

Non-Class II HLA Gene Associated With Type 1 Diabetes Maps to the 240-kb Region Near *HLA-B*

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Several studies provide evidence that in addition to the *DQ-DR* genes, HLA contains another uncharacterized gene or genes associated with type 1 diabetes. Our aim was to investigate the effect of this gene independently of the *DQ-DR* genes and to localize it with a matched case-control study. More than 1,400 patients and 30,000 control individuals from Finland were studied. They were first genotyped for the selected alleles of the *HLA-DQB1*, *-DQA1*, and *-DRB1* genes. For the *DR3/4(0404)* genotype, 75 patients and 181 control subjects were stratified, and 241 patients and 354 controls were stratified for the *DR3/4(0401)* genotype. Ten microsatellite markers in the HLA class III and I regions (D6S273, TNFa, C12A, STR MICA, MIB, C125, C143, C245, C3211, and MOGc) and selected alleles of the *HLA-A* and *HLA-B* genes were studied. In the *DR3/4(0404)*-stratified group, we found that markers located between C12A and C143 near the *HLA-B* gene confer a strong additional diabetes association. This was confirmed by the population differentiation test in both *DR3/4(0404)*- and *DR3/4(0401)*-stratified groups. Our data indicate that an additional gene associated with type 1 diabetes is located in the 240-kb region near *HLA-B*. We excluded STR MICA polymorphism as a mutation responsible for diabetes association. *Diabetes* 49:2217–2221, 2000

HLA localization of the genes predisposing to type 1 diabetes is well established. *DQB1*, *DQA1*, and *DRB1* genes are considered primary and major loci associated with type 1 diabetes. However, evidence is accumulating that the HLA class I–III region contains gene(s) associated with an increased risk

for type 1 diabetes (1–4) or its earlier age at onset (5–8). We searched for an additional HLA gene associated with type 1 diabetes among Finnish patients and control subjects stratified for the *DQ-DR* alleles.

Initially, samples from >1,400 type 1 diabetic patients and >30,000 control subjects were collected and genotyped for *DQB1* (9). *DQB1**02/0302 subjects were then studied for the *DQA1**05 and *0201 alleles and *DR4* subtypes (of which only *DRB1**0401 and *DRB1**0404 are frequent among Finns). Thus, a group stratified for the *DQA1**05-*DQB1**02/*DQB1**0302-*DRB1**0404 [*DR3/4(0404)*] genotype (75 patients and 181 control subjects) and a group stratified for the *DQA1**05-*DQB1**02/*DQB1**0302-*DRB1**0401 [*DR3/4(0401)*] genotype (241 patients and 354 control subjects) were selected. These subjects are all *DR3/DR4* haplotype heterozygotes: one group carries *DR404* and the other *DR401* haplotype. Thus, within each group we excluded the diabetogenic effect of the known *DQ-DR* markers.

In these groups, selected alleles of the *HLA-B* and *HLA-A* genes and 10 microsatellites (D6S273, TNFa, C12A, STR MICA, MIB, C125, C143, C245, C3211, and MOGc) were studied to map a non-class II HLA gene associated with type 1 diabetes susceptibility. These cover distance of ~2,300 kb and include the whole HLA class I region and the class I–III boundary (Fig. 1).

We found that in the *DR3/404*- and *DR3/401*-stratified groups there was only one allele at each microsatellite locus present in the majority of both patients and control subjects that did not confer any risk or protection (Table 1). Because *DR3* haplotype is present in both *DR3/404*- and *DR3/401*-stratified groups, these alleles were supposed to represent the extended *DR3* haplotype: *DQB1**02-*DQA1**05-D6S273*141-TNFa*2-C12A*256-STR MICA*A5.1-MIB*350-C125*194-C143*449-C245*436-C3211*217. This was confirmed by the study of parents of 20 randomly selected *DR3/404*⁺ and 20 *DR3/401*⁺ type 1 diabetic patients, in which the direct study of haplotypes was possible (data not shown). This finding implies that in the present study, the *DR3* haplotype does not contain any additional disease-associated marker and could be explained by high homogeneity of the *DR3* haplotype in the Finnish population.

In the *DR3/404*-stratified group, we confirmed strong diabetes association of *B39*, previously shown by us (3,4), and of *A24*, shown by others (7,8). There was only one allele at each microsatellite locus associated with an increased risk for

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Abbreviations: OR, odds ratio; PCR, polymerase chain reaction.

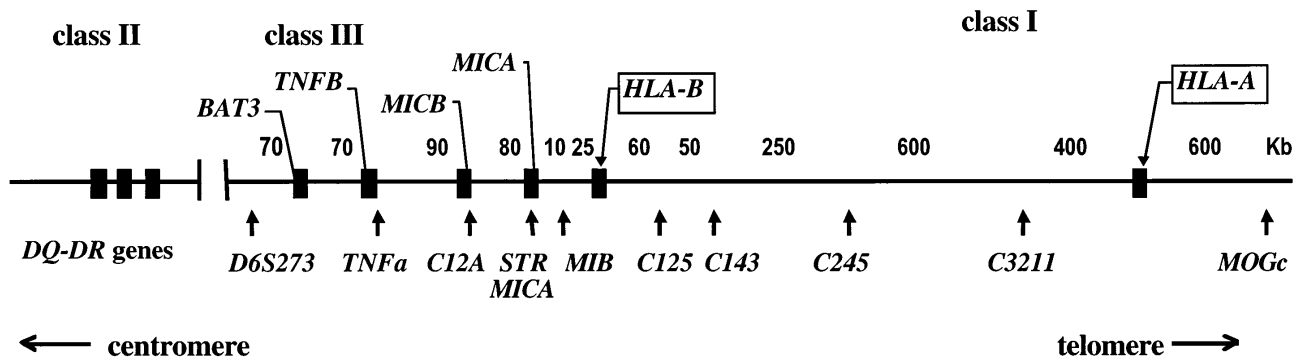


FIG. 1. Markers in the HLA region. Approximate distances (kb) between markers are shown above. ■, genes; ↑, microsatellites.

type 1 diabetes, and all of these alleles, as well as A24, were strongly associated with B39 (Table 1). None of the other alleles of the microsatellite markers studied were found to be associated with B39 (data not shown), and none were significantly increased among patients (Table 1). Based on these data and haplotype analysis in parents of 20 patients with the *DR3/404* genotype selected randomly, we reconstructed a haplotype, DQB1*0302-DRB1*0404-D6S273*137-TNF α *6-C12A*250-STR MICA*A9-MIB*332-B39-C125*200-C143*425-C245*436-C3211*207-A24-MOGc*130. This *DR404* haplotype is associated with an increased risk for type 1 diabetes, and disease risk conferred by the *DQ-DR* segment of this haplotype is increased by alleles of the D6S273-MOGc segment.

Two tests were applied to map the region of the strongest type 1 diabetes association within the D6S273-MOGc segment, which should contain the disease-predisposing gene. First, odds ratios (ORs) for the strongest associated allele of the above-described haplotype were compared (Table 1 and Fig. 2A). None of the markers were associated with higher risk than the HLA-B39 allele. Despite the fact that the 95% CIs were overlapping in all comparisons, associated alleles in the region between the C12A and C143 markers conferred higher risk than the alleles of the other markers, which suggests that the type 1 diabetes-associated gene is located in this area. Statistical evidence for this hypothesis was obtained by the population differentiation test. Thus, comparison of allele distribution in the *DR3/404*-stratified group reveals that the patient population is different from control population at the C12A, STR MICA, C125, and C143 markers, again emphasizing the importance of the region between the C12A and C143 loci (Table 2 and Fig. 2B).

In the *DR3/401*-stratified group of patients and control subjects, no single allele was associated with the significant increase or decrease of risk conferred by the *DQ-DR* markers (Fig. 3A). However, results of the population differentiation test in the *DR3/401*-stratified group demonstrate that the population of patients is different from the background population by allele distribution at the MIB locus. The test result is also very close to the significance threshold at the C12A locus (Table 2 and Fig. 3B). It is remarkable that these loci are located within the region suggested as candidate in the study of the *DR3/404*-stratified group.

The present study does not support an idea that the polymorphism of the transmembrane region of the MICA protein (STR MICA) could itself be responsible for diabetes associ-

ation. Indeed, in the *DR3/404*-stratified group, STR MICA*A9 confers high risk for type 1 diabetes (OR 4.0, 95% CI 2.1–7.6), whereas in the *DR3/401*-stratified group the same polymorphism confers significantly ($\chi^2 = 12.0$, 1 df, $P < 0.0006$) lower risk (OR 1.03, 95% CI 0.6–1.7).

Earlier studies demonstrate that the type 1 diabetes gene in the HLA class I–III regions is associated with an earlier age at onset (5–8). Hence, it is important to show that the putative polymorphism, which we map near HLA-B, confers not only an increased risk for type 1 diabetes, but also an earlier age at onset. This was tested in the group of *DR3/404*-stratified type 1 diabetic patients. The frequency of B39 among patients that were diagnosed at 10 years of age or earlier was 19 of 40 (47.5%), whereas it was 7 of 22 (31.8%) among those diagnosed at 11 years of age or later ($P = 0.18$, OR = 1.9). The difference was significant in the group of patients diagnosed between 5 and 9 years of age (9 of 13 [69.2%] vs. 17 of 49 [34.7%] diagnosed in the other age groups [$P < 0.03$, OR 4.2]). Interestingly, in the study of Japanese patients, A24 was mostly increased in the group of patients diagnosed between the ages of 6 and 12 years but not in the youngest age group (8). In the present study, non-class II HLA markers of the *DR404* haplotype, D6S273*137-TNF α *6-C12A*250-STR MICA*A9-MIB*332-B39-C125*200-C143*425-C245*436-C3211*207-A24-MOGc*130, had similar but lower effects on the age at onset than B39 (data not shown). Thus, we suggest that association of markers in HLA class I–III regions with young age at onset of type 1 diabetes is a consequence of linkage disequilibrium between these markers and the putative polymorphism located in the region near HLA-B.

Several attempts to map a non-class II HLA gene associated with type 1 diabetes have been done so far. At first, by analyzing the *DR3-B8* and *DR3-B18* haplotypes, it was predicted that the region between HLA-B and BAT3 should contain a gene associated with type 1 diabetes (10). This study is in line with our results, because suggested regions overlap between C12A and HLA-B (Fig. 1). Another study suggests that the non-class II HLA gene of type 1 diabetes susceptibility is located between D6S273 and TNF α markers (11). However, microsatellite D6S265, which was used as a marker next to TNF α is located ~1.3 Mb telomerically. Because no markers were tested between these two markers, to our mind, the telomeric boundary of the candidate region could not be specified exactly. Finally, a non-class II HLA gene associated with

TABLE 1
Frequency (%) of the HLA markers among type 1 diabetic patients and control subjects stratified for the *DR3/404* genotype

Marker*allele	Patients (n = 75)	Control subjects (n = 181)	OR	95% CI	P	P cor
D6S273*135	25.3	42.5	0.5	0.2–0.9	0.01	NS
*137†	74.7	58.0	2.1	1.1–4.1	0.02	NS
*141‡	86.7	76.2	2.0	0.9–4.6	NS	
TNFA*2‡	88.0	82.9	1.5	0.6–3.6	NS	
*6§	50.7	34.3	2.0	1.1–3.5	0.02	NS
*11	38.7	48.1	0.7	0.4–1.2	NS	
C12A*236	12.0	28.7	0.3	0.15–0.8	0.007	NS
*250§	49.3	19.3	4.1	2.2–7.6	0.000002	<0.0003
*256‡	81.3	76.2	1.4	0.7–2.8	NS	
MICA*A4	12.0	23.2	0.5	0.2–1.0	NS	
*A5.1‡	93.3	93.4	1.0	0.3–3.4	NS	
*A9§	45.3	17.1	4.0	2.1–7.6	0.000005	<0.0007
MIB*326	10.7	17.7	0.6	0.2–1.3	NS	
*332§	41.3	15.5	3.8	2.0–7.4	0.00002	0.002
*350‡	86.7	90.6	0.7	0.3–1.7	NS	
HLA-B*39§	41.3	12.7	4.8	2.5–9.6	0.000001	<0.0002
C125*194‡	78.7	74.6	1.3	0.6–2.5	NS	
*200§	41.3	14.9	4.0	2.1–7.8	0.000009	<0.002
*210	29.3	33.7	0.8	0.4–1.5	NS	
C143*425§	37.3	12.7	4.1	2.1–8.2	<0.00002	<0.002
*445	37.3	49.2	0.6	0.3–1.1	NS	
*449‡	72.0	70.2	1.1	0.6–2.1	NS	
C245*428	24.0	26.5	0.9	0.4–1.7	NS	
*436‡	90.7	80.7	2.3	0.93–6.1	NS	
*476	20.0	33.7	0.5	0.2–1.0	0.04	NS
*436/436§	36.0	21.0	2.1	1.1–4.0	<0.02	NS
C3211*207§	49.3	23.8	3.1	1.7–5.7	0.0001	<0.02
*211	21.3	35.4	0.5	0.3–1.0	0.04	NS
*217‡	58.7	61.3	0.9	0.5–1.6	NS	
HLA-A*24§	38.7	19.3	2.6	1.4–5.0	<0.002	NS
MOGc*130†	66.7	51.9	1.9	1.0–3.4	0.04	NS
*132	42.7	49.2	0.8	0.4–1.4	NS	
*148	42.7	41.4	1.1	0.6–1.9	NS	

Generally referred allele names were used for the *TNFA* and *STR MICA* microsatellites. For the other microsatellite loci, allele number corresponds to the length of the PCR product (exactly estimated by sequencing of homozygous samples). Up to three of the most common alleles of each microsatellite marker are shown. *P* cor, *P* corrected ($P \times 123$). †Allele associated with the DR404-B39 haplotype ($P < 0.0001$ for association with B39); ‡alleles associated with the DR3 haplotype; §alleles associated with the DR404-B39 haplotype ($P < 0.00000001$ for association with B39).

type 1 diabetes was mapped near D6S2223 (12), ~2.5 Mb telomeric to MOGc. Our data demonstrate that the effect seen by us is already weak at MOGc and hardly extend to D6S2223. The most probable explanation of these findings is the existence of at least three loci of type 1 diabetes susceptibility in HLA. One of them is located at the HLA class II region and consists of the *DQB1-DQA1-DRB1* genes. Another maps to the centromeric end of the class I region near *HLA-B*, whereas the third gene is located at the region flanking the HLA complex, telomeric to the class I region. This hypothesis is supported by structure of the *Idd1* locus in the NOD mice (13).

The 240-kb region mapped in the present study between *C12A* and *C143* includes the well-known *HLA-B*, *HLA-C*, *MICA*, and *MICB* genes, as well as less-characterized *P5-1* and *3.8-1.1* genes (14). Markers located in this region were associated with several autoimmune diseases (15–17). Future studies of the variants affecting protein sequence or expres-

sion of the genes in the mapped 240-kb region will identify the responsible polymorphism(s) associated with type 1 diabetes and other autoimmune diseases.

RESEARCH DESIGN AND METHODS

Subjects. Type 1 diabetic patients were recruited in Finland (mostly in Turku, Oulu, Helsinki, and Tampere) and were diagnosed before the age of 16 years. Samples from newborns were collected in Turku, Oulu, and Tampere as part of the Diabetes Prediction and Prevention project and were used as control subjects. All control subjects were tested for islet cell antibodies and positives were excluded from the analysis. The study was approved by the ethical committees of the participating hospitals. Informed consent was obtained from the participating subjects and/or their parents.

Genotyping. Samples were collected as EDTA blood, and dried blood spots of 50 μ l were prepared for screening of six DQB1 alleles, including *02 and *0302 (9), DR4 subtypes and DQA1*05 and *0201 alleles. The *DR3/4*-stratified groups were selected because this genotype is the most common among the Finnish type 1 diabetic patients and, hence, more class II-stratified patients were available. DNA was extracted using a Quiagen DNA extraction kit from the EDTA blood of the *DR3/4*⁺ subjects. The *DR404* haplotype was studied, because our previous observations demonstrate that the *HLA-B39* allele was

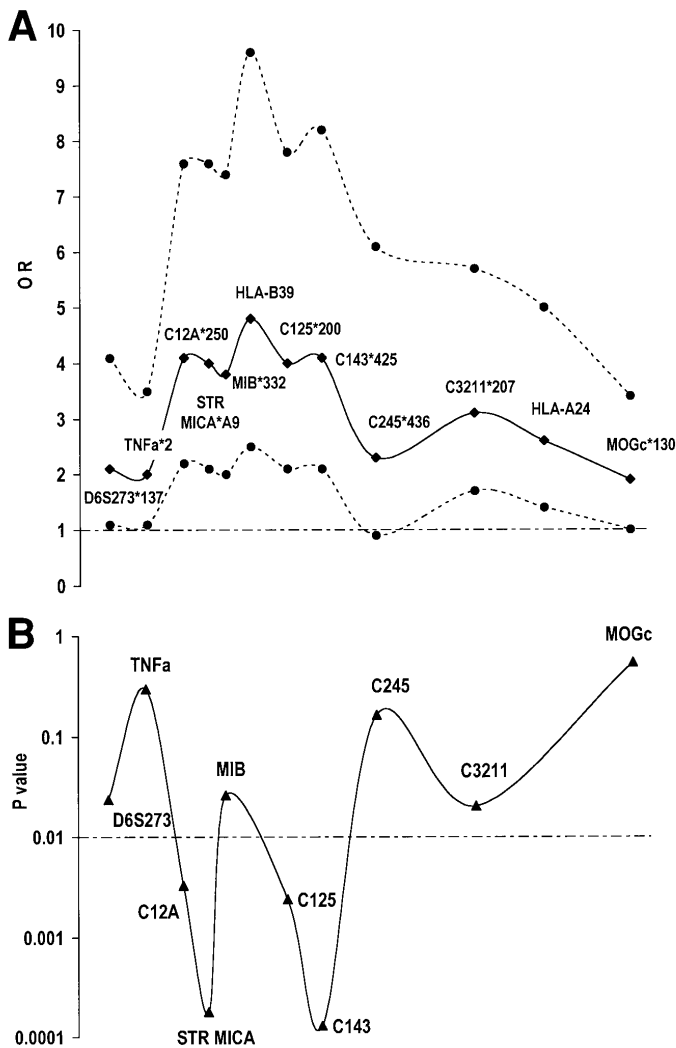


FIG. 2. ORs for the strongest associated allele (A) and the population differentiation test (B) in the *DR3/404*-stratified group. ◆, OR; ●, 95% CI for OR; ▲, *P* value for population differentiation test; —, significance threshold.

associated with a conspicuously high risk on this haplotype (3,4). At the same time, it was shown that *B39* is in linkage disequilibrium with the *HLA-A24* allele on the *DR404* haplotype (4). Our previous studies failed to identify any *HLA-B* alleles that could modify the disease risk in the *DR401* haplotype. However, the study of this haplotype also seems important, both for confirmation and fine mapping through the overlapping of the candidate regions on both haplotypes. *B62* and *A2*, the most frequent alleles of class I genes in linkage disequilibrium with *DR401* in the Finnish population (4), were also studied. So, the prevalence of *A24* and *B39* among *DR3/404*⁺ subjects and of *A2* and *B62* among *DR3/401*⁺ subjects was tested by means of the sequence-specific polymerase chain reaction (PCR) (18). Microsatellite markers were studied using PCR primers (19), labeled with fluorescent dyes, ABI 310 electrophoresis, and the Genescan software.

Statistical analysis. Allele frequencies were estimated by direct counting. χ^2 test or Fisher's exact test when appropriate were used for the calculation of statistical significance, and the level was set at $P < 0.05$. Bonferroni correction was done to correct *P* values for multiple comparisons; *P* cor is then presented. ORs were calculated according to the Woolf's formula. The Mantel-Haenszel procedure was used to test homogeneity of ORs. The Epistat program was used for calculations of these, as well as 95% CI. Population differentiation (genic) test was performed by the Genepop software package (20) with the following initial parameters: 1,000 steps of dememorization and 1,000 batches with 10,000 iterations per batch. Population differentiation test is an exact test that has an advantage of not being biased by rare alleles and

TABLE 2

Population differentiation test for comparison of type 1 diabetic patients ($n = 75$) and control subjects ($n = 181$), stratified for the *DR3/404* genotype, as well as type 1 diabetic patients ($n = 241$) and control subjects ($n = 354$), stratified for the *DR3/401* genotype

Locus	<i>DR3/404</i> -stratified group		<i>DR3/401</i> -stratified group	
	<i>P</i>	SE	<i>P</i>	SE
D6S273	0.02368	0.00040	0.09557	0.00114
TNFA	0.29543	0.00166	0.65175	0.00178
C12A	0.00326	0.00013	0.01373	0.00041
STR MICA	0.00018	0.00002	0.54007	0.00195
MIB	0.02614	0.00047	0.00463	0.00020
C125	0.00239	0.00013	0.56609	0.00229
C143	0.00013	0.00002	0.46173	0.00242
C245	0.16287	0.00117	0.27557	0.00239
C3211	0.02037	0.00042	0.14022	0.00189
MOGc	0.53826	0.00163	0.25469	0.00236

Significant *P* values of the population differentiation test (assuming $P < 0.01$ threshold) are bold. SE, standard error.

small sample size (20). Values of $P < 0.01$ were accepted as a significance threshold for rejecting the null hypothesis of the allelic distribution identity between the population of type 1 diabetic patients and the control population. Because genotyping of *HLA-A* and *HLA-B* genes was not "full-house," only microsatellite loci were analyzed by the population differentiation test.

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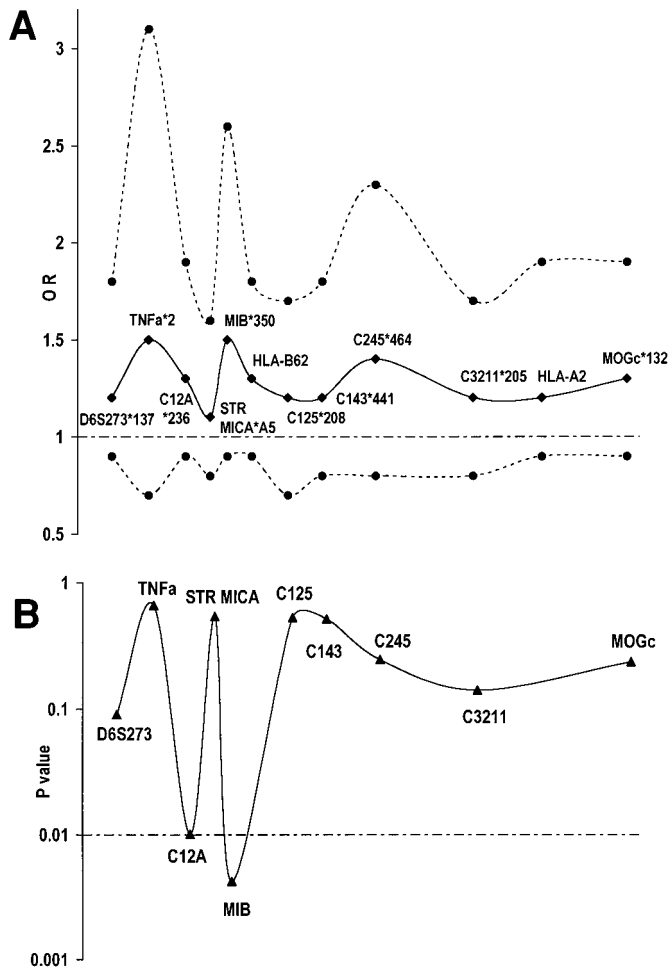


FIG. 3. ORs for the strongest associated allele (A) and the population differentiation test (B) in the DR3/401-stratified group. ◆, OR; ●, 95% CI for OR; ▲, P value for population differentiation test; —, significance threshold.

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