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

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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 of 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. χ^2 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 *LMP 7 codon 49* and *LMP7 Intron 6* G allele ($p<0.01$) were significantly increased in T1D subjects compared to controls. Haplotype analysis showed that haplotypes *GCG* and *ACT* (*LMP2 codon 60- LMP7codon 49- LMP7 intron 6*) ($p<5.9\times10^{-13}$, $p<1.9\times10^{-8}$ respectively) were significantly increased and haplotypes *GCT* and *ACG* ($p<1.9\times10^{-12}$, $p<1.9\times10^{-13}$ 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.

Introduction

Type 1 diabetes (T1D) is an incurable, multifactorial and complex autoimmune disorder. In T1D, most of the insulin producing beta cells of the pancreas are lost before the disease manifests itself in the form of abnormal glucose metabolism. Uncontrolled hyperglycemia may result in complications like ketoacidosis, retinopathy, nephropathy and even cardio-vascular diseases and pre-mature death [1].

World-wide disease affects 1 in 300-400 children [2]. The prevalence in India is 10.20/100,000 with higher prevalence of 26.6/100,000 in urban areas compared to 4.27/100,000 in rural areas [3]. While several genetic [4-6] and environmental factors have been implicated in autoimmune destruction of the insulin producing Pancreatic Beta cells, the association of the *Major Histocompatibility complex* (*MHC*) class-II alleles has been shown to be the strongest [5-7]. The function of the MHC molecule is to present antigenic peptides to the T cells for the immune response to take place. However, for the peptides to be presented on the MHC molecule, the antigenic proteins need to be processed into small peptides and loaded on to the peptide binding groove of the MHC molecule. Cytosolic or viral proteins are processed in the cytoplasm by a complex of proteosomes, which include interferon–gamma (IFN-γ) inducible low molecular mass proteases or polypeptide complex 2 and 7 (LMP2 and LMP7), also known as *proteasome subunit beta type-9* (*PSMB9*) and *Proteasome subunit beta type-8* (*PSMB8*) respectively [8-12].

LMP2 and LMP7 seem to have peptide editing function since they select the peptides to be presented on MHC class-I molecules and thus modulate the immune response against self or non-self antigens. Proteosomes have also been shown to mediate the processing and activation of the transcription factor, nuclear factor-κB (NF-κB) [13], which regulates the expression of other downstream genes involved in immune responses like cytokine and chemokine genes [13].

Because of their functional implications in terms of antigen presentation and immune responses, we studied single nucleotide polymorphisms (SNPs) in *LMP2* and *LMP7* genes to determine their association with Type 1 diabetes, age at onset and gender bias in a North Indian population.

Materials and Methods

Patients and control populations

Two hundred and thirty nine T1D subjects (105 females, mean age at onset 14.74 ± 7.57 and 134 males, mean age of onset 16.89 ± 7.25) recruited from 'Type 1 Diabetes Clinic' at All India Institute of Medical Sciences, New Delhi, India, in a consecutive manner from 2004-2008,

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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:

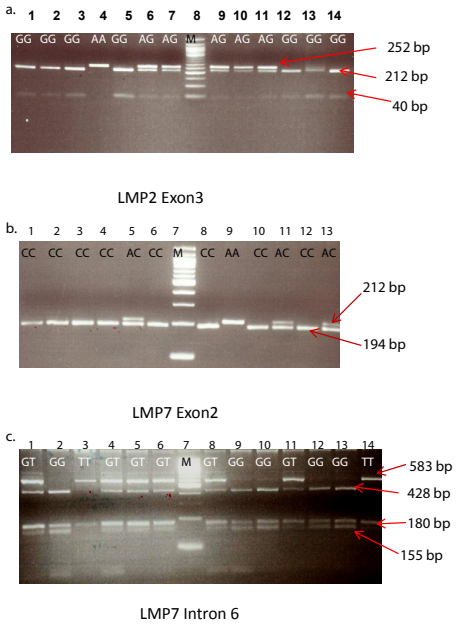
Third exon of *LMP2* and second exon and sixth Intron of *LMP7* were amplified using Polymerase Chain Reaction (PCR) using standard conditions and primers described by Casp et al. [11] listed in Table 1. SNP genotypes were determined by restriction fragment length polymorphism (RFLP) analysis of the 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*-I 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 ^{32}P- labeled sequence specific oligonucleotide probes (SSOP) 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 χ^2 tests, Odds ratios and 95% confidence intervals using Stata 9.2 software. However, whenever the numbers in any group (i.e. in cases



d.

Gene	Single nucleotide Polymorphism	Restriction enzyme	Fragment sizes
LMP2 (PSMB9)	CGC/CAC Exon 3	*Hha1*	*G*:212, 40 bp *A*: 252 bp
LMP7 (PSMB8)	CAG/AAG Exon 2	*Pst1*	*C*: 194, 18 bp *A*:212 bp
LMP7 (PSMB8)	G/T intron 6	*Hha1*	*G*: 428,180,155bp *T*:583,180 bp

Figure 1: Representative pictures of gels showing restriction digestion pattern and genotyping for single nucleotide polymorphisms (SNPs) in **a.** LMP2 exon 3 (G/A) lanes 1-3, 5, 12-14 : GG homozygous, lane 4 :AA homozygous and lanes 6,7,9-11: AG heterozygous & lane 8 M : 50bp marker, **b.** LMP2 Exon2 (C/A) lanes 1-4, 6, 8 10 and 12: CC homozygous, lanes 511 and 13 : AC heterozygous, lane 9 : AA homozygous and lane 7 M: 100 bp marker, **c**. LMP7 Intron 6 G/T) lanes 1, 4-6, 8 and 11 GT heterozygous, lanes 2,9,10, 12, 13 : GG homozygous and lanes 3 and 14 TT homozygous, lane 7 M: 100 bp marker, **d**. Table showing the SNPs on three loci, restriction enzymes used and the fragment sizes obtained after restriction digestion to decipher the SNPs.

Gene	Single nucleotide Polymorphism	PCR primers		Restriction enzyme	Fragment sizes
		Name	Sequences (5' to 3')		
LMP2 (PSMB9)	CGC/CAC codon 60 (R60H) [11,42]	LMP2-2	*GTGAACCGAGTGTTTGACAAGC*	*Hha1*	*G*:212, 40 bp
	rs17587	LMP2-1	*GCCAGCAAGAGCCGAAACAAG*	37°C for 4 hours	*A*: 252 bp
LMP7 (PSMB8)	CAG/AAG codon 49 (Q49K) [11,42]	LMP7-Z	*TCGCTTTACCCCGGGGACTG*[a]	*Pst1*	*C*: 194, 18 bp
	rs2071543	LMP7-BR	*AACTTGCACTTCCTCCTCTCAGG*	37°C for 2 hours	*A*:212 bp
LMP7 (PSMB8)	G/T intron 6 [11,25]	LMP7-7	*TTGATTGGCTTCCCGGTACTG*	*Hha1*	*G*: 428,180,155bp
		LMP7-4	*TCTACTACGTGGATGAACATGG*	37°C for 5 hours	*T*:583,180 bp

Table 1: Primers used for amplification of *LMP2 exon 3*, *LMP* 7 Exon 2 and Intron 6 for SNP genotyping [11].

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. 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 *CAC*). 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/AAG). 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.

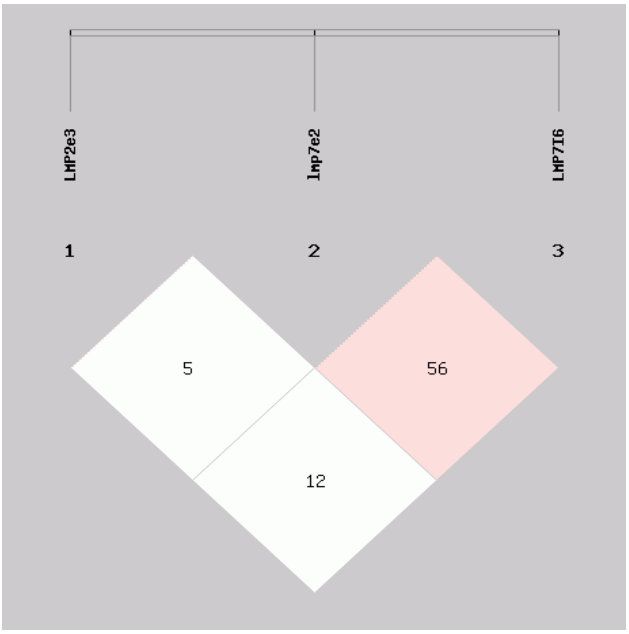
In *LMP7 Intron 6* the *G* allele was significantly increased ($p<0.01$) and *T* allele ($p<0.01$) and homozygous *TT* ($p<0.03$) were significantly reduced in T1D patients as compared to controls. The differences in allele frequencies remained significant even after the p value was corrected for multiple comparisons; however, the difference in homozygous *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 ($p=5.9\times10^{-13}$). 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 ($p=1.9\times10^{-8}$). However, haplotypes

Genotype/ allele	TID		Controls		T1D Vs Controls		
LMP2 exon3	**N=228 No.**	**%**	**N=752 No.**	**%**	**p Value**	**Odds ratio**	**(95% C.I.)**
GG (RR)	166	72.8	480	63.82	0.012	1.52	1.08-2.14
AG (HR)	57	25.00	241	32.05	0.04	0.71	0.49-0.99
AA (HH)	5	2.19	31	4.12	0.17*	0.56**	0.29-1.1
G (R)	389	85.3	1201	79.85	0.009	1.46	1.09-1.98
A (H)	67	14.7	303	20.14	0.009	0.68	0.5-0.92
LMP7 exon 2	**N=225**	**%**	**N=742**	**%**			
CC (QQ)	173	76.88	516	69.54	0.03	1.45	1.02-2.1
AC (KQ)	48	21.33	191	25.74	0.18	0.78	0.53-1.13
AA (KK)	4	1.77	35	4.72	0.03	0.4	0.2-0.81
C (Q)	394	87.55	1223	82.41	0.0098	1.5	1.1-2.1
A (K)	56	12.45	261	17.58	0.0098	0.66	0.47-0.91
LMP7 Intron 6	**N=230**	**%**	**N=738**	**%**			
GG	90	39.13	245	33.2	0.09	1.29	0.94-1.77
GT	106	46.08	332	44.99	0.77	1.04	0.77-1.4
TT	35	14.78	161	21.81	0.03	0.64	0.42-0.97
G	286	62.17	822	55.7	0.01	1.3	1.05-1.63
T	174	37.83	654	44.3	0.01	0.76	0.61-0.95
Haplotypes *LMP2 exon 3, LMP7 exon 2, LMP7 intron 6*@$							
	2N=412	**%**	**2N=1476**	**%**			
GCG	257	62.37	584	39.56	5.9×10^{-13}*	2.29	1.83-2.88
ACT	58	14.1	80	5.42	1.9×10^{-8}*	2.7	1.89-3.87
GCT	46	11.16	387	26.22	1.9×10^{-12}*	0.32	0.23-0.44
ACG	0	0	164	11.11	2.5×10^{-13}*	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 2: LMP2 and LMP7 SNPs and haplotypes in Type 1 diabetes patients compared with healthy controls.



Linkage Disequilibrium tests

D':	*LMP7e2*	*LMP7I6*
LMP2e3	0.056	0.126
LMP7e2	-	0.567

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).

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

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 *LMP 7 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 *GCG* and *ACT* were significantly increased in both early ($p<4.5\times10^{-11}$, $p<0.003$ respectively) and late age at onset ($p<2\times10^{-5}$ and $p<5.14\times10^{-8}$ 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

Genotype/allele	TID		Controls		Female T1D vs. female controls		
LMP2 exon3	**N=97** **No.**	**%**	**N=199** **No.**	**%**	**p value**	**Odds ratio**	**95% C.I.**
GG	71	73.19	117	58.79	0.016	1.91	1.09-3.39
GA	25	25.77	75	37.69	0.04	0.57	0.32-1.01
AA	1	1.03	7	3.52	0.2*	0.4**	0.11-1.4
G	167	86.08	309	77.64	0.015	1.78	1.09-2.96
A	27	13.92	89	22.36	0.015	0.56	0.34-0.91
LMP7 exon 2	**N=101**	**%**	**N=196**	**%**			
CC	79	78.22	132	67.35	0.05	1.74	0.96-3.2
AC	20	19.8	53	27.04	0.17	0.66	0.35-1.2
AA	2	1.98	11	5.61	0.12*	0.4**	0.15-1.08
C	178	88.11	317	80.87	0.02	1.75	1.05-3.01
A	24	11.88	75	19.13	0.02	0.57	0.33-0.95
LMP7 Intron 6	**N=96**	**%**	**N=196**	**%**			
GG	40	41.66	57	29.08	0.03	1.74	1.01-2.99
GT	40	41.66	95	48.47	0.27	0.75	0.45-1.28
TT	16	16.66	44	22.45	0.25	0.69	0.34-1.34
G	120	62.5	209	53.31	0.03	1.45	1.01-2.11
T	72	37.5	183	46.69	0.03	0.68	0.47-0.99
Haplotypes *LMP2 exon 3, LMP7 exon 2, LMP7 intron 6*@$							
	2N=170	**%**	**2N=390**	**%**			
GCG	111	65.29	165	42.31	7.5×10^{-6}*	2.36	1.61-3.45
ACT	21	12.35	34	8.72	0.24*	1.41	0.79-2.51
GCT	18	10.59	81	20.8	0.002*	0.43	0.25-0.74
ACG	0	0	34	8.72	4.1×10^{-5}*	0.03**	0.004-0.22

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

Genotype/allele	**TID**		**Controls**		**Male T1D vs. male controls**		
LMP2 exon3	N=131 %		N=553 %		p	Odds	95% CI
					Values	ratio	
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	13.36	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*@$							
	2N=240 %		**2N=1086 %**				
GCG	146	60.83	427	39.3	$8.5x10^{-9}$*	2.31	1.73-3.08
ACT	36	15	62	5.71	$1.3x10^{-6}$*	2.84	1.83-4.39
GCT	26	10.8	306	28.17	$8.1x10^{-9}$*	0.3	0.19-0.46
ACG	0	0	106	9.76	$3.1x10^{-7}$*	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.

*DRB1*07:01* with T1D [4,5,7]. So, to check whether the association of *LMP2* and *LMP7* SNPs in the present study were due to theirs being in Linkage Disequilibrium (LD) with the predisposing MHC allele, we calculated the co-efficient of LD (D') and co-efficient of correlation (r) between predisposing and protective *HLA* alleles with *LMP2-LMP7* haplotypes (Table 6). Haplotypes *GCG* and *GCT* were in weak linkage disequilibrium with predisposing *HLA-DRB1*03:01* in both patients (D'=0.2273, r=0.1385 and D'=0.5337, r=0.1297 respectively) and controls (D'=0.06337, r=0.1349 and D'=0.07436, r=0.0911). However, haplotype ACT was in stronger LD with protective *DRB1*07:01* allele (D'=0.1594, r=0.2134). While haplotype *GCG* and *ACT* were significantly increased, *GCT* was significantly reduced in the patients. Weak LD of both predisposing *GCG* and protective *GCT* haplotypes with predisposing *DRB1*03:01* and that of predisposing *ACT* with protective *DRB1*07:01* suggests that the association of *LMP2-LMP7* haplotypes are independent of their *HLA* alleles and not due to theirs being in LD with the predisposing *HLA* alleles. Interestingly, when we studied simultaneous presence of the predisposing and protective *LMP2-LMP7* haplotypes with predisposing and protective *HLA* alleles (Table 7), all three haplotypes *GCG, ACT* and *GCT* along with *DRB1*03:01*, were significantly increased in T1D patients compared to control. And *GCG* and *GCT* along with *DRB1*07:01* were significantly reduced in T1D compared to controls. These results indicate the dominant effect of the predisposing *HLA* allele *DRB1*03:01* which was present in more than 70% of the patients compared to only 15.7% of controls and was thus associated with both predisposing and protective *LMP2-LMP7* haplotypes suggesting the independent role of predisposing *LMP2-LMP7* haplotypes.

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) (*CGC* to *CAC*). While there was no difference in the mRNA expression of *LMP2* in the R and H alleles, their chymotrypsin-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 Type1 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*

Genotype/allele	TID Onset age ≤14	Control	Age at onset ≤ 14 Vs. Controls			TID Onset age >14	Age at onset > 14 Vs. Controls		
LMP2 exon3	**N=99 (%)**	**N=752 (%)**	**p Value**	**Odds ratio**	**95% CI**	**N=117 (%)**	**p Value**	**Odds ratio**	**95% CI**
GG	74 (74.75)	480 (63.82)	0.03	1.67	1.02-2.82	83 (70.94)	0.13	1.38	0.89-2.19
GA	23 (23.2)	241 (32.05)	0.07	0.64	0.37-1.06	31 (26.49)	0.23	0.76	0.47-1.2
AA	2 (2.02)	31 (4.12)	0.24*	0.58**	0.23-1.48	3 (2.56)	0.3*	0.7**	0.31-1.54
G	171 (86.4)	1201 (79.85)	0.029	1.598	1.04-2.54	197 (84.19)	0.12	1.34	0.92-2.0
A	27 (13.6)	303 (20.14)	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)	516 (69.54)	0.11	1.49	0.89-2.59	88 (76.52)	0.12	1.43	0.89-2.35
AC	21 (21.65)	191 (25.74)	0.38	0.79	0.45-1.35	24 (20.87)	0.26	0.76	0.45-1.25
AA	1 (1.03)	35 (4.72)	0.06*	0.31**	0.09-0.99	3 (2.61)	0.22*	0.62**	0.28-1.36
C	171 (88.14)	1223 (82.41)	0.045	1.58	0.99-2.62	200 (86.96)	0.087	1.42	0.94-2.21
A	23 (11.86)	261 (17.58)	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)	245 (33.2)	0.016	1.67	1.07-2.62	42 (35.59)	0.61	1.11	0.72-1.69
GT	40 (40.4)	332 (44.99)	0.39	0.82	0.53-1.29	57 (48.3)	0.5	1.14	0.76-1.72
TT	14 (14.14)	161 (21.81)	0.08	0.59	0.3-1.08	19 (16.1)	0.16	0.69	0.38-1.17
G	130 (65.66)	822 (55.7)	0.007	1.52	1.1-2.11	141 (59.75)	0.22	1.19	0.89-1.59
T	68 (34.34)	654 (44.3)	0.007	0.66	0.47-0.9	94 (40.25)	0.22	0.84	0.62-1.12
Haplotypes *LMP2 exon 3,LMP7 exon 2, LMP7 intron 6*@$									
	2N=174	**2N=1476**	**p Value**	**Odds ratio**	**95% CI**	**N=210**	**p Value**	**Odds ratio**	**95% CI**
GCG	119 (68.4)	584 (39.6)	$4.5x10^{-11}$*	3.05	2.16-4.29	122 (58.0)	$2x10^{-5}$*	1.89	1.41-2.54
ACT	20 (11.5)	80 (5.42)	0.003*	2.14	1.28-3.6	34 (16.2)	$5.14x10^{-8}$*	3.15	2.05-4.86
GCT	16 (9.2)	387 (26.22)	$1.96x10^{-12}$*	0.32	0.23-0.44	25 (12)	$6.85x10^{-7}$*	0.35	0.23-0.54
ACG	0 (0)	164 (11.11)	$2.47x10^{-13}$*	0.02**	0.003-0.16	0 (0)	$1.32x10^{-7}$*	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

Table 5: Association of *LMP2* and *LMP7* SNPs and haplotypes with age at onset in Type 1 diabetes patients compared with healthy controls.

(RR) genotype and *G* (R) allele to be significantly increased in the T1 D patients, Deng et al. and Ding et al. reported the homozygous RR to be protective from diabetes in Caucasian and Chinese Han populations respectively [25,26]. However, several other studies did not find *LMP2* R/H polymorphism to be associated with T1D [27,30]. A meta analysis done to resolve this problem of variable results suggested that *LMP2* RH genotype seemed to be associated with T1D [31], the results opposite to ours where we observed RR genotype to be disease conferring and heterozygous RH to be reduced in the patients compared to controls. These differences could be due to different ethnicity of the individuals studied in the present report.

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 KK (*AA*) 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

Haplotypes for LMP2 exon3-LMP7 exon2-LMP7 intron6-DRB1*03:01	Number of haplotypes observed	Observed haplotype frequency P_{ab}	Expected haplotype frequency (P_a X $P_{b)}$	D= (P_{ab} - P_a X $P_{b)}$	D'=D/ P_a (1-P_b)	$r^2=D^2/P_a$ X P_b	r = √r^2
For T1D patients 2N=398							
GCG-DRB1*03:01	148	0.3718	0.2964	0.0754	0.2273	0.0192	0.1385
GCG-DRB1*07:01	21	0.0527	0.0474	0.0054	0.00932	0.00062	0.0248
ACT-DRB1*03:01	33	0.0829	0.0746	0.0083	0.0998	0.00093	0.0305
ACT-DRB1*07:01	14	0.0352	0.0119	0.0233	0.1594	0.0455	0.2134
GCT-DRB1*03:01	30	0.0754	0.0472	0.02818	0.5337	0.0168	0.1297
GCT-DRB1*07:01	3	0.00754	0.00754	0	0	0	0
For healthy controls 2N=700							
GCG-DRB1*03:01	51	0.0728	0.04437	0.02843	0.06337	0.01822	0.1349
GCG-DRB1*07:01	61	0.0871	0.0838	0.00329	0.008	0.00013	0.0114
ACT-DRB1*03:01	9	0.01286	0.0117	0.00116	0.0098	0.00012	0.0107
ACT-DRB1*07:01	16	0.0229	0.0221	0.0008	0.0074	2.896E-05	0.00538
GCT-DRB1*03:01	18	0.0257	0.01467	0.01103	0.07436	0.00829	0.0911
GCT-DRB1*07:01	39	0.0557	0.0277	0.028	0.207	0.0284	0.1685

P_{ab} = Observed haplotype frequency counted directly and divided by 2N and the resulting haplotype frequencies were used to calculate D, P_a = haplotype frequency (hf) of GCG, ACT or GCT, P_b = gene frequency of DRB1*03:01. Haplotype frequency of (P_a) GCG=0.628, ACT=0.158, GCT=0.1, gf (P_b) of DRB1*03:01=0.472, DRB1*07:01=0.0754 in T1D. In controls hf frequency of GCG=0.493, ACT=0.13, GCT=0.163, gf of DRB1*03:01=0.09, DRB1*07:01=0.17

Table 6: Linkage disequilibrium analysis for *LMP2 exon3-LMP7 exon2-LMP7 intron6* 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	$1.5x\ 10^{-42}$	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	5.9×10^{-9}	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	0.0001	3.08	1.64-5.96
GCT-DRB1*07:01	3	0.75	39	5.57	0.00002*	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*.

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 2* Q/K polymorphism in T1D, QQ homozygosity has been shown to be associated with another autoimmune disorder, juvenile rheumatoid arthritis (JRA) [37]. *LMP7 exon 2 49*Q allele is the most frequent allele in Mexicans [38], Japanese [39], Brazilian Guarani population [39] and the north Indians in the present study and *49*K 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 *GCG* and *ACT* were significantly increased in the patients and *GCT* and *ACG* 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 *LMP2* and *LMP7* being in LD with predisposing *MHC class-II* allele *DRB1*03:01* [4,5]. Our results showed that *LMP* haplotype *GCG* was in weak linkage disequilibrium with predisposing *HLA-DRB1*03:01* and not with protective *HLA-DRB1*07:01*, both in patients and controls, however, 37.19% of the T1D patients had *GCG-DRB1*03:01* compared to 7.28% of the control and this was a highly significant difference with and Odds Ratio of 9.37. Similarly, other *LMP* haplotypes *ACT* and *GCT* along with *DRB1*03:01* were significantly increased in the patients and the same haplotypes along with *DRB1*07:01* were significantly reduced in the patients. *LMP* haplotype *GCT* by itself was significantly reduced and haplotype *GCG* was significantly increased in the patients, however, significant increase in the simultaneous presence of both these haplotypes along with predisposing *HLA DRB1*03:01* and significant decrease along

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 *GCG* and *ACT* and decrease in the frequency of *GCT* and *ACG* 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.

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