCC ACA 3°	592 bp
	Allele Specific	Forward: 5° CAG AGC ATG GAC AGG GAG CAAG 3° Reverse: 5° CAC ACTG CAG ACG TAC ATCC 3°	340 bp

Table 2: PCR Amplification Profile

	Temperature (°C)	Duration (minutes)	No of Cycles
Activation	95	5	1
Denaturation	95	1.0	30
Annealing	64	0.5	
Extension	72	1.0	
Final Extension	72	10.0	1
Soak	4	8	Hold

Table 3: Association of TaqI with

Psoriasis			
Genotypes	Controls (n)	Disease (n)	p-value
CC	48	18	0.0019
TT	17	42	
CT	45	50	
Apa-I with Psoriasis			
Genotypes	Controls (n)	Disease (n)	p-value
AA	42.72	21.82	0.0016
CC	19.10	34.55	
AC	38.12	43.64	
Vitamin D with Psoriasis			
Group	Number	Vitamin D ng/ml	p-value
Cases	110	13.5±5.34	<0.0001
Control	110	29.02±17.65	

Table 4: Odds ratio CC vs CT (TaqI) and AA vs AC (ApaI)

CC vs CT					
Genotype	Cases	Controls	CI 95%	Odds Ratio	p-value
CC	18	48	1.508 to 5.743	2.963	0.0019
CT	50	45			
AA vs AC					
Genotype	Cases	Controls	CI 95%	Odds Ratio	p-value
AA	24	47	1.22 to 4.246	2.293	0.0162
AC	48	41			

Table 5: Allele frequency of VDR Polymorphisms between cases and controls

VDR Loci	SNP	Genotype	Cases % (n = 110)	Controls% (n=110)	Vitamin D Level (ng/ml)		OR	p-value
					Cases	Controls		
Apa 1	A>C	AA	21.18	42.7	13.5	29.02	2.29	0.0162
		CC	34.5	19				
		AC	43.6	37.3				
Taq 1	C>T	CC	16.36	43.6	13.5	29.02	2.96	0.0019
		TT	38.18	15.45				
		CT	45.45	40.9				

DISCUSSION

Recent advancements in genetics have shown a relationship between psoriasis and gene mutations. Russel et al first time reported the association between HLA-B13 (Human Leukocyte Antigen serotype) and psoriasis¹⁵. Besides common type of psoriasis, genetic involvement in a rare type of psoriasis known as “Pustular psoriasis” has shown mutation in IL36RN¹⁶. This allele encodes IL36 receptor antagonist protein which reduces cytokines production. Mutation in IL36RN allele results in synthesis of abnormal protein and increase production interleukins (IL-8) from keratinocytes¹⁷. The type-2 psoriasis is a type whose genetic cause is not very much clear which shows the diversity of genetic variations associated with different types of psoriasis⁶. Previous study by Keren et al reported the role of vitamin D and its analogues in defensive mechanisms of skin and suggested the effective use of vitamin D in treatment of psoriasis¹⁸. Vitamin D influences the homeostasis of skin through its receptor i.e. vitamin D receptor.

The association between psoriasis and vitamin D polymorphisms and blood levels is not well understood as no such study had been performed previously. But vitamin D levels and VDR gene polymorphisms are associated with other autoimmune diseases like rheumatoid arthritis and multiple sclerosis. Hence, one can rationalize the possible association between psoriasis and vitamin D serum concentrations and VDR gene polymorphisms. To find possible association between psoriasis and VDR gene polymorphisms, we designed a case-control study for this purpose. The current study elucidates the role of VD and VDR gene polymorphism and showed significant difference between VD levels and genotypes of disease and control group. In our study, a total of 220 individuals of same age group were included. Out of 220, 110 were psoriatic patients and 110 were healthy controls. Level of vitamin D was analyzed for both case and control groups using commercially available kits. The levels of vitamin D were normal in control group (29.02 ± 17.65 ng/ml) while levels were below than normal in diseased patients (13.5 ± 5.34 ng/ml). The purpose of our study was to determine possible association between VDR gene polymorphism (TaqI and ApaI) and psoriasis. Genetic polymorphisms were checked by analyzing the restriction pattern of the polymerase chain reaction (PCR) products. The results were analyzed through chi-square analysis which showed a significant association between both TaqI and ApaI polymorphisms with psoriasis with a mean p-value of 0.009 (0.0019 and 0.0162). The odds ratios (95% confidence interval) for psoriasis of CC vs CT (TaqI) and AA vs AC (ApaI)

were 2.963 (1.508-5.743) and 2.293 (1.22-4.246), respectively. The odds ratios show that VDR genes polymorphisms are risk factors for the onset of psoriasis. Similarly, vitamin D levels in psoriatic cases and healthy controls were analyzed through chi-square which also showed significant association between low vitamin D level and psoriasis disease (p-value < 0.005). Hence, through our study, we have shown that VDR gene polymorphisms (TaqI and ApaI) are significantly associated with the onset and progression of psoriasis. Furthermore, levels of vitamin D are very low in our case group that further complicates the psoriasis profile of cases and may be a risk factor for development of psoriasis.

CONCLUSION

Study concludes that VDR gene polymorphisms (TaqI and ApaI) were associated with the development of psoriasis. Vitamin D receptor SNP was associated with increased risk of psoriasis. Vitamin D deficiency may be a modifiable risk factor for developing psoriasis.

Authors’ contribution: M.I.S, M.S, S.S, and M.A, conceptualized study design, collected, analyzed and interpreted data. M.A.S, and A.K interpreted data, prepared manuscript and did proof reading.

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