No Independent Associations of LMP2 and LMP7 Polymorphisms With Susceptibility to Develop IDDM

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Results from a recent study suggested that polymorphisms within the HLA class II genes LMP2 and LMP7 were associated with the susceptibility for developing IDDM, and that this association could not be explained by linkage disequilibrium to HLA-DR or -DQ genes. We typed 285 IDDM patients and 337 HLA-DRB1-DQA1-DQB1 genotypically matched control subjects from an ethnically homogeneous population for both the G/T polymorphism in intron 6 of the LMP7 gene and the Arg-His polymorphism in the LMP2 gene. In addition, we typed IDDM families in which at least one parent was homozygous for a DRB1-DQA1-DQB1 haplotype and performed a transmission/disequilibrium test of these LMP polymorphisms. Our data suggest that none of these LMP2 or LMP7 polymorphisms are independently associated with IDDM susceptibility, in contrast to what has been previously reported by others. Further, our results suggest that one partial explanation for the previously reported independent association between IDDM and these LMP polymorphisms may have been that patients and control subjects were not matched for DRB1*04 subtypes. Our results emphasize the need for a complete matching for DRB1, DQA1, and DQB1 alleles between patients and control subjects when attempting to detect independent effects of other polymorphisms in the HLA complex on IDDM susceptibility or protection. *Diabetes* 46:307–312, 1997

IDDM is a multifactorial disease with multiple genes (1,2) and as yet unknown environmental factors determining disease susceptibility. A major part of the genetic predisposition toward IDDM is encoded within the HLA complex. The HLA complex is characterized by strong linkage disequilibrium, which tends to keep specific alleles together on given haplotypes. This has made it difficult to distinguish which HLA polymorphisms are primarily involved and which ones are associated with IDDM only because of linkage disequilibrium with other genes (3). Most investigators believe HLA-DQ genes are primarily involved, based on "cross-match" haplotype analysis (4,5), transracial studies (6,7), and the observation that identical DQ heterodimers encoded either in *cis* or *trans* are associated with the disease (8). However, clearly the HLA-DQ genes cannot explain all the genetic predisposition encoded within the HLA complex. In particular, the risk encoded by DQA1*03-DQB1*0302 haplotypes has been shown by several investigators to be highly dependent on which DRB1*04 subtype is carried on these haplotypes (9–14).

Several studies have also addressed the contribution of other HLA class II genes in IDDM susceptibility. Results from studies addressing the transporter associated with antigen processing-1 (TAP1), TAP2, and DMB genes have suggested that there is no independent effect of polymorphisms in these genes on IDDM susceptibility (15–18).

The large multifunctional proteasomes (LMP) genes are encoded within the HLA class II gene region (see Fig. 1). The gene products of these genes are believed to participate in antigen processing by being part of LMPs, which digest proteins into peptides that can then bind to HLA class I molecules (for references, see 19). Deng et al. reported evidence for an independent association between IDDM and polymorphisms in the LMP2 and LMP7 genes (20). The LMP2 association was evident only on high-risk DR4-DQA1*03-DQB1*0302 haplotypes, whereas the LMP7 polymorphism was apparently associated with IDDM susceptibility independently of HLA class II background. Other studies have failed to find evidence for an independent effect of the LMP2 polymorphism tested (21,22).

To perform studies addressing the potential influence of HLA genes other than DR and DQ on IDDM susceptibility, one has to overcome the pitfalls of linkage disequilibrium. One way to do this is to study case control materials properly matched for HLA-DQ and -DR genes in a genetically homogeneous population. Another way is to find families in which at least one parent of IDDM patients is homozygous for the involved HLA class II genes and perform transmission test in such families for linkage disequilibrium (i.e., using the transmission/disequilibrium test [TDT]).

We used both these approaches in studying patients and control subjects from a genetically homogeneous Norwegian population. Initially, we limited our study to the high-IDDM

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LMP genes, large multifunctional proteasomes genes; nc, not corrected; PCR, polymerase chain reaction; PCR-RFLP, polymerase chain reaction–restriction fragment length polymorphism; TAP, transporter associated with antigen processing; TDT, transmission/disequilibrium test.

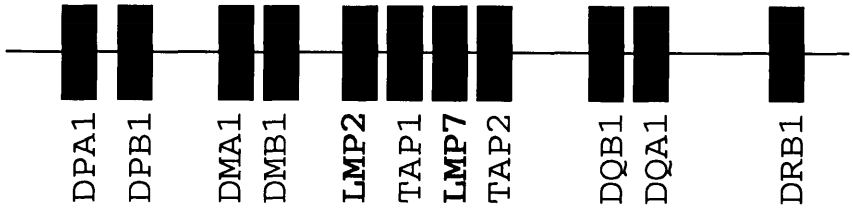

FIG. 1. Simplified schematic map of the LMP2 and LMP7 genes and some of their neighboring genes in the HLA class II region.

risk genotypes: DRB1*0301-DQA1*0501-DQB1*0201/DRB1*04-DQA1*03-DQB1*0302 (DR3-DQ2/DR4-DQ8) and DRB1*04-DQA1*03-DQB1*0302/ DRB1*04-DQA1*03-DQB1*0302 (DR4-DQ8/DR4-DQ8). When we did not find any evidence of an independent association between LMP polymorphisms and IDDM susceptibility in this subset, we then analyzed more moderate–IDDM risk genotypes: DRB1*0101-DQA1*0101-DQB1*0501/DRB1*04-DQA1*03-DQB1*0302 (DR1-DQ5/DR4-DQ8), DRB1*0701-DQA1*0201-DQB1*0202/DRB1*04-DQA1*03-DQB1*0302 (DR7-DQ2/DR4-DQ8), and DRB1*08-DQA1*0401-DQB1*0402/DRB1*04-DQA1*03-DQB1*0302 (DR8-DQ4/DR4-DQ8). We did not find any evidence of an independent association between LMP polymorphisms and IDDM susceptibility in these subgroups either.

RESEARCH DESIGN AND METHODS

IDDM patients and control subjects. The study population comprised 285 IDDM patients and 337 control subjects selected according to DRB1-DQA1-DQB1 genotype. Patients and control subjects had to have one of the following five genotypes to be included in the study: 1) DR3-DQ2/DR4-DQ8, 2) DR4-DQ8/DR4-DQ8, 3) DR1-DQ5/DR4-DQ8, 4) DR7-DQ2/DR4-DQ8, or 5) DR8-DQ4/DR4-DQ8. The reasons for only including these genotypes were twofold. First, these genotypes were the most frequent among the IDDM patients, which allowed a meaningful statistical analysis when comparing patients and HLA-matched control subjects. Second, Deng et al. (20) reported evidence for an independent association of the LMP2 and LMP7 polymorphisms on certain haplotypes, all of which are present on one or more of these genotypes. IDDM patients were all less than age 15 years at disease onset, and were recruited at pediatric departments all over Norway during the period 1992–1995. The distribution of DR4 subtypes in this material has been previously reported (14). In comparison with our previous study of DR4 subtypes, the current study included eight fewer control subjects and one fewer IDDM patient carrying the high risk DR3/4 or DR4/4 genotypes. These individuals were excluded either because of a lack of DNA or a failed polymerase chain reaction (PCR), defined as PCR having failed three times.

Families. We selected families in which one or more parent was homozygous for either the DRB1*0301-DQA1*0501-DQB1*0201 or the DRB1*04-DQA1*03-DQB1*0302 haplotypes (n = 61 families: 55 simplex and 6 multiplex). There were 24 DRB1*04-DQA1*03-DQB1*0302 homozygous parents (also homozygous for DR4 subtype) and 40 DRB1*0301-DQA1*0501-DQB1*0201 homozygous parents. Those parents who were heterozygous for the LMP2 or LMP7 polymorphisms and homozygous for DRB1-DQA1-DQB1 were subjected to TDT analysis (23) of LMP2 and LMP7 alleles, respectively.

Genotyping of LMP2 and LMP7 polymorphisms. The R/H-60 polymorphism in the LMP2 gene and the G/T 37360 polymorphism of intron 6 of the LMP7 gene were typed by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) as previously published (20).

Statistical methods. Comparison of genotype frequencies was performed using χ^2 analysis or Fisher's exact test when appropriate. $P < 0.05$ was considered significant.

RESULTS

Distribution of LMP7 genotypes among patients and control subjects with high risk DR3/4 and DR4/4 genotypes. The distribution of LMP2 and LMP7 genotypes among DR3-DQ2/DR4-DQ8– and DR4-DQ8/DR4-DQ8–positive patients and control subjects is given in Tables 1 and 2. The naming of alleles and genotypes is identical to that used by Deng et al. (20). As can be seen from Table 1, there was a decrease in the LMP7*A/B genotype among IDDM patients in comparison with control subjects in the DRB1*03-DQA1*0501-DQB1*0201/DRB1*04-DQA1*03-DQB1*0302 heterozygous group (Pnc = 0.02; nc = not corrected) before splitting for DR4 subtypes. However, this tendency (which was not significant after correction for number of comparisons) disappeared when patients and control subjects were also matched for DR4 subtypes. When comparing the allele frequencies of the LMP7*A and LMP7*B allele, respectively, we found that DRB1*0401-positive individuals (both patients and control subjects) had a significantly higher frequency of the LMP7*A allele than did DRB1*0404-positive individuals, both in the DRB1*03-DQA1*0501-DQB1*0201/DRB1*04-DQA1*03-DQB1*0302 heterozygous group and in the DRB1*04-DQA1*03-DQB1*0302 homozygous group (Pnc < 0.0001 and Pnc < 0.05, respectively, when comparing the con-

TABLE 1
LMP2 and LMP7 polymorphisms among DRB1*03-DQA1*0501-DQB1*0201/DRB1*04-DQA1*03-DQB1*0302 heterozygous Norwegian IDDM patients and healthy control subjects by DRB1*04 subtype

	All 04 subtypes			0401			0404			Other 04 subtypes	
	Patients	Control subjects	P	Patients	Control subjects	P	Patients	Control subjects	P	Patients	Control subjects
LMP2											
R/R	43 (23)	53 (23)	NS	29 (20)	32 (28)	NS	12 (33)	17 (17)	0.03	2 (20)	4 (36)
R/H	141 (74)	167 (74)	NS	111 (77)	78 (68)	NS	22 (61)	82 (80)	0.02	8 (80)	7 (64)
H/H	7 (4)	7 (3)	NS	5 (3)	4 (4)	NS	2 (6)	3 (3)	NS	0	0
LMP7											
A/A	107 (56)	107 (47)	NS	95 (66)	73 (64)	NS	12 (33)	33 (32)	NS	0	1 (9)
A/B	71 (37)	111 (49)	0.02	44 (30)	39 (34)	NS	19 (53)	63 (62)	NS	8 (80)	9 (82)
B/B	13 (7)	9 (4)	NS	6 (4)	2 (2)	NS	5 (14)	6 (6)	NS	2 (20)	1 (9)

Data are n (%). Uncorrected P values comparing genotype frequencies in patients and control subjects.

TABLE 2
LMP2 and LMP7 polymorphisms among DRB1*04-DQA1*03-DQB1*0302/DRB1*04-DQA1*03-DQB1*0302 homozygous Norwegian IDDM patients and control subjects

	All 04 subtypes			0401-0401			0401-0404			0404-0404		Other 04 homozygous	
	Patients	Control subjects	*P*	Patients	Control subjects	*P*	Patients	Control subjects	*P*	Patients	Control subjects	Patients	Control subjects
LMP2													
R/R	37 (93)	52 (93)	NS	22 (96)	19 (100)	NS	11 (100)	17 (85)	NS	2	14	2	2
R/H	3 (8)	4 (7)	NS	1 (4)	0	NS	0	3 (15)	NS	1	0	1	1
H/H	0	0	NS	0	0	NS	0	0	NS	0	0	0	0
LMP7													
A/A	20 (50)	20 (36)	NS	13 (57)	10 (53)	NS	4 (36)	6 (30)	NS	2	4	1	0
A/B	17 (43)	29 (52)	NS	9 (39)	8 (42)	NS	5 (45)	13 (65)	NS	1	5	2	3
B/B	3 (8)	7 (13)	NS	1 (4)	1 (5)	NS	2 (18)	1 (5)	NS	0	5	0	0

Data are *n* or *n* (%). Uncorrected *P* values comparing genotype frequencies in patients and control subjects.

trol groups stratified for DR4 subtypes; in the homozygous group, we compared allele frequencies in DRB1*0401 homozygous control subjects with DRB1*0404 homozygous control subjects). This reflects a higher frequency of LMP7*A on DRB1*0401-DQA1*03-DQB1*0302 haplotypes than on DRB1*0404-DQA1*03-DQB1*0302 haplotypes, whereas the reverse is true for LMP7*B.

Distribution of LMP2 genotypes among patients and control subjects with high risk DR3/4 and DR4/4 genotypes. The distribution of LMP2 genotypes among DR3-DQ2/DR4-DQ8– and DR4-DQ8/DR4-DQ8–positive patients and control subjects is reported in Tables 1 and 2. The only differences observed with *P*nc < 0.05 were an increase of the LMP2*R/R genotype and a decrease of the LMP2*R/H genotype among DRB1*0301-DQA1*0501-DQB1*0201/DRB1*0404-DQA1*03-DQB1*0302 heterozygous IDDM patients compared with HLA class II matched control subjects. This tendency, which did not reach statistical significance after correction for number of comparisons, was the opposite of that reported by Deng et al. (20), who reported an increase of the LMP2*H allele on DR4 positive haplotypes among IDDM patients when compared with control subjects.

TDT analysis of LMP2 and LMP7 alleles. The transmission of LMP7*A and LMP7*B alleles, respectively, from parents who were homozygous for DRB1*0301-DQA1*0501-DQB1*0201, DRB1*0401-DQA1*03-DQB1*0302, or DRB1*0404-DQA1*03-DQB1*0302, and who were LMP7*A/B heterozygous are reported in Table 3. We observed that LMP7*A and B were transmitted with identical frequency to children with IDDM, and that there was no significant transmission distortion to healthy children. Similarly, with the LMP2 polymorphism, we observed no evidence for transmission distortion from DRB1-DQA1-DQB1 homozygous parents to their affected children (Table 4).

Distribution of LMP2 and LMP7 polymorphisms among patients and control subjects with moderate–IDDM risk HLA genotypes. Our data on the distribution of LMP2 and LMP7 polymorphisms among DR3-DQ2/DR4-DQ8– and DR4-DQ8/DR4-DQ8–positive IDDM patients and HLA-matched control subjects, as well as the result of our TDT analysis, suggested that, among individuals with these high-risk HLA genotypes, there was no independent association between IDDM susceptibility and these LMP polymorphisms. However, Deng et al. (20) also observed evidence for an independent association of the LMP7 polymorphism on DRB1*0101-DQB1*0501 (DR1-DQ5) and DRB1*0701-DQB1*0202 (DR7-DQ2) haplotypes. We therefore went on to analyze the LMP7 and LMP2 polymorphisms in IDDM patients and HLA-matched control subjects carrying these and other haplotypes—namely, patients and control subjects being DRB1*0101-DQB1*0501/DRB1*04-DQB1*0302, DRB1*07-DQB1*0202/DRB1*04-DQB1*0302, and DRB1*08-DQB1*0402/DRB1*04-DQB1*0302 positive.

The results are given in Tables 5 and 6. Only the data including all DRB1*04 subtypes (before stratification) and the DRB1*0401 subtype are shown. The other DRB1*04 subtypes are infrequent among IDDM patients with these genotypes, and no significant deviations between patients and control subjects carrying these other DRB1*04 subtypes were found (data not shown). There was a decrease of the LMP7*A/B genotype among DRB1*08-DQB1*0402/DRB1*04-DQB1*0302 heterozygous patients compared with control subjects before stratifying for DRB1*04 subtype (Table 5; *P*nc = 0.03).

TABLE 3
Transmission/disequilibrium test analysis of LMP7 alleles from LMP7*A/B heterozygous and HLA-DRB1-DQA1-DQB1 homozygous parents to affected and healthy children

	A transmitted	B transmitted	χ^2
IDDM patients	17 (51)	16 (49)	0.03 (NS)
Healthy children	16 (49)	17 (51)	0.03 (NS)

Data are *n* (%).

TABLE 4
Transmission/disequilibrium test analysis of LMP2 alleles from LMP2*R/H heterozygous and HLA-DRB1-DQA1-DQB1 homozygous parents to affected and healthy children

	H transmitted	R transmitted	χ^2
IDDM patients	9 (50)	9 (50)	0 (NS)
Healthy children	13 (72)	5 (28)	3.56 (NS)

Data are *n* (%).

TABLE 5
LMP7 polymorphisms among selected DRB1*04-DQA1*03-DQB1*0302/X heterozygous Norwegian IDDM patients and healthy control subjects

DRB1-DQB1 genotype	LMP7	All DRB1*04 subtypes			DRB1*0401		
		Patients	Control subjects	*P*	Patients	Control subjects	*P*
DRB1*0101-DQB1*0501/ DRB1*04-DQB1*0302	A/A	2 (7)	3 (10)	NS	1	1	NS
	A/B	22 (79)	17 (57)	NS	18	8	NS
	B/B	4 (14)	10 (33)	NS	1	3	NS
DRB1*0701-DQB1*0202/ DRB1*04-DQB1*0302	A/A	0	3 (19)	NS	0	2	NS
	A/B	6 (75)	9 (56)	NS	4	6	NS
	B/B	2 (25)	4 (25)	NS	2	1	NS
DRB1*08-DQB1*0402/ DRB1*04-DQB1*0302	A/A	10 (56)	1 (13)	NS	8	0	NS
	A/B	6 (33)	7 (88)	0.03	4	2	NS
	B/B	2 (11)	0	NS	1	0	NS

Data are *n* or *n* (%). Uncorrected *P* values comparing genotype frequencies in patients and controls.

However, statistical significance was lost after correction for number of comparisons, nor was it evident after stratification for DRB1*04 subtypes (though the numbers in the latter comparisons were small). Concerning LMP2, we observed no significant differences between IDDM patients and HLA-matched control subjects (Table 6).

DISCUSSION

A recent study by Deng et al. (20) reported an association between the LMP7*A/B polymorphism, the LMP2*R/H polymorphism, and IDDM, which they explained was not due to linkage disequilibrium with high risk DRB1, DQA1, and DQB1 alleles. The LMP2 polymorphism was found to confer additional IDDM susceptibility only among DR4 positive individuals, whereas the LMP7 polymorphism conferred susceptibility independently of HLA class II genotype.

Our data did not confirm these observations. We found no evidence for any additional susceptibility conferred by these LMP polymorphisms among a large cohort of carefully DRB1-DQA1-DQB1–matched patients and control subjects, including matching for DR4 subtypes. In the study by Deng et al. (20), several methods to overcome the pitfalls of linkage disequilibrium were applied. However, in contrast to our study, Deng et al. did not match their patient and control haplotypes for DR4 subtypes that are known to be associated with IDDM susceptibility (9–14). Our data demonstrate that DRB1*0401 and DRB1*0404 are in different linkage disequilibrium with the A and B allele, respectively, of the LMP7 polymorphism. DRB1*0401 occurs more frequently together with the A allele than DRB1*0404, whereas the opposite is the case for LMP7*B. Furthermore, the DRB1*0401 allele is more frequent among DQA1*03-DQB1*0302–positive IDDM patients compared with DQ-matched control subjects, suggesting a contribution of DRB1*0401 to susceptibility (14). Thus our data indicate that these different linkage disequilibriums between different DR4 subtypes and the LMP7 polymorphisms may partly explain the observations of Deng et al. (20). When looking at our high-risk class II–matched patients

TABLE 6
LMP2 polymorphisms among selected DRB1*04-DQA1*03-DQB1*0302/X heterozygous Norwegian IDDM patients and healthy control subjects

DRB1-DQB1 genotype	LMP2	All DRB1*04 subtypes			DRB1*0401		
		Patients	Control subjects	*P*	Patients	Control subjects	*P*
DRB1*0101-DQB1*0501/ DRB1*04-DQB1*0302	R/R	26 (93)	26 (87)	NS	18	11	NS
	R/H	2 (7)	4 (13)	NS	2	1	NS
	H/H	0	0	NS	0	0	NS
DRB1*0701-DQB1*0202/ DRB1*04-DQB1*0302	R/R	8 (100)	13 (81)	NS	6	8	NS
	R/H	0	2 (13)	NS	0	1	NS
	H/H	0	1 (6)	NS	0	0	NS
DRB1*08-DQB1*0402/ DRB1*04-DQB1*0302	R/R	11 (61)	6 (75)	NS	8	2	NS
	R/H	6 (33)	2 (25)	NS	4	0	NS
	H/H	1 (6)	0	NS	1	0	NS

Data are *n* or *n* (%). Uncorrected *P* values comparing genotype frequencies in patients and controls.

and control subjects, we found that, before taking DR4 subtypes into account, there was a decrease of the LMP7*A/B genotype among patients compared with control subjects (*P*nc = 0.02; Table 1). However, when stratifying for DR4 subtypes, this difference disappeared (Table 1), suggesting that the initial observation was not due to an independent effect of this LMP7 polymorphism on IDDM susceptibility, but rather reflected a difference in the distribution of DR4 subtypes among patients and control subjects.

We found a tendency for an increase of the LMP2*R/R and a decrease of the LMP2*R/H genotype among DRB1*03-DQA1*0501-DQB1*0201/DRB1*0404-DQA1*03-DQB1*0302 heterozygous IDDM patients in comparison with control subjects (Table 1; *P*nc = 0.03 and *P*nc = 0.02, respectively). Similarly, we found a decrease of the LMP7*A/B genotype among DRB1*08-DQB1*0302/DRB1*04-DQB1*0302 heterozygous IDDM patients in comparison with control subjects before stratifying for DRB1*04 subtype (*P*nc = 0.03). However, these differences did not reach statistical significance after correction for number of comparisons. The latter difference (concerning LMP7) was not evident when stratifying for DR4 subtypes (though the numbers involved in these comparisons were small). Further, the differences observed for LMP2 were in a different direction than was found by Deng et al. (20), who observed an increase of the LMP2*H allele on DRB1*04-DQA1*03-DQB1*0302–positive haplotypes. Hence, we believe these differences were random and not indicative of LMP2 or LMP7 polymorphisms being independently associated with IDDM susceptibility.

The data concerning LMP2 are also supported by previous publications. Van Endert et al. (21) also analyzed the LMP2 polymorphism at position 60 in DR3-DQ2/DR4-DQ8 Danish IDDM patients compared with HLA-DR and -DQ matched Danish control subjects. Even though the numbers studied were small, those authors found no evidence for an independent association. Kawaguchi et al. (22) studied the same LMP2 polymorphism in Japanese IDDM patients and control subjects, and again found no evidence for an independent association. None of these latter studies, however, addressed the LMP7 polymorphism reported in our study.

The TDT analysis (Tables 3 and 4) from DRB1*04-DQA1*03-DQB1*0302 and DRB1*0301-DQA1*0501-DQB1*0201 homozygous parents confirmed the results of our case-control analysis, showing no evidence of transmission distortion of either LMP7 or LMP2 alleles to affected offspring. Thus, our data would argue against a primary role for any of these polymorphisms in IDDM susceptibility.

Neither all possible genotypes nor all haplotypes were analyzed in the present study. Hence the possibility exists that these LMP polymorphisms may confer additional susceptibility to IDDM on other HLA haplotypes. To address this will require very large data sets of IDDM patients to obtain sufficient numbers of such genotypes/haplotypes.

Some of the different results obtained in our study compared with the study by Deng et al. may relate to the fact that the latter investigators did not take DR4 subtypes into account when matching patient and control subject haplotypes. Another explanation for the different results obtained is that Deng et al.'s group, drawn from the U.S. population, was genetically heterogeneous, making artifact associations attributable to population admixture likely. Such population stratification effects are caused by sometimes subtle ethnic differences between cases and control subjects (24). In contrast to the United States, Norway is a much more genetically homogeneous population.

It is of course possible that there were real ethnic differences between our population and that studied by Deng et al., and that the LMP polymorphisms may only confer additional susceptibility in certain ethnic groups. However, Deng et al. studied predominantly U.S. citizens of Northern European ancestry (20), which should be ethnically similar to our Norwegian population. Therefore, we believe this is an unlikely explanation.

Our data for DR4 subtypes and their different linkage disequilibriums with the LMP7 polymorphism clearly demonstrated that a complete matching for DRB1, DQA1, and DQB1 alleles is essential for reporting independent associations of IDDM to alleles at other HLA complex loci. The recent history concerning the polymorphisms of the TAP genes, where one early study claimed an independent association (25), a claim that today must be considered to have been disproved (15–17,21), highlights how easily it can be to believe that polymorphisms in the HLA class II region have independent effects on IDDM susceptibility. This emphasizes the need for exercising caution and careful data interpretation before claiming independent effects.

In conclusion, our data argue against any independent effects of polymorphisms within the antigen processing genes LMP2 and LMP7 on IDDM susceptibility.

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