Haplotypes of Polymorphic Antigen Processing Genes for Low Molecular Mass Polypeptides (LMP2 and LMP7) are Strongly Associated with Type 1 Diabetes in North India

Bhukya Saida, Prachi Dani, Nachiketa Patnaik, Bharti Agrawal, T Rajarathna, Anushree Jaiswal, Alok Kumar Singh and Rajni Rani*

Molecular Immunogenetics Group, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi-110067, India

Abstract

Objective: Type 1 diabetes (T1D) is a multifactorial autoimmune disorder where several genes have been implicated. Aberrant presentation of auto-antigens on the MHC molecules is the hallmark of autoimmune disorders. The antigen is processed into small peptides by low molecular mass polypeptides, LMP2 and LMP7 before they are presented on the MHC class-I molecules. We have studied the associations of the Single nucleotide polymorphisms (SNPs) in these antigen processing genes and their haplotypes which may be detrimental for the processing of the auto-antigens in T1D.

Methods: 239 T1D subjects and 752 normal healthy controls from North India were studied for LMP2 codon 60 G/A (R/H), LMP7 codon 49 C/A (Q/K) and LMP7 Intron 6 G/T polymorphisms using PCR-RFLP. HLA class-II alleles were studied using bead based Luminex assays. Haplotypes of LMP2 and LMP7 were constructed using SHEsis online software. χ² test was used to study the significance of differences between patients and controls.

Results: The G (R) allele (p<0.009) and homozygous GG (RR) genotype (p<0.01) of LMP2 codon 60, C (Q) allele (p<0.0098) and homozygous CC (QQ) (p<0.03) of LMP7 codon 49 and LMP7 Intron 6 G allele (p<0.01) were significantly increased in T1D subjects compared to controls. Haplotypic analysis showed that haplotypes GCG and ACT (LMP2 codon 60- LMP7 codon 49- LMP7 Intron 6) (p<5.9×10⁻⁶, p<1.9×10⁻⁴ respectively) were significantly increased and haplotypes GCT and ACG (p<1.9×10⁻³, p<1.9×10⁻³ respectively) were significantly reduced in T1D patients irrespective of the gender, age at onset of T1D and the predisposing HLA DRB1*03:01.

Conclusion: Association of LMP2 and LMP7 haplotypes GCG and ACT with T1D may have a role in processing of auto-antigens to be presented by MHC class-I molecules to cytotoxic T cells in T1D.
that were part of our earlier studies [5,14] and 752 normal healthy controls (199 females and 553 males, mean age of 31.86 ± 20.03) from the same ethnic background were studied for LMP2 and LMP7 SNPs after obtaining informed written consent and Institutional Human Ethics Committee’s approval from both All India Institute of medical Sciences and National Institute of Immunology, New Delhi. All subjects i.e. patients and controls were based in Delhi, originally from three states of North India, Uttar Pradesh, Haryana and Punjab. The controls were the random healthy individuals with no disease, symptoms of a disease or family history of any autoimmune or infectious disease and comprised of students, scholars and employees of NII and AIIMS, who gave informed consent.

**PCR amplification and genotyping:**

Third exon of LMP2 and second exon and sixth Intron of LMP7 were amplified using Polymerase Chain Reaction (PCR) using standard conditions and primers described by Casp et al. [11] listed in Table 1. SNP genotypes were determined by restriction fragment length polymorphism (RFLP) analysis of the PCR products as described [11]. The digested fragments were resolved on 3% agarose gel electrophoresis in TBE buffer. The single SNP studied from LMP2 was G/A substitution at codon 60 in exon 3, studied using restriction endonuclease Hha I, which cleaves the G allele, but not the A allele (Figure 1a). LMP7 exon 2 SNP A/C at codon 49 was studied using restriction enzyme Pst-1 which cleaves the C allele and the A allele remains uncut (Figure 1b). LMP7-intron 6 SNP was studied using Hha I enzyme which cleaves the G allele but not the T allele (Figure 1c). Figure 1 shows the interpretations of different genotypes based on PCR-RFLP patterns.

**HLA-DRB1 polymorphism**

Alleles of HLA-DRB1 locus were studied for 199 T1D patients and 350 controls for whom LMP2 and LMP7 data were available as described earlier using either SSO or Luminex based HLA typing using Labtype SSO kit from One Lambda. (Canoga Park, CA, USA) according to the manufacturer’s instructions as described earlier [5,15].

**Statistical analysis**

The significance of differences in allelic and genotypic frequencies between T1D patients and controls was determined by standard χ² tests, Odds ratios and 95% confidence intervals using Stata 9.2 software. However, whenever the numbers in any group (i.e. in cases...
were significantly increased in T1D patients even after Bonferroni's correction for multiple comparisons. Linkage disequilibrium between HLA alleles and LMP haplotypes were calculated as described earlier [5]. Haplotype analysis for LMP2-LMP7 SNPs, haplotype association with disease, gender and age at onset were done using online SHEsis software [19,20] (http://202.120.31.177/myAnalysis.php).

Results

Genotype, allele and haplotype frequencies of LMP2 and LMP7 SNPs in T1D patients as compared to controls

Genotype, allele and haplotype frequencies of LMP2 exon 3 G/A, LMP7 exon 2 A/C and LMP7 intron 6 G/T are shown in Table 2. All genotype frequencies in patients as well as controls were in Hardy Weinberg equilibrium. The G to A substitution in LMP2 exon 3 leads to an amino acid change from arginine (R) to histidine (H) at codon 60 (CGC to CAG). A significant increase in the frequency of G (R) allele (p<0.009) and homozygous GG (RR) genotype (p<0.01) was observed in T1D patients compared to healthy controls. These differences were significant even after Bonferroni's correction.

In LMP 7 exon 2 substitution of C to A results in amino acid change from glutamine (Q) to Lysine (K) at codon 49 (CAG/AGG). Allele C (Q) (p<0.0098) and homozygous – CC (QQ) (p<0.03) were significantly increased in T1D. Allele A (K) (p<0.0098) and genotype AA (KK) were significantly reduced (p<0.03) in T1D patients compared to healthy controls. While the differences in allele frequencies remained significant even after the p value was corrected for multiple comparisons, the difference in genotype frequencies did not remain significant after correction.

In LMP7 intron 6 the G allele was significantly increased (p<0.01) and T allele (p<0.01) and homozygous TT (p<0.03) were significantly reduced in T1D patients as compared to controls. The differences in allele frequencies remained significant even after the p value was corrected for multiple comparisons; however, the difference in homozgyous TT genotype frequency between patients and controls did not remain significant after correction.

Since G allele of LMP2 exon 3, C alleles of LMP7 exon 2 and G allele of LMP7 intron 6 were significantly increased in T1D patients even after Bonferroni’s correction; we wanted to study if there is a Linkage Disequilibrium (LD) between these alleles. For this purpose, haplotypes were constructed using online software SHEsis [19,20] for 206 T1D and 738 healthy controls samples which were typed for all the three loci. Interestingly, as expected these SNPs were indeed in LD (Figure 2) and GCG (G allele of LMP2- C alleles of LMP7- G allele of LMP7 intron 6) was the most frequent haplotype observed with a frequency of 62.37% in the patients compared to 39.56% in the controls and this difference was highly significant (p=5.9×10⁻¹³). Haplotype ACT was observed with a frequency of 14.1% in patients compared to 5.42% in the controls and this difference was also significant (p=1.9×10⁻⁴). However, haplotypes

or controls) were less than 5 for any allele Fisher’s exact test was used. In such cases, Odds ratios were calculated using Woolf’s method [16] with Haldane’s [17] modification as described earlier [18]. p values were corrected using Bonferroni’s correction for multiple comparisons.

Table 2: Haplotype analysis for LMP2-LMP7 SNPs, haplotype association with disease, gender and age at onset.

** Odds Ratio calculated using Woolf’s formula with Haldane’s modification

\[ \text{Odds Ratio} = \frac{a \times d}{b \times c} \]

\[ \text{Confidence Interval (CI)} = \frac{a \times c}{b \times d} \]

Linkage Disequilibrium tests

\[ D' = \frac{\text{LMP7c2 * LMP7I6}}{\text{LMP2c3 * LMP7I6}} \]

\[ \text{LMP7c2} = 0.056 \]

\[ \text{LMP7I6} = 0.126 \]

\[ \text{LMP2c3} = 0.567 \]

\[ \text{Figure 2: Linkage disequilibrium analysis for construction of haplotypes for LMP2 exon 3, LMP7 exon 2 and LMP7 Intron 6 using SHEsis online software[19, 20].} \]
**Table 3:** LMP2 and LMP7 SNPs and haplotypes in female Type 1 diabetes patients compared with female healthy controls.

We further analyzed the data to check if age at onset of the disease was associated with any particular SNPs of LMP2 and LMP7 (Table 5). Patients were divided in two groups, those who were 14 years or less than 14 years old at the time of onset (considering onset of adolescence at the age of 14 years) and those above 14 years old at the time of onset of T1D. These two groups were compared with all healthy controls. The data revealed a significant increase in the frequencies of G (R) allele (p<0.029) and homozygous GG (RR) genotype (p<0.03) for LMP2 codon 60, allele C (Q) for LMP7 codon 49 (p<0.045) and G allele for LMP7 Intron 6 (p<0.007) and GG genotype (p<0.016) in T1D patients with early age at onset as compared to controls. However, there were no significant differences in the frequencies of these SNPs in patients with more than 14 years age at onset and healthy controls.

Interestingly, while individual SNPs showed significant differences only in patients with early age at onset, haplotypes GGC and ACT were significantly increased in both early (p<4.5×10⁻¹¹; p<0.003 respectively) and late age at onset (p<2×10⁻⁶ and p<5.14×10⁻⁷ respectively). Similarly, haplotypes GCT and ACG were significantly reduced in patients with both early and late age at onset (Table 5).

**Linkage Disequilibrium analysis of LMP2 and LMP7 SNPs with HLA-class-II alleles DRB1*03:01 and DRB1*07:01**

LMP2 and LMP7 are localized in the MHC class-II region on chromosome 6p21, and we and others have earlier reported a very strong association of HLA-DRB1*03:01 and negative association of

---

GCT and ACG were significantly reduced in T1D patients with a total absence of haplotype ACG. Differences in the haplotype frequencies were significant even after Bonferroni’s correction.

**Gender-wise distribution of genotype, allele and haplotype frequencies of LMP2 and LMP7 SNPs in gender matched T1D patients and controls**

To check whether there is any gender bias in the LMP2 and LMP7 alleles associated with T1D, 101 females and 134 male T1D patients’ genotypes and haplotypes were compared with 199 healthy females and 552 healthy males respectively as shown in Tables 3 and 4. The data showed that for LMP2 codon 60, allele G (R) (p<0.015) and genotype GG (RR) (p<0.016) were significantly increased and allele A (H) was significantly reduced (p<0.015) in female patients compared with female controls (Table 3) and these differences were significant even after Bonferroni’s correction. While the difference in allele frequencies of these SNPs was not statistically significant in male patients compared to male controls (Table 4), haplotype GCG was significantly increased in both female (65.29%) and male (60.83%) patients compared to female (42.31%) and male (39.3%) controls respectively. Male patients showed significant increase in frequency of haplotype ACT as compared to male controls, however, this difference was not observed in female patients. Haplotypes GCT and ACG were significantly reduced in both male and female patients compared to their control counterparts.

**Association of LMP2 and LMP7 SNPs with age at onset**

| Genotype/allele | TID | Controls | Female T1D vs. female controls |
|-----------------|-----|----------|-------------------------------|
| **LMP2 exon 3** |     |          |                               |
| N=97            |     |          |                               |
| No.             | %   | No.      | %                             |
| GG             | 71  | 73.19    | 117                           |
| GA             | 25  | 25.77    | 75                            |
| AA             | 1   | 1.03     | 7                             |
| G              | 167 | 86.08    | 309                           |
| A              | 27  | 13.92    | 89                            |
| **LMP7 exon 2** |     |          |                               |
| N=101           |     |          |                               |
| %               |     | %        |                               |
| CC             | 79  | 78.22    | 132                           |
| AC             | 20  | 19.8     | 53                            |
| AA             | 2   | 1.98     | 11                            |
| C              | 178 | 88.11    | 317                           |
| A              | 24  | 11.88    | 75                            |
| **LMP7 Intron 6** |     |          |                               |
| N=96           |     |          |                               |
| %               |     | %        |                               |
| GG             | 40  | 41.66    | 57                            |
| GT             | 40  | 41.66    | 95                            |
| TT             | 16  | 16.66    | 44                            |
| G              | 120 | 62.5     | 209                           |
| T              | 72  | 37.5     | 183                           |
| **Haplotypes LMP2 exon 3, LMP7 exon 2, LMP7 Intron 6** | | | |
| N=170          |     |          |                               |
| %               |     |          |                               |
| GCG            | 111 | 65.29    | 165                           |
| ACT            | 21  | 12.35    | 34                            |
| GCT            | 18  | 10.59    | 81                            |
| ACG            | 0   | 0        | 34                            |
| N=390          |     |          |                               |
| %               |     |          |                               |
| GCG            | 111 | 65.29    | 165                           |
| ACT            | 21  | 12.35    | 34                            |
| GCT            | 18  | 10.59    | 81                            |
| ACG            | 0   | 0        | 34                            |

**Table 3:** LMP2 and LMP7 SNPs and haplotypes in female Type 1 diabetes patients compared with female healthy controls.

* p value calculated using Fisher’s exact test
** Odds Ratio calculated using Woolf’s formula with Haldane’s modification
@ Haplotypes were made using SHEsis online software [19,20]
$ haplotypes showing significant differences are shown
| Genotype/allele | TID | Controls | Male TID vs. male controls |
|----------------|-----|----------|---------------------------|
| LMP2 exon 3 |  |  |  |
| N=131 % | N=553 % | p | Odds | 95% CI |
| GG | 95 | 72.52 | 363 | 65.64 | 0.13 | 1.38 | 0.89-2.17 |
| GA | 32 | 24.43 | 166 | 30.02 | 0.2 | 0.75 | 0.47-1.18 |
| AA | 4 | 3.05 | 24 | 4.34 | 0.35* | 0.76** | 0.37-1.57 |
| G | 222 | 84.73 | 892 | 80.65 | 0.12 | 1.33 | 0.91-1.97 |
| A | 40 | 15.27 | 214 | 19.35 | 0.12 | 0.75 | 0.5-1.09 |
| LMP7 exon 2 | N=125 % | N=546 % |  |
| CC | 94 | 75.2 | 384 | 70.33 | 0.27 | 1.28 | 0.81-2.06 |
| AC | 28 | 22.4 | 138 | 25.27 | 0.5 | 0.85 | 0.52-1.38 |
| AA | 3 | 2.4 | 24 | 4.4 | 0.22* | 0.61** | 0.27-1.36 |
| C | 216 | 86.4 | 906 | 82.97 | 0.18 | 1.3 | 0.87-1.99 |
| A | 34 | 13.63 | 186 | 17.03 | 0.18 | 0.76 | 0.5-1.15 |
| LMP7 Intron 6 | N=134 % | N=542 % |  |
| GG | 50 | 37.31 | 188 | 34.68 | 0.56 | 1.12 | 0.7-1.68 |
| GT | 66 | 49.25 | 237 | 43.73 | 0.25 | 1.25 | 0.83-1.85 |
| TT | 18 | 13.43 | 117 | 21.59 | 0.034 | 0.56 | 0.31-0.97 |
| G | 166 | 61.94 | 613 | 56.55 | 0.11 | 1.25 | 0.94-1.66 |
| T | 102 | 38.06 | 471 | 43.45 | 0.11 | 0.8 | 0.6-1.06 |

**Haplotypes LMP2 exon 3, LMP7 exon 2, LMP7 Intron 6**

| Genotype/allele | 2N=240 % | 2N=1086 % |
|----------------|--------|----------|
| GCG | 146 | 60.83 | 427 | 39.3 | 8.5x10^-8* | 2.31 | 1.73-3.08 |
| ACT | 36 | 15 | 62 | 5.71 | 1.3x10^-5** | 2.84 | 1.83-4.39 |
| GCT | 26 | 10.8 | 306 | 28.17 | 8.1x10^-5** | 0.3 | 0.19-0.46 |
| ACG | 0 | 0 | 106 | 9.76 | 3.1x10^-5** | 0.009** | 0.001-0.07 |

*p value calculated using Fisher’s exact test
**Odds Ratio calculated using Woolf’s formula with Haldane’s modification
@Haplotypes were made using SHEsis online software [19,20]
$ haplotypes showing significant differences are shown

Table 4: LMP2 and LMP7 SNPs and haplotypes in male Type 1 diabetes patients compared with male healthy controls.

**Discussion**

We show here that the haplotypes of antigen processing genes LMP2 and LMP7 may have a role in the aberrant presentation of self-antigens in T1D. LMP2 and LMP7 act as peptide editors for the appropriate peptide to be presented on the MHC molecules since they generate peptides that would better bind to MHC class-I molecules [21], and polymorphism in these genes may be detrimental for the peptides being loaded on MHC class-I molecules. There are controversial reports with respect to functional role of LMP2 exon 3 SNP at codon 60 where a single nucleotide polymorphism results in an amino acid change from arginine (R) to histidine (H) (GCG to CAC). While there was no difference in the mRNA expression of LMP2 in the R and H alleles, their chromotryptsin-like and trypsin-like activities were observed to be more in RR subjects compared to heterozygous RH subjects [22]. However, Park et al. [23] did not find any effect of the codon 60 R/H polymorphism on either expression or catalytic activity of LMP2 in some cancer cell lines. Since the cancer cell lines themselves showed a lot of variability in protein expression of LMP2, it is possible that situation may be different in normal non-cancerous cells. LMP2 codon 60 R/H (G/A) polymorphism seems to be conserved since this polymorphism is observed in different strains of mice [24] including non-obese diabetic (NOD) mice, the animal model for human Type 1 diabetes, who also have R allele at codon 60. Results of LMP2 polymorphisms in T1D are variable in different populations. While we found LMP2 GG...
### Table 5: Association of LMP2 and LMP7 SNPs and haplotypes with age at onset in Type 1 diabetes patients compared with healthy controls.

| Genotype/allele | TID Onset age ≤14 | Control | Age at onset ≤ 14 Vs. Controls | TID Onset age >14 | Age at onset >14 Vs. Controls |
|-----------------|-------------------|---------|--------------------------------|-------------------|-------------------------------|
| LMP2 exon 3     |                   |         |                                |                   |                               |
| N=99 (%)        | N=752 (%)         | p Value | Odds ratio 95% CI               | N=117 (%)         | p Value Odds ratio 95% CI     |
| GG              | 74 (74.75)        | 0.03    | 1.67 1.02-2.82                 | 83 (70.94)        | 0.13 1.38 0.89-2.19          |
| GA              | 23 (23.2)         | 0.07    | 0.64 0.37-1.06                 | 31 (28.49)        | 0.23 0.76 0.47-1.2           |
| AA              | 2 (2.02)          | 0.24*   | 0.58** 0.23-1.48               | 3 (2.56)          | 0.3* 0.7** 0.31-1.54         |
| G               | 171 (86.4)        | 0.029   | 1.598 1.04-2.54                | 197 (84.19)       | 0.12 1.34 0.92-2.0           |
| A               | 27 (13.6)         | 0.029   | 0.625 0.39-0.96                | 37 (15.81)        | 0.12 0.74 0.49-1.08          |
| LMP7 exon 2     | N=97              | N=742   |                                | N=115             |                               |
| CC              | 75 (77.32)        | 0.11    | 1.49 0.89-2.59                 | 88 (76.52)        | 0.12 1.43 0.89-2.35          |
| AC              | 21 (21.65)        | 0.38    | 0.79 0.45-1.35                 | 24 (20.87)        | 0.26 0.76 0.45-1.25          |
| AA              | 1 (1.03)          | 0.06*   | 0.31** 0.09-0.99               | 3 (2.61)          | 0.22* 0.62** 0.28-1.36       |
| C               | 171 (88.14)       | 0.045   | 1.58 0.99-2.62                 | 200 (86.96)       | 0.087 1.42 0.94-2.21         |
| A               | 23 (11.86)        | 0.045   | 0.63 0.38-1.0                 | 30 (13.04)        | 0.087 0.7 0.45-1.06          |
| LMP7 Intron 6   | N=99              | N=738   |                                | N=118             |                               |
| GG              | 45 (45.45)        | 0.016   | 1.67 1.07-2.62                 | 42 (35.59)        | 0.61 1.11 0.72-1.69          |
| GT              | 40 (40.4)         | 0.39    | 0.82 0.53-1.29                 | 57 (48.3)         | 0.5 1.14 0.76-1.72          |
| TT              | 14 (14.14)        | 0.08    | 0.59 0.3-1.08                  | 19 (16.1)         | 0.16 0.69 0.38-1.17          |
| G               | 130 (65.66)       | 0.007   | 1.52 1.1-2.11                  | 141 (69.75)       | 0.22 1.19 0.89-1.59          |
| T               | 68 (34.34)        | 0.007   | 0.66 0.47-0.9                 | 94 (40.25)        | 0.22 0.84 0.62-1.12          |
| LMP7 exon 3     |                   |         |                                |                   |                               |
| N=174           | N=1476            | p Value | Odds ratio 95% CI               | N=210             | p Value Odds ratio 95% CI     |
| GCG             | 119 (68.4)        | 584 (39.6) | 4.5x10** 3.05 2.16-4.29 | 122 (58.0) | 2x10** 1.89 1.41-2.54 |
| ACT             | 20 (11.5)         | 80 (5.42) | 0.003* 2.14 1.28-3.6 | 34 (16.2) | 5.14x10** 3.15 2.05-4.86 |
| GCT             | 16 (9.2)          | 387 (26.22) | 1.96x10** 0.32 0.23-0.44 | 25 (12) | 6.85x10** 0.35 0.23-0.54 |
| ACG             | 0 (0)             | 164 (11.11) | 2.47x10** 0.02** 0.003-0.16 | 0 (0) | 1.32x10** 0.02** 0.002-0.14 |

* p value calculated using Fisher’s exact test
** Odds Ratio calculated using Woolf’s formula with Haldane’s modification
@ Haplotypes were made using SHEsis online software [19,20]$
$ haplotypes showing significant differences are shown

In LMP7 exon 2 glutamine (Q-CAG) [5] to Lysine (K- AAG) substitution in the codon 49 has been implicated in the transcription regulation of the gene. On IFN-gamma stimulation cell lines with homozygous K/K genotype showed lower expression and reduced transcript stability compared to cell lines with LMP7 QQ (CC) genotypes and heterozygous K/Q cell lines showed intermediate expression of LMP7 [32], suggesting that the K allele may reduce the formation of immunoproteasome, and thus peptide processing followed by reduced peptide-HLA presentation [32]. In the present scenario, we observed the QQ (CC) genotype to be significantly increased in patients which may be involved in higher expression of the immunoproteasome.
and may have a role in presentation of self antigens in T1D since upregulation of LMP2 and LMP7 can result in marked improvement of antigen presentation [33]. This may greatly enhance the efficiency of intracellular T cell epitope production, establishing the cytotoxic T cell repertoire and shaping their cytotoxic immune responses [34-36]. While there is dearth of studies on LMP7 exon 3 Q/K polymorphism in T1D, QK homozygosity has been shown to be associated with another autoimmune disorder, juvenile rheumatoid arthritis (JRA) [37]. LMP7 exon 2 49K allele is the most frequent allele in Mexicans [38], Japanese [39], Brazilian Guarani population [39] and the north Indians in the present study and 49K allele has been shown to have lower frequency in most of the studies except in Caucasians from USA [11]. However, in the study by Casp et al. [11], there seems to be an error in either interpretation or typographical error for C (Q) and the A (K) alleles since the frequency of Q allele in a random population from USA has been reported to be 88.1% in another study [40] compared to 88.1% for A (K) allele in the study by Casp et al. [11].

Our results are not in concordance with the earlier studies on LMP7 intron 6 where homozygous TT at G/T at 37360 site was increased and GG was reduced in T1D [25,31,41], however, our results showed a significant decrease in the frequency of TT genotype and T allele and increase in G allele frequency in T1D from North India. The reason for non-concordance with earlier published reports could be due to different ethnicity of the subjects studied in the present report and larger numbers patients and controls studied compared to most of the earlier reports.

LMP2 and LMP7 are both immunoproteosomes involved in antigen processing and act in concert with each other and thus may have integrated and synergistic roles in generation of MHC class-I fitting peptides. So, we checked for the first time, whether there were any significant differences in the frequencies of haplotypes of LMP2 exon 3 A/G, LMP7 exon 2 A/C and LMP7 Intron 6 G/T in T1D compared to controls. Comparison of haplotypes showed that haplotypes GCC and ACT were significantly increased in the patients and the same haplotypes were significantly reduced in them, irrespective of gender of the patients or age at onset of diabetes. We further checked whether this effect could be due to gender of the patients or age at onset of diabetes. We further checked whether this effect could be due to gender of the patients or age at onset of diabetes. We further checked whether this effect could be due to gender of the patients or age at onset of diabetes. We further checked whether this effect could be due to gender of the patients or age at onset of diabetes. We further checked whether this effect could be due to gender of the patients or age at onset of diabetes.

**Table 6:** Linkage disequilibrium analysis for LMP2 exon-3 LMP7 exon-6 intron-6 haplotypes with predisposing and protective HLA alleles

| LMP2-LMP7 haplotypes+HLA DRB1 | T1D | Controls |
|--------------------------------|-----|----------|
| No. of haplotypes 2N=398 % | No. of haplotypes 2N=700 % | P value | Odds Ratio | 95% C.I. |
| GCG-DRB1*03:01 | 148 | 37.19 | 51 | 7.28 | 1.5x10⁻⁴ | 9.37 | 6.48-13.63 |
| GCG-DRB1*07:01 | 21 | 5.28 | 61 | 8.71 | 0.03 | 0.584 | 0.33-0.99 |
| ACT-DRB1*03:01 | 33 | 8.29 | 9 | 1.28 | 5.9 x 10⁻⁵ | 6.94 | 3.2-16.65 |
| ACT-DRB1*07:01 | 14 | 3.52 | 16 | 2.28 | 0.229 | 1.56 | 0.69-3.44 |
| GCT-DRB1*03:01 | 30 | 7.53 | 18 | 2.57 | 0.00002¹ | 3.08 | 1.64-5.96 |
| GCT-DRB1*07:01 | 3 | 0.75 | 39 | 5.57 | 0 | 0.148** | 0.07-0.32 |

*P value calculated using Fisher's exact test
**Odds Ratio calculated using Woolf's formula with Haldane's modification

Table 7: Simultaneous presence of predisposing and protective LMP2-LMP7 haplotypes with predisposing and protective HLA alleles.
with HLA DRB1*07:01 clearly shows that while the predisposing MHC alleles have the dominant effect, association of LMP2/LMP7 haplotypes is independent of the HLA alleles.

In conclusion, our results demonstrate that the significant increase in frequencies of haplotypes GGC and ACT and decrease in the frequency of GCT and ACC haplotypes is independent of gender, age at onset and the predisposing HLA alleles and may have a significant role in manifestation of T1D through higher presentation of self antigens, activation of early T cell responses and differentiating them into effector cells through polarizing cytokines [33,36]. While association with MHC class-II allele DRB1*03:01 [7] may be involved in generating Th1 type responses, predisposing MHC class-I alleles [5] and LMP2-LMP7 haplotypes may be involved in generating self reactive cytotoxic T cells. Since all three SNPs of LMP2-LMP7 are very closely linked and are inherited en-bloc as a haplotype and the two proteosomes may be functioning in an integrated manner, it may be more relevant to study the haplotypes rather than individual SNPs in future studies.

Acknowledgements

Authors are thankful to study participants and Dr. Ravinder Goswami, All India Institute of Medical Sciences for diagnosis of the patients and for providing blood samples without which the study would not have been possible. This work was supported by grant from Department of Science & Technology, New Delhi, India, grant No. SP/SO/B54/98 and core grant from National Institute of Immunology, New Delhi, India.

References

1. Piccoli F, McDermott MF (2002) Genetics of type 1 diabetes mellitus. Genes Immun 3: 235-249.
2. Todd JA (1995) Genetic analysis of type 1 diabetes using whole genome approaches. Proc Natl Acad Sci U S A 92: 8560-8565.
3. Ramachandran A, Snehalatha C, Krishnaswamy CV (1996) Incidence of IDDM in children in urban population in southern India. Madras IDDM Registry Group Madras, South India. Diabetes Res Clin Pract 34: 79-82.
4. Israni N, Goswami R, Kumar A, Rani R (2009) Interaction of vitamin D receptor with HLA DRB1 0301 in type 1 diabetes patients from North India. PLoS One 4: e7023.
5. Kumar R, Goswami R, Agarwal S, Israni N, Singh SK, et al. (2007) Association and interaction of the TNP-alpha gene with other pro- and anti-inflammatory cytokine genes and HLA genes in patients with type 1 diabetes from North India. Tissue Antigens 69: 557-567.
6. Bennett ST, Todd JA (1996) Human type 1 diabetes and the insulin gene: principles of mapping polymorphic genes. Annu Rev Genet 36: 343-370.
7. Rani R, Sood A, Goswami R (2004) Molecular basis of predisposition to develop type 1 diabetes mellitus in North Indians. Tissue Antigens 64: 145-155.
8. Spies T, Bresnahan M, Bahram S, Arnold D, Blanck G, et al. (1990) A gene in the human major histocompatibility complex class II region controlling the class I antigen presentation pathway. Nature 349: 744-747.
9. Van Koot L (2008) Pillars article: antigen presentation: discovery of the peptide TAP. J Immunol 180: 2723-2724.
10. Cresswell P, Ackerman AL, Giudini A, Peaper DR, Wearsch PA (2005) Mechanisms of MHC class I-restricted antigen processing and cross-presentation. Immunol Rev 207: 145-157.
11. Casp CB, She JX, McCormack WT (2003) Genes of the LMP/TAP cluster are associated with the human autoimmune disease vitiligo. Genes Immun 4: 492-499.
12. Ortiz-Navarrete V, Seelig A, Gernold M, Frenzelit S, Kloetzel PM, et al. (1991) Subunit of the 20S proteasome (multicatalytic proteinase) encoded by the major histocompatibility complex. Nature 353: 662-664.
13. Hayashi T, Faustman DL (2001) Selected contribution: Association of gender-related LMP2 inactivation with autoimmune pathogenesis. J Appl Physiol (1985) 91: 2804-2815.
14. Goswami R, Marwaha RK, Goswami D, Gupta N, Ray D, et al. (2006) Prevalence of thyroid autoimmunity in sporadic idiopathic hypoparathyroidism in comparison to type 1 diabetes and premature ovarian failure. J Clin Endocrinol Metab 91: 4256-4259.
15. Singh A, Sharma P, Kar HK, Sharma VK, Tembhire MK, et al. (2012) HLA alleles and amino-acid signatures of the peptide-binding pockets of HLA molecules in vitiligo. J Invest Dermatol 132: 124-134.
16. Woolf B (1955) On estimating the relation between blood group and disease. Ann Hum Genet 19: 251-253.
17. Haldane JB (1956) The estimation and significance of the logarithm of a ratio of frequencies. Ann Hum Genet 20: 309-311.
18. Rani R, Fernandez-Vina MA, Stastny P (1998) Associations between HLA class II alleles in a North Indian population. Tissue Antigens 52: 37-43.
19. Shi YY, He L (2005) SHEEis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. Cell Res 15: 97-98.
20. Li Z, Zhang Z, He Z, Tang W, Li T, et al. (2009) A partition-tigation-combination-subdivision EM algorithm for haplotype inference with multialemic markers: update of the SHEEis (http://analysis.bio-x.cn). Cell Res 19: 519-523.
21. Miller Z, Ao L, Kim KB, Lee W (2013) Inhibitors of the immunoproteasome: current status and future directions. Curr Pharm Des 19: 4140-4151.
22. Miao H, Bellivista E, Santoro A, Stolzing A, Ligrò C, et al. (2006) Immunoproteasome and LMP2 polymorphism in aged and Alzheimer’s disease brains. Neurobiol Aging 27: 54-66.
23. Park JE, Ao L, Miller Z, Kim K, Wu Y, et al. (2013) PSMB9 codon 60 polymorphisms have no impact on the activity of the immunoproteasome catalytic subunit B1i expressed in multiple types of solid cancer. PLoS One 8: e73732.
24. Zhou P, Cao H, Smart M, David C (1993) Molecular basis of genetic polymorphism in major histocompatibility complex-linked proteasome gene (Lmp-2). Proc Natl Acad Sci U S A 90: 2681-2684.
25. Deng GY, Muir A, Maclaren NK, She JX (1995) Association of LMP2 and LMP7 genes within the major histocompatibility complex with insulin-dependent diabetes mellitus: population and family studies. Am J Hum Genet 56: 528-534.
26. Ding HL, Cheng H, Fu ZZ, Deng QL, Yang Q, Yang YL, et al. (2000) The relationship of Map2 and Dr3 genes with susceptibility to type I diabetes mellitus in south China Han population. World J Gastroenterol 6: 111-114.
27. van Endert PM, Llabau RS, Patel SD, Fugger L, Lopez T, et al. (1994) Major histocompatibility complex-encoded antigen processing gene polymorphism in IDDM. Diabetes 43: 110-117.
28. Undlien DE, Akselsen HE, Joner G, Dahl-Jorgensen K, Sevik O, et al. (1997) No independent associations of LMP2 and LMP7 polymorphisms with susceptibility to develop IDDM. Diabetes 46: 307-312.
29. Kawaguchi Y, Ikegami H, Fukuda M, Takekawa K, Fukijoka Y, et al. (1994) Absence of association of TAP and LMP genes with type 1 (insulin-dependent) diabetes mellitus. Life Sci 54: 2049-2053.
30. Chauffert M, Cissé A, Chevenne D, You JF, Michel S, et al. (1997) Susceptibility to type 1 diabetes in the Senegalese population is linked to HLA-DQ and not TAP and LMP genes. Diabetes Care 20: 1299-1303.
31. Sia C, Weinem M (2005) Genetic susceptibility to type 1 diabetes in the intracellular pathway of antigen processing - a subject review and cross-study comparison. Rev Diabet Stud 2: 40-52.
32. Fellerhoff B, Gu S, Laumbacher B, Nerlich AG, Weiss EH, et al. (2011) The LMP7-K allele of the immunoproteasome exhibits reduced transcript stability and amino-acid signatures of the peptide-binding pockets of HLA molecules in vitiligo. J Clin Immunol 31: 1319-1326.
36. Muchamuel T, Basler M, Aujay MA, Suzuki E, Kalim KW, et al. (2009) A selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis. Nat Med 15: 781-787.

37. Prahalad S, Kingsbury DJ, Griffin TA, Cooper BL, Glass DN, et al. (2001) Polymorphism in the MHC-encoded LMP7 gene: association with JRA without functional significance for immunoproteasome assembly. J Rheumatol 28: 2320-2325.

38. Vargas-Alarcón G, Gamboa R, Vergara Y, Rodríguez-Zepeda JM, de la Peña A, et al. (2002) LMP2 and LMP7 gene polymorphism in Mexican populations: Mestizos and Amerindians. Genes Immun 3: 373-377.

39. Faucz FR, Probst CM, Peltz-Erler ML (2000) Polymorphism of LMP2, TAP1, LMP7 and TAP2 in Brazilian Amerindians and Caucasoids: implications for the evolution of allelic and haplotypic diversity. Eur J Immunogenet 27: 5-16.

40. Lim JK, Hunter J, Fernandez-Vina M, Mann DL (1999) Characterization of LMP polymorphism in homozygous typing cells and a random population. Hum Immunol 60: 145-151.

41. Ding H, Cheng H, Fu Z, Yan L, Yang G (2001) Relationship of large multifunctional proteasome 7 gene polymorphism with susceptibility to type 1 diabetes mellitus and DR3 gene. Chin Med J (Engl) 114: 1263-1266.

42. Deshpande A, Wheeler CM, Hunt WC, Peyton CL, White PS, et al. (2008) Variation in HLA class I antigen-processing genes and susceptibility to human papillomavirus type 16-associated cervical cancer. J Infect Dis 197: 371-381.