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Andleeb Batool

Government College University, Lahore, Pakistan, andleeb.batool@gcu.edu.pk

Maryam Mukhtar

Government College University, Lahore, Pakistan, m.mukhtar29@gmail.com

Iram Qayyum

Government College University, Lahore, Pakistan, iram.qayyum03@yahoo.com

Romessa Shakeel

Government College University, Lahore, Pakistan

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Association of rs2476601 and rs1544410 with Onset of T1D in Youngsters of Lahore, Pakistan.

Andleeb Batool¹, Maryam Mukhtar*¹, Iram Qayyum¹, Romessa Shakeel¹

¹*Department of Zoology, Government College University, Lahore, Pakistan.*

Corresponding author*: Maryam Mukhtar

Email: m.mukhtar29@gmail.com

Contact no: 92-3400150119

Andleeb Batool: andleeb.batool@gcu.edu.pk

Iram Qayyum: iram.qayyum03@yahoo.com

ABSTRACT

The *PTPN22* gene plays vital role in T1D onset by encoding Lymphoid-specific phosphatase (LYP) that lead to T-cell receptor-associated CsK kinase inactivation and preventing T-cell spontaneous activation by dephosphorylation. *VDR* gene encoded for *VDR* receptor is involved indirectly in prevention of T1D onset by promoting insulin production. The present study was conducted to determine the mutations on rs2476601 and rs1544410 polymorphic sites on the *PTPN22* and *VDR* genes respectively. We genotyped 50 patients and 50 control subjects from Lahore by using sequencing and Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (RFLP) techniques. It was observed that the allelic frequency of r and b were higher in patients as compared to controls and significantly associated with the onset of T1D. The genotype distribution frequencies varied significantly among patients and controls ($p < 0.01$). The mutation on the rs2476601 polymorphic site led to the change of Isoleucine to tryptophan in patients. In conclusion, compelling evidence was found of T1D onset association with the polymorphism at *RsaI* on rs2476601 and *BsmI* on rs1544410 on *PTPN22* and *VDR* genes.

KEY WORDS

T1D, *PTPN22*, *VDR*, SNP, Polymorphism, PCR-RFLP.

INTRODUCTION

The autoimmune destruction of β -cells caused by genetic susceptibility leads to the development of a complex disorder named as Type 1 Diabetes mellitus (T1D) (Pociot and Mcdermott, 2002). Although the genetic association of T1D was reported in various populations, major Histocompatibility Complex (*MHC*), Insulin (*INS*) and Cytotoxic T Lymphocyte Associated Protein 4 (*CTLA4*) genes were confirmed in several independent studies (Undlien *et al.*, 2001; Anjos *et al.*, 2004)

Polymorphism in *PTPN22* and *VDR* genes are an important predictor of T1D. *PTPN22* is located on the short arm of chromosome 1 near telomere (1p.13.2). It contained 57,898 bases and encodes a protein (lymphoid-specific phosphatase) of 807 amino acids with 91705 Da Molecular weight (Presscott *et al.*, 2005; Vang *et al.*, 2007). Tyrosine phosphatase expressed primarily in lymphoid tissues and was reported to be involved in several signaling pathways associated with the immune response (Bottini *et al.*, 2004). *PTPN22* specifically expressed in lymphocytes and through the formation of a complex with C-terminal Src Kinase (CSK) suppressed the downstream mediators of T-cell receptor signaling (Cloutier *et al.*, 1996). *VDR* is highly polymorphic and is located on 12 q chromosome (12-12q14) (Stene *et al.*, 2000). Vitamin D performed an immunosuppression and inhibited the production of cytokine and immunoglobulin and the activation of lymphocyte (Sandler *et al.*, 1994). Therefore, infants who have vitamin D intake in early childhood have a lower incidence of T1DM in adulthood (Dalquist *et al.*, 1999).

Several SNPs potentially contribute to susceptibility to T1D and among them, rs2476601 on *PTPN22* and rs1544410 on *VDR* were most reported SNP (Ban *et al.*, 2001; Dharia *et al.*, 2011). Single-nucleotide polymorphism (rs2476601, A/G) located on exon 14 resulted in arginine (R) substitution by tryptophan (W) at LYP codon 620 (R620W) and showed greater T-cell receptor

signaling inhibition (Begovich *et al.*, 2004). In the rs1544410, A/G is located on Intron 8 of *VDR* gene, but its function is still not clearly understood (Uitterlinden *et al.*, 2004)

T1D is a disease affecting children, especially the young Pakistani population because of common intracaste marriages that made a narrow genetic pool. The reported cases of T1D are increasing with the passage of time throughout the world, as well as in Pakistan. Therefore, the present study was conducted to evaluate the strength of *PTPN22* and *VDR* polymorphisms in Pakistani Youngsters with T1D.

MATERIAL AND METHOD

Subjects

Blood samples were collected after informed consent from 50 T1D and 50 healthy control subjects. Patients and controls were age and sex matched. The current study was approved by the Board of Studies at GC University, Lahore and the Ethical Committee of Shalamar Hospital, Lahore. The patients the participated in the current study were diagnosed with T1D below 10 years of age and depended on insulin treatment from the time of diagnosis.

DNA Isolation and Genotyping

DNA was isolated from each sample through the modified organic method (Sambrook *et al.*, 1989). The SNPs were selected on the basis of the SNP reported associated with T1D in various populations. PCR of the region with the polymorphic site (rs2476601) was performed by using F.P: ACCAGCTTCCTCAACCACAA and R.P: AGAATTCCTTTGGATTGTT and the polymorphic site (rs1544410) by using F.P: CAACCAAGACTACAAGTACCGCGTCAGTGA and R.P: AACCAGCGGGAAGAGGTCAAGGG. The PCR conditions were as follows: initial denaturation (95°C for 5 min), then 35 cycles with following temperature profile: denaturation (95°C for 45 sec), annealing (rs2476601: 63.4°C and rs1544410: 55.9°C for 30 sec), extension (68°C for 45 sec), and final elongation for 10 min.

Restriction Fragment Length Polymorphism (RFLP)

The PCR products of rs2476601 (218 bp) and rs1544410 (825 bp) were digested with *RsaI* and 65°C respectively at 37°C for 3h and then subjected to 2% agarose gel electrophoresis. The PCR Products were sent to ABI (Korea) for sequencing to determine the genotype. The sequences were visualized using BioEdit software.

Statistical Analysis

Hardy-Weinberg equilibrium was determined using Transposer software.8. Allelic and Genotypic associations of both SNPs was tested by using the Fisher exact test through online SHEsis software (<http://analysis.bio-x.cn/SHEsisMain.htm>). Changes in Amino Acid Alignment were determined using MEGA6 Software.

RESULTS

A total of 50 T1D patients participated. The mean age was 18, with 12 years in males and 20.21 years in Females. All patients had a positive maternal and paternal family history of T1D. On the Other hand, the mean age of males and female controls was 17.41 years and 19.91 years respectively with a negative family history of T1D. The mean concentration of isolated DNA samples were 50 ng/ μ l and quality (260/280 value) 1.8 shown in Figure 1.

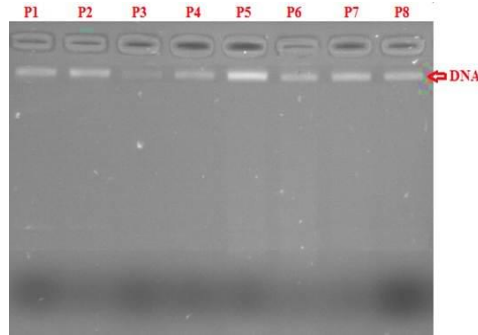


Figure 1: DNA isolated from P (Patients) blood sample.

The *PTPN22* and *VDR* gene polymorphisms (R/r and B/b) were assigned in the patients and controls respectively by using the PCR-RFLP method. It was observed that the Digestion of the PCR product with *RsaI* and *BsmI* endonuclease enzyme produced three different sized bands, i.e. 218bp, 176bp, 42bp and 825bp, 650bp, 175bp respectively shown in Figure 2 and 3.

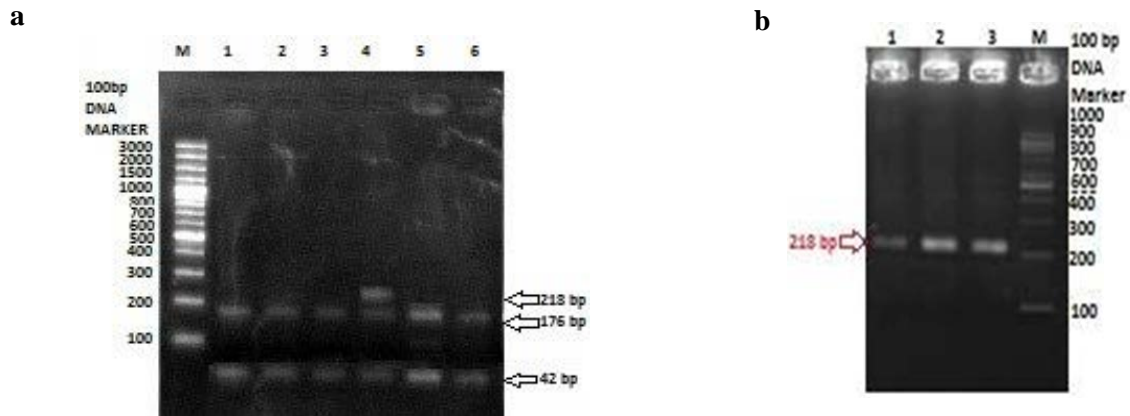


Figure 2: RFLP of *PTPN22* (rs2476601) on 2% agarose gel. (a) Patients. Lanes 1-3 and 5-6 shows homozygous genotype (rr) with 176bp and 42bp and Lane 4 shows heterozygous genotype with 218bp, 176bp and 42bp (Rr). (b) Controls: Lanes 1-3 shows homozygous genotype with 218bp (RR).

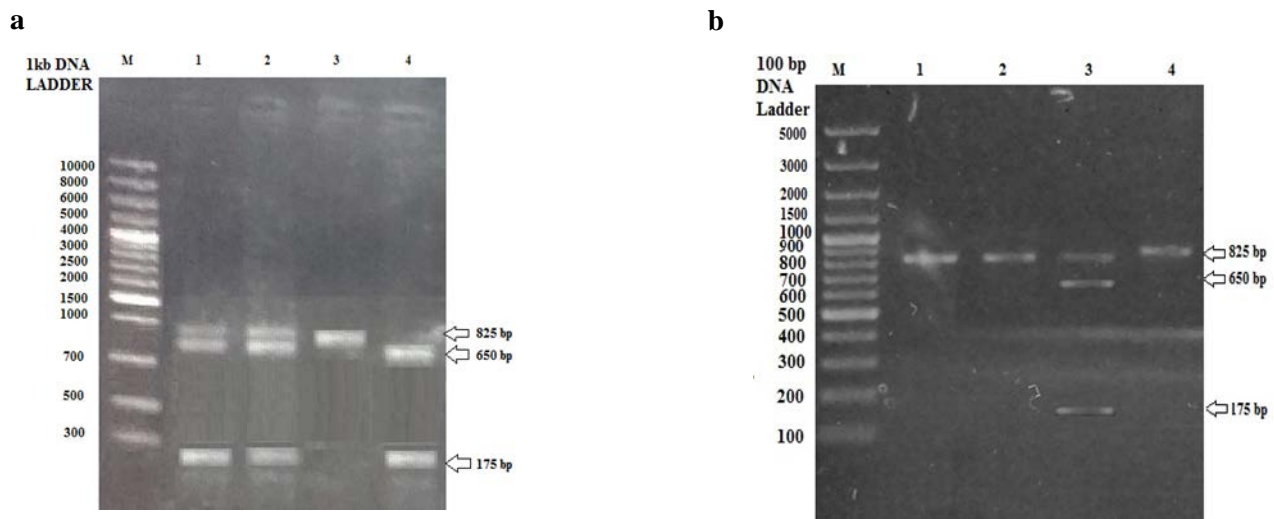


Figure 3: RFLP of *VDR* (rs1544410) on 2% agarose gel. (a) Patients. Lanes 1-2 shows heterozygous genotype (Bb) with 825bp, 650bp and 175bp. Lane 3 shows homozygous genotype with 825bp (BB) and Lane 4 shows homozygous genotype with 650bp and 175bp (bb). (b) Controls: Lanes 1, and 4 shows homozygous genotype with 825bp (BB) and Lane 3 shows heterozygous genotype with 825bp, 650bp and 175bp (Bb).

RsaI and BsmI polymorphisms did not deviate from Hardy Weinberg equilibrium ($p > 0.05$). The frequency of r and b alleles was higher in patients as compared to controls (Table 1). The genotype distribution frequencies vary significantly among patients and controls ($p < 0.01$). The rr, Rr, bb and Bb genotype occurred more frequently in patients, whereas RR and BB were more common in controls (Table 2). Therefore, it was found that the onset of T1D was significantly associated with the polymorphism at RsaI on rs2476601 and BsmI on rs1544410.

Table 1: Allele frequency with T1D in patients and controls.

Gene	SNP No	Alleles	Patients: n	Control: n	p-value
<i>PTPN22</i>	rs2476601	R	2	98	0.002**
		r	92	8	
<i>VDR</i>	rs1544410	B	38	100	0.015*
		b	62	0	

*represent $p < 0.05$ and ** represents $p < 0.01$

Table 2: Genotype frequency with T1D in patients and controls.

Gene	SNP No	Genotype	Patients: n (%)	Controls: n (%)	X ²	p-value df (2)
<i>PTPN22</i>	rs2476601	Rr	44 (88)	0 (0)	86.98	0.002**
		rR	4 (8)	2 (4)		
		RR	2 (4)	48 (96)		
<i>VDR</i>	rs1544410	Bb	15 (30)	0 (0)	88.67	0.011*
		Bb	32 (64)	1 (2)		
		BB	3 (6)	49 (98)		

*represent $p < 0.05$, ** represents $p < 0.01$, R = A, r = G, B = A, b = G

The rr and bb genotype conferred a higher risk for type 1 diabetes with an odds ratio of 10.42 [95% confidence interval: 11.6±27.2] for rr and 6.41 [95% confidence interval: 4.54±8.94] for bb.

By using BioEdit software, sequences were visualized and genotype was shown in Figure 4.

The alignment of DNA sequences of patients on the MEGA-6 software showed that a change in the nucleotide on the rs2476601 polymorphic site lead to the change of Isoleucine tryptophan (Figure 5).

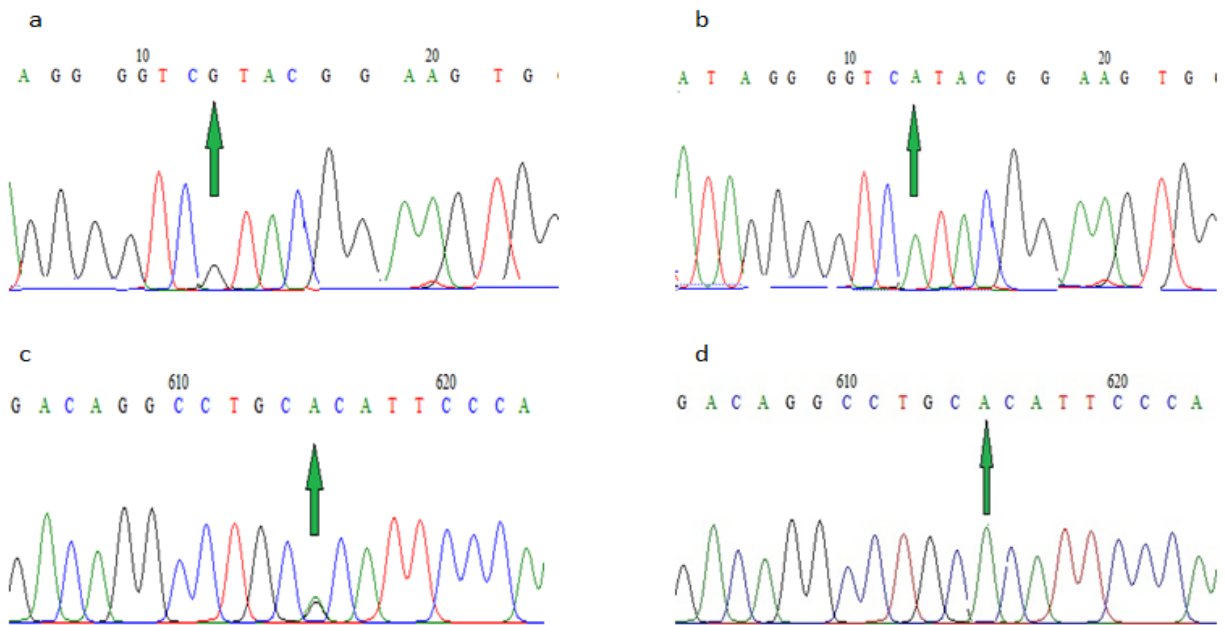


Figure 4: Chromatographic representation of rs2476601 (a) Patient Genotype (GG) (b). Control Genotype (AA) and rs1544410 (c) Patient Genotype (GA) (d). Control Genotype (AA).

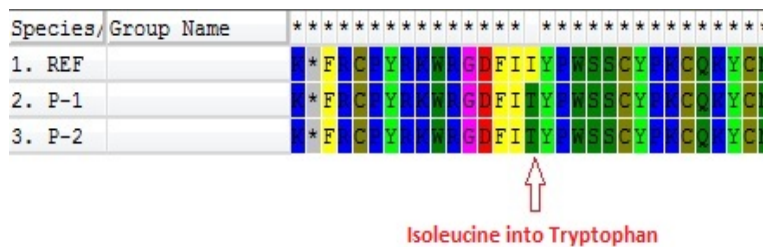


Figure 5: Change in Nucleotide (Cytosine into Thymine) lead to the change of amino acid sequence (Isoleucine into Tryptophan).

DISCUSSION

The current study demonstrated that *PTPN22* and *VDR* gene polymorphism was associated with T1D onset in Pakistani population. In human genomes, single nucleotide polymorphisms (SNPs) are common and that provides evidence for specific genes involvement in the onset of human diseases. A genetic variant that appears in at least 1 % of the population is called a polymorphism. Alteration in the gene regulatory parts could affect the gene expression level, and lead ultimately to the level of proteins (Vilarino *et al.*, 2011). Genetics plays a vital role in the early onset and progression of the disease (Gyrus, E. 2012). Insulin secretion is influenced by the Vitamin D hormone, as well as *PTPN22*.

The current study confirmed the significant association of rs2476601 with T1D that results in a substitution of arginine (R) by tryptophan (W) residue at LYP codon 620(R620W) with the tryptophan variant showing greater inhibition of T-cell receptor signaling. A study conducted by Chelala *et al.* (2007) supported that rs2476601 was the major risk determinant at *PTPN22* for T1D onset. Recently, Maah *et al.* (2014) showed evidence that the *PTPN22* C1858T variant regulates type 1 diabetes-specific autoimmunity and strongly affects the progression from preclinical to clinical diabetes.

Allelic frequencies of r and b were higher in patients as compared to control subjects. Similarly, polymorphisms within the *VDR* gene that may modify T1D susceptibility were reported in Southern Indian subjects through the transmission disequilibrium test (McDermott *et al.*, 1997). McDermott *et al.* (1997) reported that allele 'b' of BsmI was found frequently in affected subjects. In line with the present study, polymorphism on BsmI is present in intron 7 of *VDR* gene and therefore, no direct change is expected in the coding sequences. Hence, the BsmI polymorphism role on the susceptibility to T1D remained unknown. Farrow (1994) and Barker *et al.* (1988), reported that the regulation of protein synthesis might be mediated by especially elongated 30 untranslated regions in the *VDR* gene of mRNA that ultimately lead to the change in the gene expression of stability of mRNA. Some groups tried to relate stability and levels of *VDR* mRNA to *VDR* genotypes and reported that the stability of *VDR* mRNA was not influenced by polymorphism within the *VDR* 30 untranslated region (Verbeek *et al.*, 1997).

CONCLUSION

In conclusion, the present study demonstrates that T1D is a chronic disorder affecting the health of young adults and causing huge losses to Pakistani society. Polymorphism at the rs2476601 and rs1544410 site on *PTPN22* and *VDR* gene respectively are significantly associated with the onset of T1D in Pakistan. A large scale systematic survey is required to get the clearer picture of disease and the genetic susceptibility of a population.

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