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Association of Protein Tyrosine Phosphatase Non-receptor, Type 22 *(PTPN22) C1858T* Polymorphism with Type 1 Diabetes in North India: A Replication Study

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Abstract

Objective: Type 1 diabetes (T1D) is a multifactorial autoimmune disorder where several genes have been associated with the disease. While the major histocompatibility complex has been shown to be the major locus, contributions of other loci in different combinations seem to have synergistic effects. *PTPN22* (Protein tyrosine phosphatase non-receptor, type 22) gene encoding lymphoid tyrosine phosphatase (LYP) is a negative regulator of T cell signaling. A gain of function mutant at nucleotide position *1858 C>T* has been associated with diabetes, however, it is reported to be absent in Asians. We have studied *PTPN22 C1858T* polymorphism in T1D patients from North India since there are no reports from this region.

Methods: *PTPN22 C1858T* polymorphism was studied in 250 T1D patients and 480 healthy controls using polymerase chain reaction followed by restriction digestion (PCR-RFLP). Alleles of *HLA-DRB1* locus were studied using PCR followed by hybridization with sequence specific oligonucleotide probes using a bead based assay on Luminex platform.

Results: In spite of reports of absence of *1858T* allele in Asians, we observed this allele to be present in North Indians, albeit with low frequency (1.98%). However, T1D patients from the same ethnic background showed significantly higher frequency of the allele and heterozygous genotype *1858CT* as compared to controls. Patients with both *1858CT* and *1858CC* genotypes had predisposing MHC alleles.

Conclusion: The association of *PTPN22 1858CT* genotype with Type 1 diabetes was independent of the predisposing Human leukocyte antigen (*HLA*) alleles *DRB1*03:01*, *DRB1*04:01*, *DRB1*04:05* in North Indian patients, suggesting their integrated roles in manifestation of T1D. Based on the reported role of *PTPN22 1858CT* genotype in defective innate immune responses against viral infections, and defects in early T cell signaling, it is tempting to speculate that it may be detrimental for the destruction of pancreatic beta cells in the present scenario.

Keywords: *PTPN22*; Major histocompatibility complex; Type 1 diabetes

Introduction

Type 1 diabetes (T1D) is a multifactorial autoimmune disorder where insulin producing pancreatic beta cells are lost before the manifestations of the disease. It has an incidence of 10.5/100,000/year in India [1]. Several genetic and environmental factors have been implicated as the causative factors, however, a role of complex interaction of these factors leading to the autoimmune destruction of the pancreatic beta cells cannot be ruled out [2]. The cell-mediated autoimmune destruction of pancreatic beta cells is brought about by infiltrating macrophages and lymphocytes in the pancreatic islets, the phenomenon known as Insulitis. While the function of most T-cells is to mount host-defense response, regulatory T-cells are programmed to moderate the reactivity of effector T-cells, in order to protect the body from autoimmune responses. It has been proposed that an increase in the ratio of effector T-cells to regulatory T-cells may result in autoimmunity in T1D [3]. Both $CD4^+$ helper T cells and $CD8^+$ Cytotoxic T cells are the effector T cells required to induce beta cell autoimmunity [4]. Defects in T cell signaling may be involved in altering the ratios of effector and regulatory T cells.

Of the 20 genomic intervals implicated in the predisposition for development of T1D [5], we and others have reported association of T1D with the major histocompatibility complex *(MHC)* alleles on chromosome 6p21 and the 5' regulatory region of the insulin (INS) gene on 11p15.5 in North Indians and other ethnic groups [6-8]. Besides, we have also reported association and interaction of *TNF-α* gene with other cytokine genes [9], Vitamin D receptor gene (*VDR*) and their role in expression of predisposing *MHC* allele in manifestation of T1D [10]. Interestingly, it seems that several genes have integrated roles in manifestation of the disease since simultaneous presence of different alleles of two or more genes seem to be significantly increased in T1D patients. For instance, simultaneous presence of HLA *DRB1*0301*, *DRB1*0401* and/or *DRB1*0405* along with class-I alleles of INS-linked variable number of tandem repeats (VNTR) were very significantly associated with T1D [6]. Similarly an interaction was observed between *VDR* gene and predisposing *HLA* alleles, and this interaction of *VDR* with *DRB1*0301* was through the vitamin D response element (VDRE) present in the promoter region of the predisposing HLA allele and was involved in regulating the expression of *HLA-DRB1*0301* [10]. Besides

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MHC, *INS*, cytokine and *VDR* genes, there are several other genes that have been shown to be associated with T1D which include *CTLA4* gene located on chromosome 2q33 [11,12] and *PTPN22* gene located on chromosome 1p13 [4].

The *PTPN22* (Protein tyrosine phosphatase non-receptor, type 22) gene codes for lymphoid tyrosine phosphatase (LYP) which is an 807 amino acid residue protein with a molecular weight of 110 kDa and is involved in negative regulation of T cell signaling [13-15]. A single nucleotide polymorphism (SNP) in the *PTPN22* gene at nucleotide position *1858 C>T* results in the amino acid change from arginine (R) to tryptophan (W) (*CGG* to *TGG*) at codon 620 which has been shown to be a gain-of-function mutant [16]. The negative regulation of T cells by LYP is brought about by its dissociation from C-terminal Src kinase (CSK) and recruitment of LYP to the plasma membrane where it down-regulates TCR signaling. LYP*W620, however, cannot bind CSK and negatively regulates LCK-mediated phosphorylation of the TCRξ chain, reduces tyrosine phosphorylation of LAT and reduces activation of ERK2 as compared to LYP*R620. In essence, LYP*W620 has been reported be more efficient at dephosphorylation than LYP*R620 [15,16]. Several autoimmune disorders like systemic lupus erythematosus [17], rheumatoid arthritis [18,19] and type 1 diabetes [20-22] have been shown to be associated with the mutant *T* allele i.e., LYP*W620.

The allele frequency of the minor *1858T* allele varies from 0% in some Asian populations [23] to 8-17% in different ethnic groups of European origin [17,18,24]. Africans [24] also have a very low frequency of the minor *1858T* allele [10]. Such ethnic differences make it important to study the role of the minor allele in manifestations of autoimmune disorders in different populations. While earlier studies reported absence of *1858T* allele in Asia [23], we had reported a very low frequency (2.03%) of this allele in normal healthy individuals [25]. In spite of this low frequency in the normal population, we observed a disease specific increase in the frequency of *1858T* allele in leprosy patients. So, we investigated if the same was true for T1D as well, since several reports have shown association of *1858T* allele with Type 1 diabetes in different ethnic groups [4,20-22], however, there are no studies reported so far from the Indian sub-continent with respect to *PTPN22* polymorphism in T1D, probably due to presumption that the allele is absent in Asian populations. Here, we report the association of *PTPN22 1858T* allele and its association with predisposing *MHC class-II* alleles in T1D in patients from North India.

Materials and Methods

Two hundred and Fifty T1D patients and 480 normal healthy controls based in Delhi, originally from North Indian states of Uttar Pradesh, Haryana and Punjab, were studied after obtaining informed consent from patients and controls and Institutional Human Ethics Committee's clearance from National Institute of Immunology and All India Institute of Medical Sciences, New Delhi, India. The patient group consisted of 106 females and 144 males with a mean age of 25.96 ± 9.79 and age at onset of diabetes<30 years (Mean age at onset ± SD=15.995 ± 7.43), attending 'Juvenile Diabetes Clinic' at the All India Institute of Medical Sciences, Delhi, India. All the patients attending 'Juvenile Diabetes Clinic' were carefully assessed and categorized as type 1, type 2 and fibrocalculous pancreatopathy according to the classification of the American Diabetes Association expert committee [26] as reported earlier [9,10]. Controls included 480 healthy subjects (135 women and 345 men), with a mean age ± SD of 30.16 ± 9.02.

For controls, we preferred higher age group to rule out their possibility of developing T1D at a later date after the collection of their blood samples as normal healthy controls. Controls were asked for their clinical history and only those without any history of infectious or autoimmune diseases were included in the study.

DNA was extracted using a standard protocol from 10 ml of blood from T1D patients and healthy controls from the same ethnic background after obtaining informed consent and Institutional Ethics Committee's approval.

PTPN22 Genotyping using PCR-RFLP Polymerase Chain Reaction followed by restriction fragment length polymorphism

The genotyping for *PTPN22* single nucleotide polymorphism at position *C1858T* in the codon 620 was carried out using PCR-RFLP methods where standard PCR protocol was followed by restriction digestion of the PCR amplified product with restriction enzyme Rsa1. Primers used for amplification were forward primer (5'-ACTGATAATGTTGCTTCAA-3.) and reverse primer (5'-TCACCAGCTTCCTCAACCA-3'), as described by Zheng et al., 2005 (Genbank accession # AL 137856). Briefly, 100 ng of genomic DNA (5 µl) was mixed with 2.5 µl of 10X buffer, 0.25 µl of dNTPs (100 mM), 2.5 µl $MgCl_2$ (25 mM), 2.0 µl (5 pm/µl) each of the primers, and 0.125 µl (5 U/µl) of Taq polymerase in 25 µl reaction volume. After initial denaturation at 94°C for 2 minutes, 35 cycles of denaturation at 94°C, annealing at 60°C and extension at 72°C, each for 30 seconds, were carried out followed by final extension cycle at 72°C for 7 minutes in Gene Amp 2700 thermocycler (Applied Biosystems, CA, USA). After confirmation of the amplified products on 1% Agarose gel, 10 µl each of the amplified products were digested with 5 U of RsaI enzyme (New England Biolabs) overnight at 37°C. The digested PCR products were run on 2.5% agarose gel in 0.5X TBE buffer along with 50 bp ladder. The restriction endonuclease *Rsa 1* cleaves the DNA strand which contains the *C* nucleotide at 1858th position and gives two bands of 176 bp and 42 bp sizes, the mutant *1858T*, however, is not digested by *Rsa 1* and gives a single band of 218 bp size (Figure 1).

HLA-DRB1 polymorphism

Alleles of *HLA-DRB1* locus were determined for 243 T1D patients

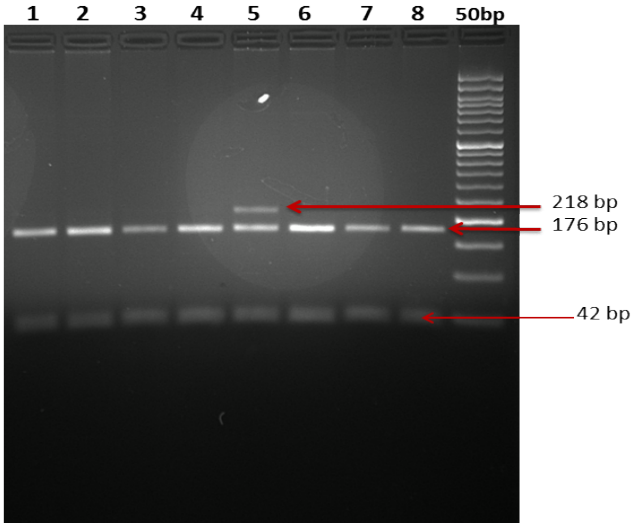

Figure 1: A representative picture of PCR-RFLP of *PTPN22 C1858T* on 2.5% Agarose gel: lanes 1-4 and 6-8 show homozygous *1858CC* genotype with 176 bp and 42 bp bands. Lane 5 shows heterozygous *1858CT* genotype with 218 bp, 176 bp and 42 bp bands, last lane is 50 bp ladder.

and 327 controls for whom *PTPN22 C1858T* data was available. For 100 patient samples and 94 controls samples, the second exons of the *DRB1* genes were PCR amplified using standard conditions and hybridized with ^{32}P-labeled sequence specific oligonucleotide probes (SSOP) as described earlier [6,9]. For additional 143 patients and 233 control samples, Luminex based typing was done using Labtype SSO kit from One Lambda, (Canoga Park, CA, USA) according to the manufacturer's instructions as described earlier [9]. Briefly, 40 ng of DNA was amplified in master mix, primers and 2 µl of Taq DNA polymerase in Gene Amp 2700 thermocycler (Applied Biosystems, CA, USA). After confirmation of amplification, on 1% agarose gel, 5 µl of amplified product was denatured and hybridized with appropriate amount of multiplex beads conjugated with oligonucleotide probes, in hybridization buffer at 60°C for 15 minutes followed by washings and incubation with 50 µl 1X SAPE solution (Steptavidin Phycoerythin) at 60°C for 5 minutes. Fluorescence of the labeled beads was acquired on Luminex 2.2 flow cytometer (Luminex corporation, Austin, TX). Acquired data was analysed using Labtype software provided by One Lambda for analysis of *MHC* alleles. Reproducibility of the Luminex based HLA typing method had been checked by us earlier [9].

Statistical analysis

Chi-square test was used to examine the statistically significant differences between patients and controls using Stata 9.2 software. Odds Ratios and 95% confidence Intervals were also calculated using the same software. *p* values were corrected using Bonferroni's correction for multiple comparisons. Fisher's exact test was used whenever the numbers in any group were less than 5 i.e. in cases or controls for any allele. In such cases, Odds ratios were calculated using Woolf's method [27] with Haldane's [28] modification as described earlier [29].

Results

We have genotyped 250 T1D patients and 480 healthy controls from the same ethnic background for *C1858T* alleles of *PTPN22* as shown in Figure 1. Table 1 shows that that the mutant *1858T* allele is significantly increased in patients with T1D as compared to controls (p<0.0004, Odds Ratio=2.82, 95% confidence Interval=1.49-5.4). While homozygous genotype *CC* was significantly reduced (p<0.0003, Odds Ratio=0.34, 95% confidence Interval=0.18-0.65), heterozygous *CT* was significantly increased in T1D patients compared to controls (p<0.0003, Odds Ratio=2.93, 95% confidence Interval=1.53-5.71). And these differences remained significant even after Boneferroni's correction. All genotype frequencies in patients as well as controls were in Hardy Weinberg equilibrium.

To study if there was any gender bias, we stratified the samples for association of *1858CT* genotype with gender of the patients. While genotype *CT* and *T* allele were significantly increased in both males and females when compared to male and female controls respectively, the association was stronger in females (for *CT* p<0.002, OR=6.4 and for *T* p<0.003, OR=6.12) since the frequencies of *CT* genotype and *T* allele were much less in female controls (1.5% and 0.75% respectively) compared to male controls (4.9% and 2.51% respectively), although the same were comparable in male and female patients (Table 2).

We further checked whether *CT* genotype and *T* allele had any significant association with age at the onset of T1D (Table 3). For this we divided the patients in two groups based on their ages at the onset of the disease i.e., more than (>) 14 years and less than or equal to (≤) 14 years. Interestingly, while both age groups had significant association with the predisposing genotype *CT* and allele *T,* patients who had early age at onset i.e. ≤ 14 years showed more significant association (for *CT* p<0.0007, OR=3.13, for *T* p<0.0008, OR=3.15).

We had earlier reported *HLA-DRB1*03:01*, **04:01* and *04:05* to be predisposing for T1D in North Indians [6], To study the role of predisposing HLA alleles in combination with the *PTPN22* alleles, 243 T1D patients and 327 normal healthy controls who were studied for *PTPN22 C1858T* were also typed for *HLA-DRB1* locus alleles. Our results were in accordance with the earlier results i.e. all three alleles, *HLA-DRB1*03:01*, **04:01* and *04:05* were significantly increased in T1D patients (Table 4) and *DRB1*07:01* was significantly reduced in the patients as compared to healthy controls. Simultaneous presence of *PTPN22 1858CT* and *1858CC* along with predisposing *HLA* alleles was investigated and interestingly both *PTPN22 1858CT* and *1858CC* along with *HLA-DRB1*03:01* were significantly increased in the T1D patients

Allele/Genotype	T1D Patients N=250		Controls N=480		T1D vs. Controls		
	n	%	n	%	p	Odds Ratio	95% C.I.
Allele*1858C*	473	94.6	941	98.02	0.0004	0.35	0.18-0.67
Allele*1858T*	27	5.4	19	1.98	0.0004	2.82	1.49-5.4
Genotype *CC*	223	89.2	461	96.04	0.0003	0.34	0.18-0.65
Genotype *CT*	27	10.8	19	3.96	0.0003	2.93	1.53-5.71

Table 1: Allele and genotype frequencies of *PTPN22 C1858T* in T1D as compared to normal healthy controls.

Allele/Genotype	T1D Male N=144	T1D Female N=106	Controls Male N=345	Controls Female N=135	Male T1D vs. Male Controls			Female T1D vs. Female Controls		
	n (%)	n (%)	n (%)	n (%)	p value	Odds Ratio	95% C.I.	p value	Odds Ratio	95% C.I.
Allele*1858C*	272 (94.4)	201 (94.8)	673 (97.5)	268 (99.25)	0.015	0.43	0.2-0.9	0.003*	0.16**	0.06-0.43
Allele*1858T*	16 (5.6)	11 (5.2)	17 (2.5)	2 (0.75)	0.015	2.33	1.08-4.97	0.003*	6.12**	2.3-16.26
Genotype *CC*	128 (88.9)	95 (89.6)	328 (96.1)	133 (98.5)	0.013	0.41	0.19-0.9	0.002*	0.15**	0.05-0.42
Genotype *CT*	16 (11.1)	11 (10.4)	17 (4.9)	2 (1.5)	0.013	2.4	1.1-5.2	0.002*	6.4**	2.4-17.2

*Fisher's exact test was used to calculate p values.
**Haldane's modification was used to calculate the Odds Ratio.

Table 2: Gender-wise distribution of allele and genotype frequencies of *PTPN22 C1858T* in T1D as compared to matched normal healthy controls (07.01.14).

Allele/ Genotype	T1D >14 years N=133	T1D ≤ 14 years N=117	Controls N=480	Age at onset>14 years vs. controls			Age at onset ≤ 14 years vs. controls		
	n (%)	n (%)	n (%)	p value	Odds Ratio	95% C.I.	p value	Odds Ratio	95% C.I.
Allele*1858C*	253 (95.1)	220 (94.0)	941 (98.02)	0.008	0.39	0.18-0.88	0.0008	0.32	0.15-0.69
Allele*1858T*	13 (4.9)	14 (5.98)	19 (1.98)	0.008	2.54	1.1-5.5	0.0008	3.15	1.43 -6.73
Genotype *CC*	120 (90.2)	103 (88.1)	461 (96.04)	0.0076	0.38	0.17-0.86	0.0007	0.3	0.14-0.68
Genotype *CT*	13 (9.8)	14 (11.9)	19 (3.96)	0.0076	2.62	1.1-5.8	0.0007	3.3	1.5-7.2

Table 3: Genotype frequencies of *PTPN22 C1858T* in T1D based on age at onset as compared to normal healthy controls (07.01.14).

Allele/Genotype	T1D Patients N=243		Controls N=327		p	Odds Ratio	95% C.I.
	n	%	n	%			
*DRB1*03:01*	188	77.4	52	15.9	6.58×10^{-49}	18.07	11.61-28.21
*DRB1*04:01*	28	11.5	8	2.45	0.00001	5.19	2.24-13.4
*DRB1*04:03*	8	3.29	29	8.87	0.0075	0.35	0.13-0.8
*DRB1*04:05*	22	9.05	1	0.31	$5.71 \times 10^{-8*}$	22.11**	6.82-71.59
*DRB1*07:01*	30	12.35	103	31.5	8.96×10^{-8}	0.31	0.19-0.49

* Fisher's exact test was used to calculate p values.
** Haldane's modification was used to calculate the Odds Ratio.

Table 4: Frequencies of predisposing *HLA-DRB1* alleles In T1D patients and controls typed for *PTPN22 C1858T.*

PTPN22 CC/CT with predisposing and protective *HLA* alleles	T1D N=243		Controls N=327		p	Odds ratio	95% C.I.
	n	%	n	%			
*CT- HLA-DRB1*03:01*	24	9.9	3	0.91	$6.34 \times 10^{-7*}$	10.34**	4.64-23.05
*CC- HLA-DRB1*03:01*	164	67.9	49	14.98	1.36×10^{-37}	11.77	7.71-18.05
*CT- HLA-DRB1*04:01*	2	0.82	0	0	0.18*	6.78**	0.78-58.22
*CC- HLA-DRB1*04:01*	26	10.7	8	2.453	0.00003*	4.78**	2.05-12.41
*CT- HLA-DRB1*04:03*	0	0	0	0	-	-	-
*CC- HLA-DRB1*04:03*	8	3.3	29	8.86	0.0075	0.35	0.14-0.8
*CT- HLA-DRB1*04:05*	2	0.82	0	0	0.18*	6.78**	0.78-58.22
*CC- HLA-DRB1*04:05*	20	8.23	1	0.31	$3.17 \times 10^{-7*}$	19.96**	6.14-64.87
*CT- HLA-DRB1*07:01*	3	1.23	5	1.53	0.53*	0.85**	0.33-2.22
*CC- HLA-DRB1*07:01*	27	11.11	98	29.97	7.39×10^{-8}	0.29	0.17-0.47

*Fisher's exact test was used to calculate p values
**Haldane's modification was used to calculate the Odds Ratio.

Table 5: Association of predisposing *PTPN22 C1858T* allele with predisposing *HLA* alleles.

as compared to controls (Table 5), suggesting that while predisposing HLA alleles have a dominant effect, association of *PTPN22CT* genotype is independent of *MHC* associations.

Discussion

Several genes have been implicated for predisposition to develop T1D. While the *MHC* region has been shown to account for 50% of the inherited risk [8], an integrated role of other genes like *INS-VNTR*, *CTLA-4*, Vitamin D receptor *(VDR)*, *cytokine genes* and *PTPN22* amongst a number of other genes cannot be ruled out [6,9,10,12,30,31] since it is a very complex autoimmune disorder where most of the implicated genes have some functional relevance. For example, *INS-VNTR class-I* alleles have been shown to have poor expression of insulin in thymii of fetuses as compared to those with *Class-III* alleles [32,33] which may be responsible for poor thymic education for insulin resulting in autoimmunity against insulin producing pancreatic beta cells [32,33]. Similarly, the products of *CTLA-4* and *PTPN22* genes have roles in negative T cell signaling. CTLA-4 is constitutively expressed on regulatory T cells [34,35] and defect in CTLA-4 expression may be detrimental for the manifestations of autoimmunity since autoimmune responses may not be regulated in these cases [35].

The other negative regulator of T cell signaling, *PTPN22* with SNP *C1858T,* has been implicated in many autoimmune disorders [18,19,36] and was first reported by Bottini et al. in Type 1 diabetes patients from two independent populations [4,37], followed by several reports where the associations were reported with either T1D per se, or with female gender or with beta cell dysfunction or autoantibodies and even with early age at onset in children fed with cow milk formula, in different populations [20-22,38-41]. Our observations replicated earlier reports, and show a significantly higher number of T1D patients from North India to have *1858CT* genotype and *T* allele as compared to controls [37,39,42,43]. Fedetz et al. [42], observed a significantly higher number of T1D patients from Ukraine to have *TT* genotype and *T* allele as compared to healthy controls. They also observed the association was stronger in female patients and in those at younger age at onset, similar to our findings where *1858CT* genotype showed stronger association in female patients and early age at onset.

While we do find a significant increase of *T* allele and *CT* genotype in T1D patients, total absence of homozygous *1858TT* genotype was surprising. Under normal Mendelian inheritance pattern, one would expect some homozygous *TT*s as well since 10.8% of the patients had heterozygous *CT* genotype. However, since the frequency of the *T* allele is very low in healthy controls, the chances of getting homozygous *TT* would probably be very low. While no association of *1858T* allele was observed with T1D in Japanese population, another SNP in the region, rs1310182, was significantly associated in them. In fact, this nucleotide position *C1858T* was reported to be non-polymorphic in Japanese [44]. Frequency of *1858T* alleles varies in different populations. While we observed a very low frequency of *1858T* allele in North Indians [25], earlier studies reported a total absence of *T* allele in Asian populations [23]. Our study suggests that Asian populations cannot be generalized as one ethnic group since the earlier reports of absence of the *T* allele have been from Asian populations from Japan, Korea and China, which being Mongoloid in origin, are ethnically different from Indians who are basically Caucasoid with racial admixture of Mongoloid and Negroid elements [45].

Our data further suggests that association of *PTPN221858CT* genotype was independent of the predisposing MHC alleles, suggesting that the primary association is with *DRB1*03:01* which was observed in 77.4% of T1D patients, compared to 10.8% of the patients having *PTPN221858CT* genotype. Recently several papers have been published on functional relevance of the *PTPN22 1858T* or LYP*W620 allele, where *PTPN22* encoded LYP has been shown to have roles in both innate and adaptive immune responses. Expression of LYP*W620 has been associated with reduced TLR signaling resulting in defective type 1 IFN-mediated host response to infection which may have role in initiation of T1D that is associated with viral infection [46]. Also, LYP negatively regulates T cell functions by dissociating from C-terminal Src tyrosine kinase (CSK) which is essential for recruitment of LYP to the lipid rafts where it regulates TCR mediated signaling. And the disease associated LYP*W620 (*1858T* allele) was shown to partition into lipid rafts more efficiently than LYP*R620 due to its inefficiency to bind CSK and was thus shown to be more potent inhibitor of TCR induced responses [15].

Type 1 diabetes is a multifactorial disease with several components like a jigsaw puzzle with several genomic loci implicated in the manifestation of the disease. We have been trying to put some of the pieces of the puzzle together where some components are inter-related while others are independently affecting the manifestations in the presence of other predisposing factors. We have reported earlier that simultaneous presence of *DRB1*0301* along with homozygous *INS-VNTR class-I* alleles was significantly increased ($p < 10^{-8}$) in T1D patients, giving a relative risk of 70.81 [6]. We also provided evidence of statistically significant interaction between the predisposing *HLA* allele and high producer alleles of *VDR* which may be detrimental for the manifestation of T1D in the absence of 1,25-$(47)_2D_3$ in early childhood and/or *in-utero*. Our studies on *HLA-DRB1*03:01* homozygous cell lines showed enhanced expression of HLA-DR on the B-LCLs stimulated with 100 nM calcitriol as compared to the unstimulated ones confirming that indeed the interaction of *VDR* with *HLA-DRB1*03:01* is occurring through the VDRE present in the promoter region of the gene [10]. Based on these studies we speculated that in the absence of required amount of Vitamin D in early life in the predisposed individuals with *HLA-DRB1*0301*, the expression of the allele may be impaired in the thymus [47,48] resulting in escape from thymic deletion of autoreactive T cells leading to T1D manifestations. Poor thymic education for insulin and HLA-DRB1*03:01 proteins along with environmental factors like viral infections, vitamin D deficiency and some milk proteins may be involved in initiation of the autoimmune responses against the pancreatic beta cells. While predisposing HLA class-II molecules may be involved in auto-antigen presentation to T helper cells, higher producing genotypes of pro-inflammatory cytokines like *IFN-gamma* and *TNF-alpha* may be involved in enhancing the cell mediated immune responses through proliferation of $CD4^+$ and $CD8^+$ T cells, and higher producing genotypes of *IL-10* and *TGF-beta* may have a role in recruitment of these autoreactive T cells in the pancreas through ICAM-1 and Integerin dependent pathways [9]. Additionally, *PTPN22 1858CT* genotype may have a role in defective innate immune responses against viral infections that may trigger islet cell autoimmunity [46] combined with defects in early T cell signaling due to which autoreactive T cells may not be deleted and may lead to insufficient activity of regulatory T cells, thus leading to autoimmune [4,16] destruction of pancreatic beta cells which may occur through $CD4^+$ and $CD8^+$ T cells and nitric oxide production.

Author Contribution

RR conceived the project and critically evaluated the data, NI, AK SV and JS were involved in generating the Type1 diabetes and control data for *PTPN22* and *HLA*. RR analysed the data and wrote the manuscript.

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