Interaction of Vitamin D Receptor with HLA DRB1*0301 in Type 1 Diabetes Patients from North India

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Abstract

Background: Type 1 diabetes (T1D) is a multifactorial autoimmune disorder where interaction and integration of immune response genes along with environmental factors play a role in autoimmune destruction of the insulin producing Pancreatic Beta cells.

Methodology/Principal Findings: We have studied four single nucleotide polymorphisms (FokI site in Exon 2, BsmI and Apal sites in Intron 8 and TaqI site in exon 9) in the vitamin D receptor (VDR) gene using PCR-RFLP and HLA-DRB1 alleles using PCR and hybridization with sequence specific oligonucleotide probes and studied their interaction using LD based statistics for non-linked loci followed by sequence analysis of the vitamin D response element (VDRE) present in the promoter region of HLA-DRB1*0301. Haplotypes, constructed using SHEsis program for four single nucleotide polymorphisms in the VDR gene, were studied for their interaction with HLA-DRB1 alleles in 233 T1D patients and 191 healthy controls from North India. A significant increase of haplotypes FBAT and FBAT (p<0.02, OR = 1.44 and p<0.002, OR = 3.23 respectively) was observed in the patients. Both the haplotypes FBAT and FBAT were significantly increased in male patients with age at onset less than 18 years; however, FBAT was significantly increased in female patients irrespective of their age at onset. LD based statistics showed significant interaction between the high producer F and T alleles with HLA-DRB1*0301. F and T alleles of VDR have been shown to contribute to VDR mRNA independently. The promoter sequence analysis of HLA-DRB1*0301 showed presence of VDRE involved in higher expression of HLA-DRB1*0301, which was confirmed by flow cytometry and real time PCR analysis.

Conclusions/Significance: These data suggest that the interaction between VDR and HLA alleles is mediated by VDRE present in the promoter region of HLA-DRB1*0301 allele, which may be detrimental for the manifestation of T1D in the absence of 1,25-(OH)2D3 in early childhood due to poor expression of DRB1*0301 in the thymus resulting in autoimmunity.

Introduction

Type 1 diabetes (T1D) is a multifactorial, autoimmune disorder where the insulin producing pancreatic beta cells are destroyed by one’s own immune system. The disorder occurs with an incidence of 10.5/100,000/year in India [1]. T1D develops as a result of complex interaction of many genetic and environmental factors leading to autoimmune destruction of the insulin producing Pancreatic Beta cells. While 20 genomic intervals have been implicated for the manifestation of the disease, role of an intricate network of the products of these genes cannot be ruled out. We have shown earlier that simultaneous presence of DRB1*0301 along with homozygous INS-VNTR class-I was significantly increased (p<10^-6) in T1D patients, giving a relative risk of 70.81 [2]. Simultaneous presence of high secretor genotypes of TNF-x -308 (Ga + Aa) along with high secretor genotypes of IL-6, IL-10 and TGF-β1 were also significantly increased in T1D patients. Low secretor genotype of TNF- x -308 GG along with low secretor genotypes of IFN-γ, high secretor genotypes of IL-6, and TGF- β1 were protective. This effect of TNF-x high secretor genotype was independent of predisposing HLA-DRB1*0301 [3]. To further understand the intricate network of genes regulating the immune responses, we have studied the interaction of vitamin D receptor (VDR) polymorphic alleles with predisposing HLA alleles in T1D patients using Linkage Disequilibrium (LD) based statistics between two unlinked loci.

Vitamin D Receptor (VDR) is a ligand dependent transcription factor that belongs to the super family of the Nuclear Hormone Receptors [4]. The ligand for VDR is Vitamin D3 i.e., 1,25-(OH)2D3 which mediates its biological actions through VDR. Binding of 1,25-(OH)2D3 induces conformational changes in VDR which promotes its hetero-dimerization with Retinoid X Receptor (RXR), followed by translocation of this complex into the nucleus. The RXR-VDR heterodimer binds to the vitamin D3 responsive elements (VDRE) in promoter regions of 1,25-(OH)2D3 responsive genes[5], which in turn results in the regulatory function of 1,25-
(OH)_2D_3. In the absence of classical responsive elements, 1,25-(OH)_2D_3 may control the expression of some genes like cytokine genes by targeting inducible transcription factors like NFAT in IL-2 in a sequence specific manner [6]. 1,25-(OH)_2D_3 has been shown to have an important immuno-modulatory role since it represses transcription of IL-2 [7-8], IFN-γ [9], GM-CSF [10] and IL-12 [11] and up regulates the production of Th2 cytokines IL-4 and TGF-β1 [12], thereby inhibiting the overall Th1 responses. It has been shown to enhance the development of TH2 cells via a direct effect on naïve CD-4 cells [5]. Besides, 1,25-(OH)_2D_3 has also been shown to modulate the expression of HLA class-II alleles on monocytes and human bone cells [13,14].

In NOD mice, administration of 1,25-(OH)_2D_3 before the onset of Insulitis, has been effectively shown to prevent the disease progression. However, this treatment was found to be ineffective when Insulitis had already been established. Treatment of adult NOD mice with 1,25-(OH)_2D_3 analog has also been shown to be effective [15-18]. Similarly, in humans, vitamin D supplementation in early childhood has been shown to reduce the incidence of T1D [19,20]. Since 1,25-(OH)_2D_3 is a VDR ligand, we have studied the VDR gene polymorphisms and their interaction with the most predisposing MHC alleles to investigate their role, if any, in the pathophysiology of T1D. The VDR single nucleotide polymorphisms (SNPs) studied include the T>C SNP in exon2 initiation codon detected with FokI restriction enzyme [21], the A>G SNP detected with BsmI [22] and G>T SNP detected with ApaI [23] located in Intron 8, and a silent C>T SNP [24] detected with TaqI, located in Exon 9. We studied the interaction between VDR alleles and predisposing HLA alleles using LD based statistics [25] and subsequently sequenced the promoter region of the predisposing MHC allele to detect the VDRE sequence which has been shown to modulate the expression of the HLA alleles [26], suggesting the functional implications of the statistically significant interaction.

Results

VDR FokI, BsmI, Apal and TaqI Genotypes and Haplotypes in T1D Patients

Table 1 shows the frequencies of FokI, BsmI, Apal and TaqI genotypes (figure 1A-D) in the T1D patient group as compared to the control group. FokI, BsmI and TaqI sites were found to be in Hardy Weinberg equilibrium both in patients as well as controls and Apal site was in Hardy Weinberg disequilibrium in both patients and controls. While there were no significant differences in the genotypes of Apal and TaqI in patients and controls, FokI ‘ff’ was significantly increased in the patient group as compared to controls (p<0.047) and BsmI ‘bb’ was significantly decreased in the patient group (p<0.04). However, these differences did not remain significant after Bonferonni’s correction.

Haplotype analysis was carried out for the four restriction sites studied in the VDR gene in patients and controls using SHEsIs software (http://202.120.7.14/analysis/myAnalysis.php) [27]. Additionally, Famhap (http://famhap.meb.uni-bonn.de) was used to confirm the frequencies of the haplotypes. Since both Famhap and SHEsIs were giving similar results, we carried out the rest of the analysis using SHEsIs only. Table 2 shows the haplotype frequencies of VDR in T1D patients and controls. The software was instructed to drop frequencies less than 0.03 in the analysis. The analysis showed that FokI site was in a very weak linkage disequilibrium (LD) with BsmI, Apal and TaqI with D’ values of 0.004, 0.01 and 0.04 respectively. BsmI, however, was in strong LD with Apal and TaqI with D’ values of 0.91 and 0.93 and Apal was also in strong LD with TaqI with a D’ value of 0.97. Significant differences in terms of haplotypes was observed between patients and controls with a global chi-square of 20.9 for 6 degrees of freedom with a p value of 0.002. Individually, haplotype FBAI and FBAT were significantly increased in T1D patients (p<0.02 and <0.001 respectively) and FbaT was significantly reduced in them as compared to controls (p<0.036).

Association of VDR FokI-BsmI-Apal-TaqI Haplotypes with Age at Onset

To study if the VDR haplotypes are associated with age at onset, we divided the patients into two groups adult onset i.e., above 18 and young onset as below or equal to 18 years (Table 3 shows data on 223 samples in whom the exact age at onset was known). The analysis showed that haplotypes FBAI and FBAT to be significantly increased in patients who developed diabetes before 18 years of age (p<0.0002 and 0.0077 respectively) and haplotype FbaT seemed to be protective from younger onset of T1D (p<0.037). However, adult onset was not significantly associated with any VDR haplotypes in T1D patients.

Association of VDR FokI-BsmI-Apal-TaqI Haplotypes with Gender and Age at Onset of T1D Patients

To study if this association of VDR haplotypes had any gender bias, comparisons were done between female/male patients and female/male controls and also with all controls (female + males). Table 4 shows the gender-wise analysis of VDR haplotype using SHEsIs software. While FBAT was significantly increased in female patients as compared to controls (both female controls and all controls), FBAT was significantly increased in males as compared to controls (both male controls and all controls). FbaT was significantly reduced in female patients when compared to female controls and FbaT was significantly reduced in male patients as compared to controls. A comparison of female patients with male patients did not reveal any significant differences. Since the patients with younger age at onset were showing significant differences, we analysed the data with respect to age at onset in the two genders (data not shown). The haplotypes FBAI and FBAT were significantly increased in male patients below the age of onset of 18 years as compared to controls (p<0.0024 and p<0.0017 respectively) and FBAT was significantly reduced (p<0.0038) in the male patients below the age at onset of 18 years as compared to controls. No significant differences were observed in the VDR haplotypes of male patients with age at onset above 18 years when compared to controls. However, in the females, the haplotype FBAT was significantly increased in patients irrespective of their age at onset whether early or adult onset and haplotype FbaT was significantly reduced in female patients with less than 18 years age at onset.

Gene to Gene Interaction of VDR Haplotypes with Predisposing HLA Alleles

Based on the SHEsIs and Famhap analysis, haplotypes were constructed manually for each sample. The haplotype with the highest frequency as calculated by SHEsIs was constructed from the genotype of each sample and the second haplotype was constructed by subtraction of the first haplotype. Simultaneous presence of different haplotypes with the predisposing HLA alleles DRB1*0301, DRB1*0401, DRB1*0402 and DRB1*0405 (listed as DR3 for the sake of simplicity in Table 5 which includes all these alleles) in patients was studied. Interestingly, haplotypes FBAI, FbaT, whose frequencies were not significantly different in patients and controls, showed a significant increase in patients when present along with the predisposing
Table 1. Comparison of genotype frequencies of VDR SNPs at Fok1, Bsm1, Apa and Taq1 sites in T1D patients with controls.

| SNP | T1D | Controls | T1D vs Controls | 95% CI | Global p value |
|-----|-----|----------|-----------------|-------|--------------|
| FF  | 142 | 116      | FF vs FF        | 0.483 | 0.774–1.79   | 0.122          |
| Ff  | 79  | 76       | Ff vs ff        | 0.03**| 0.346        | 0.109–1.086    |
| ff  | 15  | 5        | ff vs ff        | 0.06**| 0.41         | 0.126–1.246    |
| BB  | 79  | 56       | BB vs Bb        | 0.735 | 0.698–1.75   | 0.134          |
| Bb  | 120 | 94       | Bb vs bb        | 0.055 | 0.638        | 0.4–1.017      |
| bb  | 37  | 47       | bb vs bb        | 0.04  | 0.996–3.23   |               |
| AA  | 85  | 60       | AA vs Aa        | 0.52  | 0.757–1.82   | 0.09           |
| Aa  | 133 | 110      | Aa vs aa        | 0.09  | 0.907–3.64   |               |
| aa  | 18  | 27       | aa vs aa        | 0.04  | 2.125        | 1.02–4.45      |
| TT  | 91  | 80       | TT vs Tt        | 0.93  | 0.651–1.523  | 0.38           |
| Tt  | 112 | 98       | Tt vs Tt        | 0.246 | 0.658        | 0.335–1.285    |
| tt  | 33  | 19       | TT vs tt        | 0.253 | 0.655        | 0.329–1.299    |
| HWE p = 0.38@ | HWE p = 0.07 | FF vs Ff+ff   | 0.862 | 0.704–1.58   |               |
| bb vs Bb+bb | 0.305 | 1.3       | 0.82–1.95       |       |               |
| N = 236 | N = 197 | N = 236 | N = 197 |
| AA  | 85  | 60       | AA vs Aa        | 0.52  | 0.757–1.82   | 0.09           |
| Aa  | 133 | 110      | Aa vs aa        | 0.09  | 0.907–3.64   |               |
| aa  | 18  | 27       | aa vs aa        | 0.04  | 2.125        | 1.02–4.45      |
| TT  | 91  | 80       | TT vs Tt        | 0.93  | 0.651–1.523  | 0.38           |
| Tt  | 112 | 98       | Tt vs Tt        | 0.246 | 0.658        | 0.335–1.285    |
| tt  | 33  | 19       | TT vs tt        | 0.253 | 0.655        | 0.329–1.299    |
| HWE p = 0.0005 | HWE p = 0.04 | AA vs Aa+aa    | 0.263 | 0.84–1.96    |               |
| aa vs AA+Aa. | 0.056 | 0.52      | 0.264–1.02      |       |               |
| N = 236 | N = 197 | N = 236 | N = 197 |

*Corrected p (pc) value is not significant.
**Calculated using Fisher’s exact test.
@HWE p value calculated using SHEsis program.
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**DRB1** alleles while the same haplotypes were protective when associated with non-predisposing alleles of **DRB1**. Similar results were obtained with other haplotypes like **FBAT**, **fbAT** and **fbaT** in association with the predisposing **HLA-DRB1** alleles, as shown in Table 5.

These results prompted us to study the interaction between two unlinked loci i.e., **VDR** and the predisposing **HLA-DRB1** alleles since it was crucial to study the LD patterns of these two unlinked loci between patients and controls. For this kind of analysis we used LD based statistics as described by Zhao et al (22). The analysis has been shown in Table 6. Two most frequent haplotypes of **VDR**, **FbaT** and **FBAT** and a relatively less frequent haplotype in patients **fbaT** do not show any significant difference between patients and controls when studied by themselves. However, when studied in association with the predisposing **HLA-DRB1** alleles, these VDR haplotypes show significant linkage disequilibrium. Since all these haplotypes had consistently **T** allele at **Taql** site and either of the two alleles at **FokI**, **BsmI** and **ApaI** sites, in order to decipher which alleles were actually involved, we studied the LD pattern between the predisposing **HLA-DRB1** with each of these alleles at the four SNP sites separately.

**Figure 1. The genotypes for the four SNPs were determined by PCR amplification and restriction digestion of the PCR products with enzymes **FokI**, **BsmI**, **ApaI** and **Taql**.**

- **A**: **FokI** digestion: SNP C/T in exon 2 was studied by amplifying a 265 bp fragment using primers 5'AGCTGCCCTGGCACTGACTTGGCTCT 3' and 5'ATGGAAACCCCTTGGCTTCTCCT 3' with 68°C as annealing temperature and digestion by **fok1** at 37°C for 3 hours. Presence of restriction site is denoted by 'T' while absence of restriction is denoted by 'C'. Results show CC (CC) i.e., 265 bp, 196 bp and 69 bp bands, FF (CT) i.e., 265 bp and 196 bp bands, and FF (CT) i.e., 265 bp and 196 bp bands. M is the 100 bp ladder.
- **B**: **BsmI** digestion: SNP A/G in Intron 8 was studied by amplifying an 825 bp fragment using primers 5'CAACAGACTACAAGTACCGGTCAATGA 3' and 5'AACACGGGGAGAGGTCAAGGGG 3' with 65°C as annealing temperature and digestion by Bsm1 at 65°C for one hour. Presence of restriction site is denoted by 'B' and absence of restriction is denoted by 'A'. The results show BB (AA) i.e., 825 bp band, Bb (AG) i.e., 825 bp, 650 bp and 175 bp and bands and bb (GG) 650 bp and 175 bp bands. M is 25 bp ladder.
- **C**: **ApaI** digestion: SNP T/G in Intron 8 was studied by amplifying an 740 bp fragment using primers 5'CAACACTCTCTAGGGCTAGCTCTCA 3' with 65°C as annealing temperature and digestion by Apa1 at 37°C for one hour. Presence of restriction site is denoted as 'a' and absence of restriction is denoted by 'A'. The results show AA (TT) i.e., 740 bp band, Aa (AT) i.e., 740 bp, 520 bp and 220 bp bands and aa (GG) 520 bp and 220 bp bands. M is 100 bp ladder.
- **D**: **Taql** digestion: SNP T/C in exon 9 was studied by amplifying an 265 bp fragment using primers 5'AGCTGGCCCTGGCACTGACTCTTGCTCT 3' and 5'CAACACTCTCTAGGGCTAGCTCTCA 3' with 65°C as annealing temperature and digestion by Taq1 at 65°C for one hour. Presence of restriction site is denoted as 't' and absence of restriction is denoted by 'T'. The results show TT (TT) i.e., 265 bp and 220 bp bands Tt (TC) i.e., 265 bp, 245 bp and 220 bp bands and tt (CC) 245 bp, 220 bp and 205 bp bands. M is 100 bp ladder.

Table 7 shows the LD pattern of the **FokI**, **BsmI**, **ApaI** and **Taql** alleles with predisposing **HLA-DRB1** alleles with predisposing **HLA-DRB1** alleles (listed as DR3 in tables 5–7) in TID patients. F and T alleles in the exons 2 and 9 for **FokI** and **Taql** alleles respectively show significant interactions (p<0.0002 and 0.0003 respectively). However, for **BsmI** and **ApaI** sites both the alleles were significantly associated with predisposing HLA alleles suggesting their null effect.

**Sequence Analysis of HLA DRB1*0301 Promoter Region**

Since 85.9% of the patients had **DRB1** alleles, to study if the interaction of **VDR** with predisposing HLA alleles was mediated by **VDRE** present in the promoter region of the allele, we amplified the promoter region of 3 TID subjects and 3 healthy controls homozygous for **HLA-DRB1** alleles. Sequencing of the amplified promoter region was done to determine the **VDRE** variants in the North Indian population. Sequences were aligned using ClustalW2, and the presence of a **VDRE** was confirmed using JASPAR CORE version 3.0 database using default conditions [28]. Figure 2 shows the **HLA-DRB1** promoter sequences showing the localization of vitamin D response element (VDRE) in the promoter region of **HLA-DRB1** from the 6 subjects. Important regulatory elements like S-box, X-box, Y-box,
CCAAY-box, TATA-box and VDRE are highlighted in the figure. Interestingly, the alignment showed exactly the same sequence of VDRE in the promoter region of HLA-DRB1*1501 recently by Ramagopalan et al [26] suggesting the bases for interaction of VDR with HLA-DRB1*1501.

### Table 2. Haplotype Analysis for Fok1, Apa1, Bsm1 and Taq1 loci using SHEsis software [27]. (http://202.120.7.14/analysis/myAnalysis.php).

| Haplotype (Nucleotides) | 2N = 472 | Controls 2N = 394 | T1D Vs Controls | γ2 | Pearson’s p value | Odds Ratio | 95% C.I. |
|-------------------------|---------|------------------|-----------------|----|-------------------|-----------|--------|
| FBAT (CATC)              | 0.303   | 0.233            |                 | 5.340 | 0.0208            | 1.436     | 1.056–1.953 |
| FBAT (CATT)              | 0.142   | 0.146            |                 | 0.032 | 0.8582            | 0.966     | 0.659–1.416  |
| FbAT (CGGT)              | 0.262   | 0.317            |                 | 3.311 | 0.0688            | 0.759     | 0.563–1.022  |
| FbAT (CGTT)              | 0.045   | 0.065            |                 | 1.611 | 0.2042            | 0.683     | 0.378–1.234  |
| FBAT (TATT)              | 0.063   | 0.099            |                 | 4.402 | 0.0359            | 0.586     | 0.354–0.970  |
| FBAT (TATC)              | 0.065   | 0.021            |                 | 9.564 | 0.0019            | 3.227     | 1.478–7.049  |
| fbat(TGGT)               | 0.079   | 0.074            |                 | 0.066 | 0.7976            | 1.068     | 0.645–1.768  |

1Haplotype Frequencies less than 0.03 have not been shown in the analysis.

95% C.I. = 95% confidence interval. Global Chi² = 20.9 with Pearson’s p value = 0.002.

Linkage disequilibrium test using SHEsis (D').

D': Fok1-Bsm1 = 0.004, Fok1-Apa1 = 0.010, Fok1-Taq1 = 0.04, Bsm1-Apa1 = 0.91, Bsm1-Taq1 = 0.93, Apa1-Taq1 = 0.97.

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### Table 3. Association of VDR haplotypes with age at onset in T1D patients.

| VDR Haplotype | FBA | FBAT | FbAT | FbAT | FbAT |
|---------------|-----|------|------|------|------|
| Nucleotides   | CATC| CATT | TATT | CGGT | TATC |
| Age at Onset  |     |      |      |      |      |
| Patients≤18 years |   |   |   |   |   |
| HF* 2N = 268 | 0.33 | 0.11 | 0.08 | 0.25 | 0.06 |
| Patients>18 years | | | | | |
| HF 2N = 178 | 0.27 | 0.19 | 0.04 | 0.27 | 0.06 |
| Controls | | | | | |
| HF 2N = 394 | 0.23 | 0.15 | 0.02 | 0.32 | 0.1 |
| Patients age at onset≤18 years Vs. Controls | | | | | |
| p value | 0.0077 | 0.17 | 0.0002 | 0.0367 | 0.082 |
| Odds Ratio | 1.6 | 0.718 | 4.16 | 0.69 | 0.59 |
| 95% CI | 1.13–2.27 | 0.45–1.15 | 1.84–9.4 | 0.483–0.99 | 0.33–1.08 |
| Patients age at onset >18 years Vs. Controls | | | | | |
| p value | 0.274 | 0.21 | 0.112 | 0.273 | 0.169 |
| Odds Ratio | 1.26 | 1.35 | 2.2 | 0.8 | 0.62 |
| 95% CI | 0.84–1.89 | 0.84–2.17 | 0.81–5.93 | 0.54–1.19 | 0.31–1.23 |

Adult onset was considered above 18 years of age and child onset as 18 years or below 18 years. The two groups were compared with controls using SHEsis software.

Linkage Disequilibrium tests for patients below 18 years of age (SHEsis).

D': Fok1-Bsm1 = 0.048, Fok1-Apa1 = 0.005, Fok1-Taq1 = 0.054, Bsm1-Apa1 = 0.914, Bsm1-Taq1 = 0.949, Apa1-Taq1 = 0.955.

Linkage Disequilibrium tests for patients above 18 years of age (SHEsis).

D': Fok1-Bsm1 = 0.004, Fok1-Apa1 = 0.009, Fok1-Taq1 = 0.104, Bsm1-Apa1 = 0.886, Bsm1-Taq1 = 0.93, Apa1-Taq1 = 0.946.

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Altered Expression of HLA-DRB1*0301 by 1,25-(OH)2D3 (Calcitriol)

Flow cytometry. HLA-DRB1*0301 homozygous B-lymphoblastoid cell lines (B-LCL) VAVY (International Histocompatibility Workshop cell line Number IHW09023) and DUCAF (International Histocompatibility Workshop cell line Number IHW09019) were treated with 100 nM of calcitriol (Sigma) for 24 hours and stained with anti-HLA DR-PE antibody (BD Biosciences) to study the expression of HLA-DR on B-LCL cells treated with or without calcitriol and acquired on BD-LSR flowcytometer. The data was analysed using WinMDI 2.9 software.

The results showed 1.2-1.3 fold higher expression of HLA-DR in the B-LCLs treated with calcitriol as compared to the vehicle controls in three independent experiments (Figure 3). Two tailed Paired T test shows the difference to be significant with p<0.001 suggesting enhanced expression of HLA-DR in B-LCLs treated with calcitriol as compared to untreated ones.

Real Time PCR

RNA was extracted from the B-LCLs VAVY and DUCAF after 24 hour treatment with calcitriol and vehicle control, reverse transcribed and real time PCR was performed for HLA-DR to study its expression. The data shows an average of 1.79±0.28 (mean±S.D. of three independent experiments) fold increase in the HLA-DRB1 transcripts from B-LCL treated with calcitriol as compared to the vehicle control. We also confirmed these results using peripheral blood mononuclear cells (PBMCs) derived from a normal healthy control homozygous for HLA-DRB1*0301 and observed a 1.77 fold increase in expression of HLA-DR when treated with calcitriol as compared to untreated PBMCs.

Discussion

In this study we show that the F and the T alleles of FokI and TaqI site of VDR interact with predisposing HLA DRB1*0301 through VDRE present in the promoter region of the DRB1*0301 allele. VDR-1,25-(OH)2D3 complex has been shown to play a significant role in interfering with the signaling of transcription factors (like NFAT, NF-kB and AP-1) involved in the regulation of immunomodulatory genes [6,29–33] as well as expression of HLA class-II alleles on monocytes and human bone cells [13,14]. Polymorphisms in the VDR gene have been shown to influence FDR mRNA and protein levels [34], which in turn may influence...
the immunomodulatory function of VDR. We have studied four single nucleotide polymorphisms (FokI, BsmI, ApaI and TaqI sites) in the VDR region in TID and compared them to normal healthy controls. The SNP detected by FokI digestion is the only polymorphisms at the translation start site where a ‘T’ to ‘C’ substitution alters the VDR protein in such a way that there are either two ATG start sites separated by six nucleotides or only one start codon if the ‘T’ is substituted by ‘C’. The former gives rise to a restriction site for FokI and is designated as ‘f’ allele which encodes a 427 amino acid protein, while the latter is designated as ‘F’ allele encoding a 424 amino acid protein. [35–37]. The shorter ‘F’ allele has been reported to have emerged later after the divergence of hominids from Apes [38] and a higher percentage of the allele suggests an evolutionary advantage [39]. Recently, ‘F’ allele has also been shown to affect the immune system with a more active immune system for short ‘F’-VDR suggesting its role in the immune-mediated diseases [40]. The gene to gene interaction analysis shows allele F and predisposing MHC alleles to have significant interaction suggesting the integrated roles of the MHC alleles in antigen presentation and VDR-F allele in enhanced autoimmune responses since shorter F-VDR has been shown to result in higher NF-kappaB- and NFAT-driven transcription as well as higher IL-12p40 promoter-driven transcription [40].

In the present study, we observed homozygous ‘bb’ to be significantly reduced in the patients in contrast to a report by McDermott et al [41] in Southern Indian families where ‘b’ allele was shown to be excessively transmitted to the affected offsprings. This difference could be due to different ethnicity of south and north Indian populations since the B/b polymorphism does not have any functional significance. Individual VDR polymorphisms have been studied in different populations with conflicting reports. Some reports find a positive association of VDR polymorphism with T1D [41–46], while there are a considerable number of reports where no association of VDR polymorphism with T1D was

| VDR Haplotype | FBAt | FBAT | FbAT | FbaT | fBAt |
|---------------|------|------|------|------|------|
| Female (HF)* | CATC | CATT | TATT | CGGT | TATC |
| Patients 2N = 210 | 0.267 | 0.173 | 0.084 | 0.252 | 0.067 |
| Male (HF) | 0.333 | 0.119 | 0.048 | 0.268 | 0.056 |
| Patients 2N = 262 | 0.255 | 0.166 | 0.012 | 0.348 | 0.114 |
| Female (HF) Controls 2N = 162 | 0.231 | 0.128 | 0.030 | 0.314 | 0.096 |
| Male (HF) Controls 2N = 232 | 0.233 | 0.146 | 0.021 | 0.317 | 0.099 |
| All (HF) Controls 2N = 394 | 0.233 | 0.146 | 0.021 | 0.317 | 0.099 |

Female Patients vs. Controls

| p value | Odds Ratio | 95% C.I. |
|---------|------------|----------|
| 0.41 | 1.18 | 0.8–1.74 |
| 0.42 | 1.21 | 0.76–1.9 |
| 0.003 | 4.21 | 1.81–9.8 |
| 0.074 | 0.71 | 0.48–1.03 |
| 0.17 | 0.64 | 0.34–1.21 |

Female Patients vs. Female Controls

| p value | Odds Ratio | 95% C.I. |
|---------|------------|----------|
| 0.42 | 1.22 | 0.49–1.27 |
| 0.95 | 1.02 | 0.97–5.8 |
| 0.0025 | 0.61 | 0.56–1.12 |
| 0.0298 | 0.61 | 0.29–1.1 |
| 0.095 | 0.54 | 0.26–1.12 |

Male Patients vs. Controls

| p value | Odds Ratio | 95% C.I. |
|---------|------------|----------|
| 0.004 | 1.67 | 0.54–1.57 |
| 0.33 | 0.75 | 0.64–4.27 |
| 0.051 | 0.29 | 0.54–1.19 |
| 0.185 | 0.27 | 0.28–1.11 |
| 0.052 | 0.09 | 0.09 |

Male Patients vs. Male Controls

| p value | Odds Ratio | 95% C.I. |
|---------|------------|----------|
| 0.01 | 1.69 | 0.59–2.56 |
| 0.75 | 0.94 | 0.84–3.75 |
| 0.29 | 0.59–1.36 |
| 0.27 | 0.55–2.52 |

Female Patients vs. Male Patients

| p value | Odds Ratio | 95% C.I. |
|---------|------------|----------|
| 0.089 | 0.71 | 0.113 |
| 0.113 | 1.5 | 0.13 |
| 0.13 | 1.77 | 0.61 |
| 0.61 | 0.67 |
| 0.67 | 0.67 |

95% C.I. | 0.8–1.74 | 0.76–1.9 | 1.81–9.8 | 0.48–1.03 | 0.34–1.21 |

95% C.I. | 0.49–1.27 | 0.97–5.8 | 0.56–1.12 | 0.29–1.1 |

95% C.I. | 0.54–1.57 | 0.64–4.27 | 0.54–1.19 | 0.28–1.11 |

95% C.I. | 0.94–3.75 | 0.84–3.75 | 0.59–1.36 | 0.55–2.52 |

HF = Haplotype frequency.

Linkage Disequilibrium tests for Female patients using SHEsis software.

D: Fok1-Bsm1 = 0.066, Fok1-Apa1 = 0.042, Fok1-Taq1 = 0.048, Bsm1-Apa1 = 0.9, Bsm1-Taq 1 = 0.966, Apa1-Taq 1 = 0.946.

Linkage Disequilibrium tests for Male patients using SHEsis software.

D: Fok1-Bsm1 = 0.015, Fok1-Apa1 = 0.03, Fok1-Taq1 = 0.014, Bsm1-Apa1 = 0.898, Bsm1-Taq 1 = 0.923, Apa1-Taq 1 = 0.957.

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Table 4. Comparisons of frequencies of VDR haplotypes in male and female patients with male and female controls, all controls and between male and female patients using SHEsis software.
Table 5. Simultaneous presence of different VDR haplotypes along with predisposing HLA-DRB1*0301, *0401, *0402 and *0405 alleles.

| VDR Haplotype | T1D (N = 233) | Controls (N = 191) | T1D vs CONTROLS |
|---------------|-------------|-----------------|-----------------|
|               | No. | %   | No. | %   | p Value | OR  | 95% CL |
| FBAT-DR3-ve   | 0   | 0.00 | 1   | 0.52 | 0.45   | 0.27 | 0.007–3.0 |
| FBAT-DR3+ve   | 3   | 1.29 | 0   | 0.00 | 0.16   | 5.9  | 0.7–58.8 |
| FBAT-DR3-ve   | 1   | 0.43 | 3   | 1.57 | 0.241  | 0.34 | 0.07–1.5 |
| FBAT-DR3+ve   | 107 | 45.92| 20  | 10.47| <10^-4*| 7.2  | 4.15–12.8 |
| FBAT-DR3-ve   | 27  | 11.58| 60  | 31.41| <10^-4*| 0.29 | 0.16–0.49 |
| FBAT-DR3+ve   | 48  | 20.60| 7   | 3.66 | <10^-6 | 6.8  | 2.9–16.9 |
| FBAT-DR3-ve   | 4   | 1.72 | 34  | 17.80| <10^-8 | 0.07 | 0.03–0.2 |
| FBAT-DR3+ve   | 103 | 44.21| 24  | 12.57| <10^-6 | 5.5  | 3.25–9.39 |
| FBAT-DR3-ve   | 16  | 6.87 | 105 | 54.97| <10^-6 | 0.05 | 0.03–0.09 |
| FBAT-DR3+ve   | 1   | 0.43 | 0   | 0.00 | 0.5   | 2.5  | 0.23–84.82 |
| FBAT-DR3-ve   | 0   | 0.00 | 1   | 0.52 | 0.45   | 0.27 | 0.007–3.0 |
| FBAT-DR3+ve   | 14  | 6.01 | 3   | 1.57 | 0.02**| 3.7  | 1.5–9.6  |
| FBAT-DR3-ve   | 3   | 1.29 | 18  | 9.42 | 0.0003^ | 0.12 | 0.04–0.46 |
| FBAT-DR3+ve   | 3   | 1.29 | 1   | 0.52 | 0.4   | 1.9  | 0.45–14.5 |
| FBAT-DR3-ve   | 0   | 0.00 | 1   | 0.52 | 0.45   | 0.27 | 0.007–3.0 |
| FBAT-DR3+ve   | 14  | 6.01 | 9   | 4.71 | 0.7   | 1.3  | 0.5–3.3  |
| FBAT-DR3-ve   | 1   | 0.43 | 29  | 15.18| <10^-6 | 0.03 | 0.01–0.1 |
| FBAT-DR3+ve   | 34  | 14.59| 7   | 3.66 | 0.0003^ | 4.5  | 1.8–11.4 |
| FBAT-DR3-ve   | 7   | 3.00 | 13  | 6.81 | 0.11   | 0.42 | 0.15–1.2 |
| FBAT-DR3+ve   | 29  | 12.45| 0   | 0    | <10^-8 | 55.2 | 8.2–173.4 |
| FBAT-DR3-ve   | 8   | 3.43 | 9   | 4.71 | 0.67   | 0.72 | 0.25–2.1 |
| FBAT-DR3+ve   | 3   | 1.29 | 0   | 0.00 | 0.16   | 6    | 0.7–58.8 |
| FBAT-DR3-ve   | 1   | 0.43 | 1   | 0.52 | 0.7   | 0.81 | 0.13–5.1 |
| FBAT-DR3+ve   | 7   | 3.00 | 2   | 1.05 | 0.15   | 2.6  | 0.9–8.2  |
| FBAT-DR3-ve   | 1   | 0.43 | 8   | 4.19 | 0.009^ | 0.13 | 0.04–0.5 |

No. shows the number of individual positive for the indicated VDR haplotype and DR3 allele.

#DR3-ve includes all the Predisposing Alleles i.e. DRB1*0301, *0401, *0402 and *0405.

^Corrected P(c) value is significant.

^Corrected P(c) value is not significant.

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Table 6. LD based statistics to study the linkage disequilibrium between two unlinked loci (VDR haplotypes and predisposing HLA-DRB1*0301, *0401, *0402 and *0405 alleles shown collectively as DR3 in the table 6).

| VDR haplotype-DR3^# | T1D (N=233) | Controls (N=191) | V_A | V_N | T1 | p value |
|----------------------|-------------|-----------------|-----|-----|----|--------|
| FBAT-DR3            | 0.035       | 0.0056          | 0.0004 | 0.000035 | 11.52 | 0.0006 |
| FBAT-DR3            | 0.042       | 0.03            | 0.0001 | 0.000075 | 0.82  | 0.365  |
| FBAT-DR3            | 0.0576      | 0.0233          | 0.000081 | 0.000069 | 7.84  | 0.005  |
| FBAT-DR3            | 0.0222      | 0.01            | 0.000323 | 0.00003 | 2.39  | 0.122  |
| FBAT-DR3            | 0.01        | 0.013           | 0.000016 | 0.000043 | 0.15  | 0.698  |
| FBAT-DR3            | 0.0146      | -0.0028         | 0.000032 | 0.0000085 | 9.195 | 0.002  |

^#DR3-ve includes all the Predisposing Alleles i.e. DRB1*0301, *0401, *0402 and *0405.

^#δ_m, δ_n, V_A, V_N and T1 calculated as shown in statistical methods.

Frequencies of DR3 = 0.6, FBAT = 0.12, FBAT-DR3 = 0.107, FBAT = 0.33, FBAT-DR3 = 0.34, FbaT = 0.264, FbaT-DR3 = 0.216, FBAT = 0.088, FBAT-DR3 = 0.075, FBAT = 0.034, FBAT-DR3 = 0.03, FbaT = 0.079, FbaT-DR3 = 0.062 in patients and DR3 = 0.107, FBAT = 0.118, FBAT-DR3 = 0.0183, FBAT = 0.233, FBAT-DR3 = 0.055, FbaT = 0.369, FbaT-DR3 = 0.063, FBAT = 0.052, FBAT-DR3 = 0.0157, FBAT = 0.099, FBAT-DR3 = 0.0236, FbaT = 0.026 and FbaT-DR3 = 0.0 in controls. Frequencies of the VDR haplotypes are marginally different from Table 2 as these have been calculated from the reconstructed haplotypes based on SHEsis program to study interaction with predisposing HLA alleles, whereas Table 2 shows the frequencies as calculated by SHEsis analysis.

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observed [47–49]. In a meta-analysis Guo et al. [50] did not see any evidence of significant association between VDR polymorphism and T1D in either case-control or family transmission. Yet another meta-analysis [51] concluded that F and the B alleles at FokI and BsmI site showed an increased relative risk for T1D as regional winter UVR levels increased, however, the association of TaqI T allele with T1D decreased with winter suggesting that the environmental UVR conditions may influence the association between VDR genotype and T1D risk. Hence, the contradictory reports in the literature with respect to VDR associations in T1D could be due to ethnic differences in the frequencies of different SNPs in different populations, the environmental UVR and the pleiotropic behaviour of VDR.

It is important that all the SNPs of the gene constituting a haplotype be studied as representative of the allele or the so-called “pseudo-allele” as such rather than individual SNPs since it is possible that the 5’ and 3’ polymorphisms may be functionally linked to each other either through their independent influences on VDR activity or by physical interaction [52]. SHEsis and Famhap analysis showed BsmI site was in strong LD with ApaI site and TaqI site with D’ values of 0.91 and 0.93 and ApaI site was also in strong LD with TaqI site with a D’ value of 0.97, however, the FokI site was in a very weak linkage disequilibrium (LD) with BsmI, ApaI and TaqI sites with D’ values of 0.04, 0.01 and 0.04 respectively. The haplotypes, with FokI alleles, however, could still be constructed because 60.2% of the patients had FF genotype and 6.4% had Ff genotype. SHEsis analysis in the present study showed haplotype ‘FfBAT’ and ‘fBAT’ to be significantly increased in patients as compared to controls. Both these haplotypes were increased in patients with less than 18 years as age at onset. However, there was no significant difference in patients with adult age at onset as compared to controls. We also observed a gender-wise distribution of these haplotypes. While ‘fBAT’ was significantly increased in female patients as compared to all controls and female controls, ‘FfBAT’ was significantly increased in male patients as compared to all controls and male controls. To study the distribution of these haplotypes in the two genders with the age at onset, we divided the males and females into two groups each i.e., with adult onset (above 18 years) and younger onset (≤18 years). Interestingly, both ‘FfBAT’ and ‘fBAT’ were significantly increased in male patients with younger age at onset and not in the male patients with adult onset of T1D. However, ‘fBAT’ was significantly increased in female patients in both adult onset as well as younger onset of the disease. These data suggest that different haplotypes with ‘F’ or ‘T’ allele may be involved in differential effects on the immune systems in males and females patients since both FokI and TaqI sites have been implicated in transcription of VDR [34].

We have shown earlier that HLA-DRB1*0301, *0401 and *0405 are predisposing for T1D [2]. Association of MHC class-II alleles with an autoimmune disease could be due to its antigen presenting function and VDR may have a role in regulation of autoimmune responses through VDR −1,25 (OH)2D3 complex. Hence, we sought to study whether interaction of two independently assorting genes i.e. predisposing HLA alleles and VDR alleles have a role to play in the precipitation of the disease. T1D is a complex, multi-factorial disease where individual factors may not show any significant difference, however, when studied in association with other predisposing factors, integration of different factors may be implicated. To study the interaction of two genes which are not linked i.e., VDR (on chromosome 12q 13–14) and predisposing HLA alleles (on chromosome 6p21.3), we used “LD based statistics” as described by Zhao et al. [25]. We tested the interaction by comparing the difference in the LD levels between VDR haplotypes and HLA-DR3 (HLA-DRB1*0301, *0401, *0402 and *0405 collectively) between cases and controls. Since there is a possibility of background LD between the two unlinked loci in the population, we used more robust case-control formula as Zhao et al. [25] suggest that case-only formula may lead to type 1 error. Moreover, Zhao et al. [25] argue that LD based statistics has much higher power in detecting interaction than does the logistic regression. The analysis revealed FBAT-DR3, FbaT-DR3 and fBAT-DR3 to show significant interactions in the patients as compared to controls. It is obvious from these data that ‘T’ allele is common in all the haplotypes which showed interaction with pre-disposing HLA alleles, while haplotypes with both ‘F’ and ‘f’ alleles were associated with HLA-DR3 allele, so to dissect out which of these alleles were actually interacting with DR-3 allele, we applied LD based statistic to individual FokI, BsmI, ApaI and TaqI alleles (Table 7) and the data suggested that ‘F’ allele at FokI

### Table 7. LD based statistics to study the linkage disequilibrium between two unlinked loci (VDR alleles F/f, B/b, A/a and T/t and predisposing HLA-DRB1*0301, *0401, *0402 and *0405 alleles shown collectively as DR3 in the table).

| VDR allele-DR3 | T1D (N = 233) | Controls (N = 191) | V_A | V_B | T1 | p value |
|----------------|---------------|-------------------|-----|-----|-----|---------|
| F-DR3          | 0.063         | 0.022             | 0.00011 | 0.00016 | 13.3 | 0.0002  |
| f-DR3          | 0.042         | 0.0245            | 0.000078 | 0.000069 | 2.08 | 0.149   |
| B-DR3          | 0.071         | 0.03              | 0.00012 | 0.000058 | 9.497 | 0.002   |
| b-DR3          | 0.07          | 0.0136            | 0.00011 | 0.000062 | 17.84 | 0.00002 |
| A-DR3          | 0.075         | 0.031             | 0.00012 | 0.000046 | 11.66 | 0.0006  |
| a-DR3          | 0.058         | 0.021             | 0.0001  | 0.00006  | 8.65  | 0.003   |
| T-DR3          | 0.0696        | 0.024             | 0.00012 | 0.000039 | 13.07 | 0.0003  |
| t-DR3          | 0.049         | 0.02              | 0.00011 | 0.000073 | 2.29  | 0.13    |

\[\text{LD based statistics includes all the Predisposing Alleles i.e. DRB1*0301,*0401,*0402 and *0405.} \]

\[\delta_A, \delta_B, V_A, V_B \text{ and } T1 \text{ calculated as shown in statistical methods.} \]

\[\text{Frequencies of DR3=0.6, F=0.76, F-DR3=0.519, f=0.23, FDR3=0.18, B=0.59, B-DR3=0.425, b=0.41, b-DR3=0.315, A=0.642, A-DR3=0.46, a=0.358, a-DR3=0.273,} \]
\[f=0.624, t=0.444, \text{ t-DR3=0.275 in patients and DR3=0.107, f=0.78, F-DR3=0.105, f=0.21, f-DR3=0.047, B=0.52, B-DR3=0.086, b=0.48, b-DR3=0.065,} \]
\[A=0.59, A-DR3=0.094, a=0.411, a-DR3=0.065, T=0.66, T-DR3=0.0942, t=0.34, t-DR3=0.065 \text{ in controls.} \]
site and ‘T’ allele at the TaqI site show significant interaction with the predisposing HLA-DR3. However, both ‘B’ and ‘b’ alleles and ‘A’ and ‘a’ alleles at BsmI and ApaI sites respectively showed interaction suggesting their null effect.

An association of VDR genotypes with VDR mRNA and VDR protein has been demonstrated [21,34,53] in peripheral blood mononuclear cells providing functional relevance to the VDR polymorphisms. FokI and TaqI genotypes were observed to be independent determinants of insulin secretion and VDR mRNA and protein levels [34]. Gross et al [53] showed an increased transcription rate of the VDR gene in cells with ‘FF’ genotype. ‘F’ allele which encodes the shorter protein has been shown to be transcriptionally 1.5 to 2.5 fold more active than ‘f’ allele and has also been shown to associate more avidly with Transcription factor II B (TF II B) [39]. Interestingly, Jurutka et al [39] show that vitamin-D3 mediated transcription requires specific physical interaction of VDR with TF II B which involves both C and N terminal domains in the receptor. Moreover, while FokI site is situated in the N-terminal end of the VDR molecule, the ligand binding domain is situated in its C-terminal [41], which explains why FokI and TaqI sites may be playing detrimental roles in the manifestation of T1D in the presence of predisposing MHC alleles.

FokI and TaqI genotypes have been shown to contribute to VDR mRNA and protein levels independently[54]. While the promoter
the other hand, the VDR ligand 1,25(OH)2D3 has been shown to inhibit these pro-inflammatory immune responses by binding to VDR which induces conformational changes in VDR, promoting its heterodimerization with Retinoid X Receptor (RXR), followed by translocation of this complex into the nucleus which in turn binds to the vitamin D3-responsive elements (VDRE) or the DNA binding domains in promoter regions of 1,25(OH)2D3-responsive genes [4], thereby down regulating transcription of the pro-inflammatory cytokines like IL-2, IFN-γ and IL-12 [7–9,11], thus delaying the pathogenesis of type 1 diabetes.

Additionally, presence of VDRE in the promoter region of the predisposing HLA-DRB1*0301 may also have a significant role in manifestation of the disease since a direct interaction between HLA-DRB1 and vitamin D has been demonstrated recently [26]. VDRE is shown to have subsequence differences and different HLA alleles may have different VDRE sequences in their promoter regions influencing the expression of HLA alleles [26]. Gene expression of vitamin D regulated genes has been shown to be influenced by these subsequence differences within the classical VDRE [5]. Hence, we sequenced the promoter region of normal and affected individuals homozygous for HLA-DRB1*0301 and discovered that VDRE sequence observed in DRB1*0301 promoters from North Indians is homologous to the VDRE sequence which had been shown to up-regulate the expression of HLA DRB1*1501 allele in Multiple Sclerosis [26]. Ramagopalan et al [26] showed that the VDRE corresponding to the Multiple Sclerosis (MS) associated DRB1*1501 haplotype bound to recombinant VDR/RXR with high specificity in vitro in contrast to relatively lower affinity for the VDRE variants observed in the non-MS associated HLA-DRB1 haplotypes which were not responsive to vitamin D3 [26]. 85.9% of the patients in the present study had HLA-DRB1*0301 with 27.7% being homozygous and the rest heterozygous with DRB1*04 or any other alleles (data not shown). These data suggested that the interaction of VDR with DRB1*0301 is through the VDRE present in the promoter region of the HLA allele. Interestingly, both Multiple Sclerosis and T1D have HLA as the main susceptibility locus and vitamin D a strong environmental element since supplementation of 1,25(OH)2D3 analog in NOD mice before the onset of Insulitis, has been effectively shown to prevent the disease progression. However, this treatment was ineffective when Insulitis had already been established [15–18]. Similarly, in humans, vitamin D supplementation in early childhood has been shown to reduce the incidence of T1D [19]. To study whether the interaction of VDR with the HLA-DRB1*0301 is through VDRE present in the promoter region of the HLA-DRB1*0301, we stimulated the International Histocompatibility Workshop's DRB1*0301 homozygous B-lymphoblastoid cell line VAVY and DUCAF cells were treated with 100 nM calcitriol or equal volume of alcohol as vehicle control. Both VAVY and DUCAF cells show a significant increase in surface HLA-DR expression as compared to untreated ones. B: Line graph showing the extent of enhanced HLA-DR expression in three independent experiments. doi:10.1371/journal.pone.0008023.g003

Figure 3. Flow cytometric analysis of HLA-DR expression. B-LCLs VAVY and DUCAF cells were treated with 100 nM calcitriol or equal volume of alcohol as vehicle control. Both VAVY and DUCAF cells show a significant increase in surface HLA-DR expression as determined by the geometric mean fluorescence intensity of staining with HLA-DR-PE antibody. A. The figure shows mean±S.E.M. of three independent experiments. Two tailed Paired T test shows the difference to be statistically significant with p<0.001 i.e., increased amount of VDR protein resulting in increased IL-12 production which in turn may lead to other downstream pro-inflammatory immune responses against auto-antigens in T1D. And administration of 1,25(OH)2D3 in type 1 diabetes may be able to inhibit these pro-inflammatory immune responses by binding to VDR which induces conformational changes in VDR, promoting its heterodimerization with Retinoid X Receptor (RXR), followed by translocation of this complex into the nucleus which in turn binds to the vitamin D3-responsive elements (VDRE) or the DNA-binding domains in promoter regions of 1,25(OH)2D3-responsive genes [4], thereby down regulating transcription of the pro-inflammatory cytokines like IL-2, IFN-γ and IL-12 [7–9,11], thus delaying the pathogenesis of type 1 diabetes.

Additionally, presence of VDRE in the promoter region of the predisposing HLA-DRB1*0301 may also have a significant role in manifestation of the disease since a direct interaction between HLA-DRB1 and vitamin D has been demonstrated recently [26]. VDRE is shown to have subsequence differences and different HLA alleles may have different VDRE sequences in their promoter regions influencing the expression of HLA alleles [26]. Gene expression of vitamin D regulated genes has been shown to be influenced by these subsequence differences within the classical VDRE [5]. Hence, we sequenced the promoter region of normal and affected individuals homozygous for HLA-DRB1*0301 and discovered that VDRE sequence observed in DRB1*0301 promoters from North Indians is homologous to the VDRE sequence which had been shown to up-regulate the expression of HLA DRB1*1501 allele in Multiple Sclerosis [26]. Ramagopalan et al [26] showed that the VDRE corresponding to the Multiple Sclerosis (MS) associated DRB1*1501 haplotype bound to recombinant VDR/RXR with high specificity in vitro in contrast to relatively lower affinity for the VDRE variants observed in the non-MS associated HLA-DRB1 haplotypes which were not responsive to vitamin D3 [26]. 85.9% of the patients in the present study had HLA-DRB1*0301 with 27.7% being homozygous and the rest heterozygous with DRB1*04 or any other alleles (data not shown). These data suggested that the interaction of VDR with DRB1*0301 is through the VDRE present in the promoter region of the HLA allele. Interestingly, both Multiple Sclerosis and T1D have HLA as the main susceptibility locus and vitamin D a strong environmental element since supplementation of 1,25(OH)2D3 analog in NOD mice before the onset of Insulitis, has been effectively shown to prevent the disease progression. However, this treatment was ineffective when Insulitis had already been established [15–18]. Similarly, in humans, vitamin D supplementation in early childhood has been shown to reduce the incidence of T1D [19]. To study whether the interaction of VDR with the HLA-DRB1*0301 is through VDRE present in the promoter region of the HLA-DRB1*0301, we stimulated the International Histocompatibility Workshop's DRB1*0301 homozygous B-lymphoblastoid cell line VAVY and DUCAF cells were treated with 100 nM calcitriol or equal volume of alcohol as vehicle control. Both VAVY and DUCAF cells show a significant increase in surface HLA-DR expression as determined by the geometric mean fluorescence intensity of staining with HLA-DR-PE antibody. A. The figure shows mean±S.E.M. of three independent experiments. Two tailed Paired T test shows the difference to be statistically significant with p<0.001. Ramagopalan et al [26] have also shown 1.3 fold increase in expression of HLA-DRB1 in DRB1*15 homozygous cell line PGF on addition of calcitriol and checked for the expression of DR using flow cytometry and real time PCR. The B-LCLs showed 1.2–1.3 fold enhanced expression on flow cytometry, probably because of constitutively high levels of HLA-DR expression on the cell lines tested. However, this enhanced difference was statistically significant on two tailed paired T test (p<0.001). Ramagopalan et al [26] have also shown 1.3 fold increase in expression of HLA-DRB1 in DRB1*15 homozygous cell line PGF on addition of calcitriol and they too found it to be statistically significant. Interestingly, however, real time PCR showed 1.8±0.28 fold increase in the HLA-DR expression in unstimulated one confirms that indeed the interaction of VDR with HLA-DRB1*0301 is occurring through the VDRE present in the promoter region of the HLA allele. Based on the earlier studies and the present data one can speculate 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 [26] resulting in escape from thymic deletion of autoreactive T cells leading to T1D manifestations. We have shown earlier that simultaneous presence of DRB1*0301 along with homozygous INS-VNTR class-I was significantly increased (p<10−5) in T1D patients, giving a relative risk of 70.81 [2]. INS-
VTR class-I has also been shown to be associated with lower expression of Insulin in thymi of fetuses as compared to Class-III alleles [55,56] which may be responsible for poor thymic education for insulin resulting in autoimmunity against pancreatic beta cells. The present study provides additional evidence based on the 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-(OH)2D3 in early childhood and/or in utero and this interaction is mediated by VDRE present in the promoter region of DRB1*0301.

Materials and Methods

Genomic 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 written consent and Institutional Human Ethics Committee’s approval from both All India Institute of Medical Sciences (AIIMS) and National Institute of Immunology (NII). 236 T1D subjects and 197 normal healthy controls based in Delhi, originally from three states of North India, Uttar Pradesh, Haryana and Punjab, were studied for VDR polymorphisms. The patients studied in the current study were recruited from ‘Type 1 Diabetes Clinic’ at All India Institute of Medical Sciences, New Delhi, India, in a consecutive manner from 2004–2008. Most of these patients were part of our earlier studies [3,57] and were recruited based on their availability in follow up in the clinic and informed consent and were representative of a general population of T1D patients in North India. All the patients were carefully assessed (by RG) and categorized as type 1, type 2 and fibrocalculous pancreatopathy according to the recent classification of the American Diabetes Association expert committee [58]. All of the subjects included in the study required insulin for glycemic control. Insulin requiring patients with fibrocalculous pancreatopathy and subjects with diabetes in whom glycemic control was achieved with diet and oral anti-diabetic drug were excluded from the study as described earlier [3]. The T1D group consisted of 105 females with the mean age at onset 14.74±7.57 and 131 males with a mean age of onset 16.89±7.25. The control group consisted of 81 females and 116 males with a mean age of 30.1±10.2.

Normal healthy controls from the same ethnic background with no history (of self or family) of any autoimmune or infectious diseases were included in the study. They represent the same source population because the controls too, like the patients, belonged to three states of North India, Uttar Pradesh, Haryana and Punjab. The controls were the students, scholars and employees of NII and AIIMS who belonged to these three states. They were not selected in particular but were random individuals who gave informed consent to draw blood and did not have any history of infectious or autoimmune diseases. Controls were not clinically tested; however, they were asked if they had any infectious, autoimmune or any other disease. Only healthy individuals with no disease, symptoms of a disease or family history of any autoimmune or infectious disease, were included in the study. The response rate was about 85%. There was no statistically significant difference between the numbers of Males and Females in the patient and the control group. Higher age group of controls were, however, preferred to rule out their possibility of developing T1D at a later date after the collection of their blood samples as normal healthy controls. Since the control samples have been collected over a period of 5–6 years, all the controls who had the predisposing DRB1*0301 allele and were below the age of 30 years at the time of blood collection have been followed up till the date of analysis to make sure that they did not develop diabetes.

Genotypic Analysis of VDR Polymorphisms

The genotypes for the four SNPs were determined by PCR amplification and restriction digestion of the PCR products with enzymes FokI, BsmI, ApaI, and TaqI as described earlier [23,59]. Briefly, 500 ng of DNA was amplified in 5 µl of 10X Thermopol reaction buffer supplemented with 2 mM MgSO4 (New England Biolabs), 5 µM of each primers, 0.25 mM dNTPs, and 1.25 U of Taq DNA Polymerase (New England Biolabs), under standard conditions for 35 cycles in Perkin Elmer 2700 thermocycler. 1% agarose gel was run to confirm the amplification. To determine the presence of restriction site within an amplified product, a 5 µl aliquot of respective PCR product was digested with the respective restriction endonucleases. Figures 1A–D show the sizes of the restriction fragments obtained after digestion with the respective enzymes, the primers, their annealing temperatures for the PCRs and the temperatures at which restriction digestion was carried out. The interpretation of restriction fragments were done as shown in figures 1A–D.

HLA-DRB1 Polymorphism

Alleles of HLA-DRB1 locus were determined for 233 TID patients and 191 controls. For 100 patient samples and 94 controls samples, the second exon of the DRB1 gene was PCR amplified using standard conditions and hybridized with sequence specific oligonucleotide probes (SSOP) as described earlier [2,60,61]. The PCR products were run on 1% agarose gel to check for amplification. The amplified PCR products were dot blotted on Zeta probe membranes (Biorad), UV cross-linked (Syngene, USA) and hybridized with 32P labeled probes for DRB1- generic, DR2, DR4 and DR52- associated alleles.

For the remaining 133 patients and 97 control samples a Labtype SSO kit from One Lambda, (Canoga Park, CA, USA) was used according to the manufacturer’s instructions as described earlier [2]. Briefly, 40 ng of DNA was amplified in master mix, primers and 2 µl of Taq DNA polymerase in Perkin Elmer 2700 thermal cycler. The amplified product was run on 1% agarose gel to confirm amplification. 5 µl of amplified product was hybridized with appropriate amount of multiplex beads conjugated with oligonucleotide probes, in hybridization buffer at 60°C for 15 minutes followed by washing and incubation with 50 µl 1X SAPE solution (Streptavidin 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 HLA alleles. Some of these samples that were typed using PCR-SSOP earlier were re-typed using Luminex method to study the concordance using Luminex multiple beads conjugated with oligonucleotide probes, since this was a new method being adopted in the lab to avoid using radioactivity. With 98% concordance between the two methods, Luminex method was used for the rest of the samples. A few samples were typed a second time using Luminex, for HLA-DR alleles, just to confirm the reproducibility.

HLA-DRB1 Promoter Sequencing

Sequencing of the DRB1 promoter region was done to determine the VDRE variants in the North Indian population. The HLA-DRB1 promoter region was amplified from 3 T1D subject samples and 3 control samples homozygous for HLA-DRB1*0301 using the primers described earlier, 5'-TTTCAGAAGAGGACACTT-3' and 5'-CTTACGTCGGGTGTCCGGC3'-3' [62]. Briefly, 100 ng of DNA was amplified in 2.5 µl of 10X standard Taq Buffer, supplemented with 3 mM MgCl2 (New England Biolabs), 2 pmol of each primer, 0.25 mM dNTPs, and 1.25 U of Taq DNA polymerase (New England Biolabs), under standard conditions at 54°C annealing, for
Expression Analysis Using Flow Cytometry and Real Time PCR

*HLA-DRB1*°0301 homozygous B-lymphoblastoid cell line (B-LCL, IHW09023) VAVY and DUCAF (B-LCL IHW09019) were cultured in RPMI 1640 with L-glutamine, supplemented with 15% fetal bovine serum and 100 nM sodium pyruvate at 37°C in humidified 5% CO2. Cells were incubated with Calcitriol (Sigma) at a final concentration of 100 nM for 24 hours from a 10 μM stock in ethanol. Control cells were treated with the same volume of absolute ethanol alone.

Flow Cytometry

Flow cytometry was performed using anti-human HLA-DR antibody conjugated with PE (BD biosciences). 7×10^6 Cells were stained with anti-HLA-DR antibody as per manufacturer's recommendations. Briefly, the cells were washed with Dulbecco’s Phosphate buffered saline (DPBS) twice and resuspended in 100 μl of staining buffer containing 0.5% BSA in DPBS. To this 10 μl of anti-DR antibody-PE was added and incubated on ice for 30 minutes. The cells were washed with 0.5% BSA in DPBS, fixed in 2% paraformaldehyde for 10 minutes on ice and acquired on a BD-LSR flow cytometer. Data analysis was performed on Win-MDI 2.9 software.

Real-Time Analysis

Total RNA was isolated from cells using Trizol (Invitrogen) method. The RNA was purified using Qiagen RNeasy Mini column purification kit, and reverse transcribed using Superscript III First Strand Kit (Invitrogen). Real Time PCR analysis was performed on the ABI 7000 cycler (95°C 10 min Activation, 40 cycles; 95°C 15 secs Denaturation, 60°C 1 min Annealing/Extension), using Maxima SYBR Green qPCR Master Mix (Fermentas) with the following primers:

- 18s r RNA: Forward 5’ CGAAAGCATTGGCCAGAAT 3’
- Reverse 5’ AGTCGGCATCGTTTATGGTC 3’
- HLA-DRB1: Forward 5’TGTTCCTCGGACATGTTGTTGTC 3’
- Reverse 5’TGAAGGATCCCGAGAAGAGG 3’

Expression level changes were calculated as fold-change in the expression level between treated and untreated samples and normalized to 18s rRNA from that of the corresponding HLA-DR. Fold change was calculated by using 2^-ΔΔCt, where ΔΔCt represents ΔCt of treated - ΔCt of untreated control.

Statistical Analysis

Chi-Square test or Fisher’s exact test (whenever the numbers were five or less), were used to determine the significance of differences between the patient and control groups. p values were corrected using Boneferroni’s correction by multiplying the p values with the number of alleles tested for the locus. Odds ratios were calculated using Woolf’s method, with Haldane’s modification wherever the numbers were five or less as described earlier [63]. Further, the Haplotype analysis was done using the SHEsis software freely available at [http://202.120.7.14/analysis/myAnalysis.php]. [27] Hardy-Weinberg equilibrium was also calculated for both patients and controls using SHEsis software [27]. Additionally, Famhap (http://famhap.meb.uni-bonn.de) was used to confirm the frequencies of the haplotypes. Since both Famhap and SHEsis were giving similar results, we carried out the rest of the analysis using SHEsis only.

Interaction of two unlinked loci was tested using “LD based statistics” [25] using the following formulae

\[
T_1 = \frac{(\delta_A - \delta_B)^2}{V_A + V_N}
\]

Where \(\delta_A\) is the measure of LD between two unlinked loci a and b in cases and is calculated as

\[
\delta_A = P_{ab} - P_a \times P_b
\]

For controls

\[
V_N = \frac{P_b(1-P_b)(1-2P_a)(1-2P_b) \delta_A - \delta_A^2}{2N_G}
\]

N_A and N_G are the number of samples tested in patients and control groups respectively. The test Statistic T1 is asymptotically distributed as a central \( \chi^2 \) distribution under the null hypotheses of no interaction between the two unlinked loci [25].

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Author Contributions
Conceived and designed the experiments: RR. Performed the experiments: NI AAK. Analyzed the data: NI AAK RR. Contributed reagents/materials/analysis tools: RG AAK RR. Wrote the paper: NI RR. Diagnosed the patients: RG. Provided the clinical data, ages at onset of the disease and blood samples for the study: RG.

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