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SHORT COMMUNICATION

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*D6S265*15* marks a *DRB1*15, DQB1*0602* haplotype associated with attenuated protection from type 1 diabetes mellitus

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Abstract *Aims/hypothesis:* The HLA class II *DQB1*0602* allele confers strong dominant protection against type 1 diabetes but protection is not absolute. The aim of this study was to identify markers within the HLA region that differentiate *DQB1*0602* haplotypes and show different associations with disease risk. *Methods:* We defined alleles at eight microsatellite markers spanning the HLA region in a case-control cohort from Sweden. *Results:* We found that allele 15 at marker *D6S265* (109 kb centromeric of *HLA-A*) was overrepresented among patients carrying *DRB1*15, DQB1*0602*. A detailed haplotype analysis showed that *DRB1*15, DQB1*0602* haplotypes carrying *D6S265*15* have a tenfold higher odds ratio (OR) than those carrying other alleles and thus confer reduced protection [OR *D6S265*15*+=0.186 (95% CI 0.074, 0.472) vs OR *D6S265*15*−=0.017 (95% CI 0.005, 0.062), $p<0.001$]. *Conclusions/interpretation:* Our data support the existence of a locus that modifies the protective effect associated with *DQB1*0602*. Typing for allele *D6S265*15* can identify a less protective *DQB1*0602* haplotype, thereby allowing a more accurate prediction of type 1 diabetes risk.

Keywords *D6S265* · *DQB1*0602* · *HLA-DQ* antigens · IDDM · Type 1 diabetes mellitus

Abbreviations SSP: sequence-specific primers · SSOP: sequence-specific oligonucleotide probes

Introduction

Among four common *HLA-DR2* haplotypes observed in Caucasians, the *DQA1*0102, DQB1*0602, DRB1*15* hap-

★ For full lists of members of these study groups, see Electronic Supplementary Material

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lotype is negatively associated with type 1diabetes and is extremely rare among patients in most populations studied (reviewed in [1]). The available evidence suggests that the diabetes-protective effect associated with *DR2* haplotypes may mostly map to the *HLA-DQ* locus and in particular to the *DQB1*0602* allele. Protection appears to be dominant since *DQB1*0602* protects from diabetes even in the presence of high-risk HLA alleles. It also confers dominant protection in autoantibody-positive first-degree relatives of affected individuals, who usually express fewer autoantibodies than relatives without *DQB1*0602* [2]. Although diabetic patients carrying *DRB1*15, DQB1*0602* are extremely rare, their existence indicates that the protective effect of this haplotype is not absolute. Sequence analysis of rare patients and autoantibody-positive first-degree relatives with *DQB1*0602* have demonstrated that they carry normal alleles lacking any mutations that would affect the peptide binding site [2, 3].

In a cohort of patients from Sweden, a country with one of the highest incidences of type 1 diabetes, the *DQA1*0102, DQB1*0602, DRB1*15* haplotype was found in 1% of patients. Most of these patients (87.5%) belonged to an older age group (14–34 years old) [3, 4], suggesting that the protection associated with this haplotype may attenuate with age in this population. Many studies have indicated that genes in the HLA region other than *DR* and *DQ* also contribute to diabetes susceptibility. It is possible that rare patients with *DQB1*0602* carry a haplotype in which the protective effect of *DQB1*0602* is overridden by another gene. If this is the case, it should be possible to identify markers that identify *DQB1*0602* haplotypes with different associations with diabetes risk.

As part of the activities of the 13th International Histocompatibility Working Group we genotyped, in the Swedish case-control cohort described above, the *DRB1* and *DQB1* alleles, as well as eight microsatellite markers spanning the HLA complex, from *D6S291* and mapping beyond the *DPB1* gene to *D6S2239*, which is located near the haemochromatosis locus. Using this approach, we tested the hypothesis that microsatellite markers could help identify less protective *DQB1*0602* haplotypes and help map additional loci that modify diabetes risk.

Subjects, materials and methods

Swedish case-control study The population-based matched case-control study used here has been described elsewhere [3, 4]. Patients were diagnosed with type 1 diabetes according to the World Health Organization criteria. Two main age groups were defined in this study: the first group included patients who were younger than 15 years at the time of diagnosis, which lay between 1 September 1986 and 31 December 1987. Matched control subjects were children of the same sex, born on the same day and in the same county as the index case [5]. The second group comprised patients who were aged 15 to 34 years at diagnosis and developed diabetes between 1 January 1987 and 31 December 1988; matched control subjects were also ascertained. The combined data set includes a total of 971 type 1 diabetic patients and 702 control subjects. Of the diabetic patients with *DQB1*0602*, eight had sufficient DNA available and were included in this analysis. In these eight patients, the mean age at diagnosis was 23.1 years (SD=8.4, range 11–34). Information about the Swedish case-control study is available at the Multi-locus Age-dependent Genetic Effects On Type 1 Diabetes website (http://stat-db.stat.sfu.ca/magenta/). In total, data from this study included 552 patients and 433 control subjects with *HLA DR-DQ* and microsatellite marker genotypes. All subjects provided informed consent. The Ethics Committee at the Karolinska Institute, Stockholm, Sweden, ap-

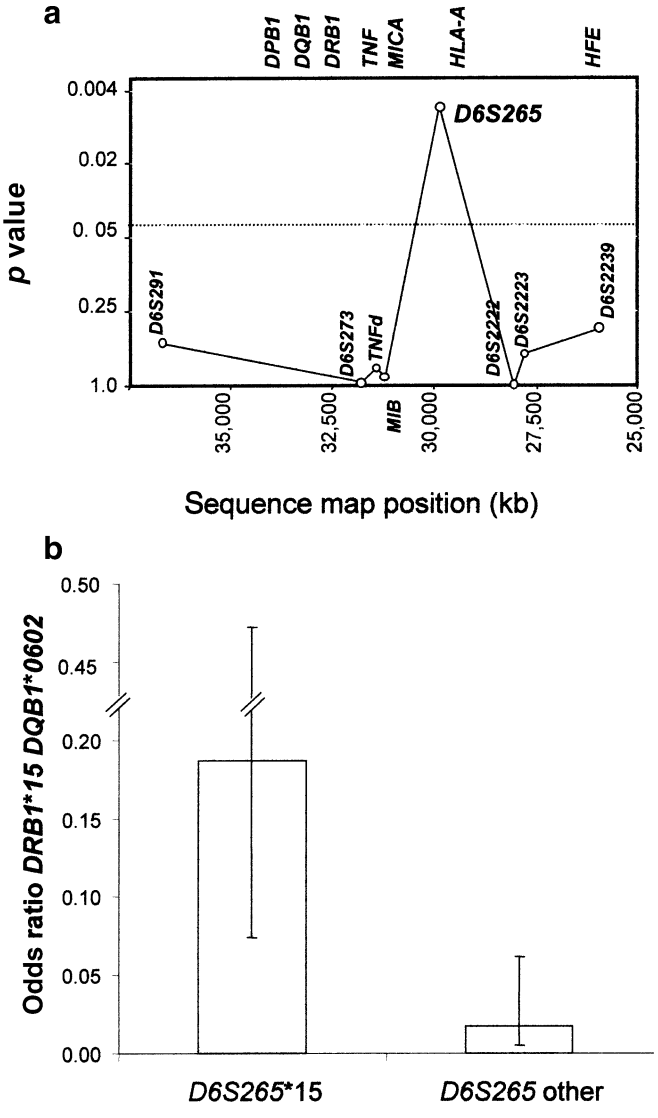

Fig. 1 **a** Sequence map position of the markers genotyped, relative to the position of some HLA genes. The *p* value (log 10 scale) was derived by comparing the allele frequencies between patients and control subjects at eight microsatellite markers in the HLA complex region among subjects that carried one or two copies of the *DRB1*15 DQB1*0602* haplotype. Source of sequence map position: http://www.ncbi.nlm.nih.gov/mapview. **b** Odds ratio for type 1 diabetes of *DRB1*15 DQB1*0602* haplotypes according to allelic variation at the *D6S265* locus in the Swedish case-control study

proved the study, which was carried out in accordance with the principles of the Declaration of Helsinki.

HLA genotyping methods Molecular HLA typing data were generated by previously described PCR methods using sequence-specific primers (SSP), RFLP and sequence-specific oligonucleotide probes (SSOP) [3, 6]. We did not identify subjects carrying *DQB1*0602* in cis with *DRB1*11*, *13* or *14*. Thus, all *DQB1*0602*-positive subjects identified carried *DRB1*1501*. The presence of *DQB1*0602* was confirmed by PCR-SSP/RFLP and PCR-SSOP methods, both of which clearly distinguish *DQB1*0602* from *DQB1*0603*.

Microsatellite genotyping DNA fragments containing the polymorphic sequences were amplified independently using the PCR primers listed below. For each marker one of the primers was labelled with a fluorescent dye. The primer sequences and dyes used were: *D6S291*: 5′:gtttc ttggggatgacgaattattcactaact, 3′:*ggcattcaggcatgcctggc, dye used FAM; *D6S273* 5′:*gcaacttttctgtcaatcca, 3′:gtttcttacca aacttcaaattttcgg, dye used FAM ; *TNFd*: 5′:*catagtgggact ctgtctccaaag, 3′:gtttcttagatccttccctgtgagttctgct, dye used NED; *MIB* 5′:gtttcttctaccatgacccccttcccc 3′:*ccacagtctctat cagtcca, dye used HEX; *D6S265* 5′:*acgttcgtacccattaacct 3′:gtttcttatcgaggtaaacagcagaaa, dye used HEX; *D6S2222*: 5′:*agtcatctgaagagttgg, 3′:gtttttcttgcatgtcttctttgttaagg, dye used NED; *D6S2223*: 5′:gtttcttaataatgttaagtaacaaactagagtac, 3′:*actcaagcctgggcaatagagc, dye used HEX; and for *D6S 2239:* 5′:*gttggaagcaatggattagatgtcc, 3′:gtttcttctacctgccag gaacaatatacac, dye used FAM; * indicates the fluorescently labelled primer. Dye-labelled fragments were separated according to size on 96-capillary sequencers (MegaBACE 1000; Amersham Biosciences, Piscataway, NJ, USA). Genotypes were called by using the Genetic Profiler software (version 1.1; Amersham Biosciences,) applied to the raw MegaBACE data. Microsatellite alleles were defined by fragment size analysis in a reference panel of 50 cell lines from the International Histocompatibility Working Group (http://www.ihwg.org). Alleles were given a numerical value designated as the normalised fragment index and based on a ranking of relative fragment size ranging from smallest to largest. The *D6S265*15* allele corresponds to a cytosine-adenine (ca) core repeat motif (15 repeats) (http://www.ihwg.org/shared/micros.htm).

Haplotype estimation and statistical methods Haplotype frequencies were computed on the basis of the probabilities for all possible haplotype phases for each individual, which were assessed using the Bayesian algorithm implemented by phase [7]. A permutation test weighting the probability of each haplotype assignment was used to compare haplotype frequencies. Allele and haplotype frequencies between groups were compared using a Pearson's chi square test.

Results

We first compared the case and control allele frequencies of all markers among individuals who carried one or two copies of the *DRB1*15, DQB1*0602* haplotype (Fig. 1a). The only significant difference was found at marker *D6S265* ($p<0.005$). The difference was significant after correcting for multiple comparisons ($p<0.045$). One allele in particular was found to be more common in *DRB1*15, DQB1*0602* carriers who had type 1 diabetes cases than in control subjects: thus 75% of the *DRB1*15, DQB1*0602* carrier patients had allele *D6S265*15* compared with 47% of control subjects. However, given the small sample size, this difference is not statistically significant (χ^2 [1 df]=2.40). We then estimated *DRB1*, *DQB1*, *D6S265* haplotype

Table 1 *DRB1*15 DQB1*0602 D6S265* haplotypes in Swedish type 1 diabetes cases and control subjects

Microsatellite (NFI)[a]	Estimated frequency of *DR15, DQB1*0602* haplotypes with *D6S265* alleles[b]				Haplotype counts (using the most likely haplotype assignment[b])	
	Overall frequency		As % of *DRB1*15 DQB1*0602*			
D6S265	Controls	Cases	Controls	Cases	Controls	Cases
11	2.9%	0.1%	21.9%	15.3%	28	1
12	0.0%	0.07%	0.0%	10.8%	0	1
13	4.4%	0.04%	31.5%	2.5%	39	0
14	1.2%	0.0%	7.9%	0.0%	9	0
15	2.9%	0.5%	19.4%	71.8%	22	6
16	2.5%	0.0%	18.1%	0.0%	22	0
19	0.04%	0.0%	0.30%	0.0%	1	0
n	866	1104	121	8	121	8

[a]Microsatellite alleles were given a numerical value designated as the normalised fragment index based on a rank order of relative fragment size ranging from smallest to largest. The *D6S265*15* allele corresponds to cytosine–adenine (ca) core repeat motif (15 repeats) (http://www.ihwg.org/shared/micros.htm)

[b]Haplotype frequency estimates reflect all possible haplotype phase assignments for every sample. Haplotype counts assign the pair of haplotypes with the highest likelihood to each individual. Only individuals who are homozygous at one or both loci have a probability of 1.0 for a given haplotype assignment. In all other cases, there is a degree of uncertainty. Therefore other possible haplotype assignments with a non-zero probability exist. These are weighted and included in the frequency estimates, but not in haplotype assignment columns [7]. Thus, the haplotype counts given in the far right columns do not correspond to the percentages

frequencies in the patient and control sets (Table 1). A comparison of the haplotype frequency distribution of *D6S265*15* between patients and control subjects who were carriers of *DRB1*15, DQB1*0602* haplotypes resulted in a Pearson's chi square value of 10.69 (1 df) ($p<0.001$); a Fisher's exact test on the same contingency table yielded $p=0.0013$. Moreover, the haplotype odds ratios (OR) are significantly higher, namely ten-fold higher for *DRB1*15, DQB1*0602* haplotypes that carry allele *D6S265*15* than for those carrying other alleles at this marker (Fig. 1b). Thus, we have identified a locus that marks *DQB1*0602* haplotypes associated with increased risk of type 1 diabetes.

Discussion

Previous investigations had identified very rare type 1 diabetic patients who carried normal *DQB1*0602* sequences, and thus suggested that the protective effect associated with *DRB1*15, DQB1*0602* haplotype is remarkably strong, but not absolute [2, 8]. We have identified a less protective *DRB1*15, DQB1*0602* haplotype in a population-based case-control study of patients from Sweden. This haplotype is marked by allele 15 at the *D6 S265* locus. The finding that allelic variation at this locus can override the dramatic protective effect associated with *DQB1*0602* provides further evidence that this locus or a locus nearby may have an important effect on type 1 diabetes risk. It will be important to assess whether polymorphisms at *D6S265* mark less protective haplotypes in populations with different *DRB1*15, DQB1*0602* frequencies.

Marker D6S265 maps 100 kb telomeric of *HLA-A*, which has been previously associated with diabetes susceptibility [9]. However, earlier studies dealt mainly with class I alleles, which influence the disease susceptibility associated with the *DRB1* and *DQB1* predisposing haplotypes such as *DR3* or *DR4*. Molecular typing of the *HLA-A* locus will be necessary to determine whether the influence of *D6S265* on the risk associated with the *DRB1*15 DQB1*0602* haplotype is independent of the class I *HLA-A* locus. However, associations between *D6S265* and other autoimmune diseases have been reported (see references in [10]). Of particular interest is the observation by Harbo and coworkers [10] of an association between susceptibility to multiple sclerosis and *D6S265* specifically on *DRB1*15, DQB1*0602* haplotypes. Taken together, their data and our findings indicate that genetic variation at *D6S265* can influence or is linked to a locus that can influence susceptibility to or protection from the autoimmunity conferred by *DRB1*15, DQB1*0602* haplotypes. Known genes that may be marked by *D6S265* include *HLA-A*, *HLA-B*, *MICA*, *TNF* and *BAT1*. Polymorphisms at these loci may have important effects on the function of cytotoxic T cells and cytokine secretion. Moreover, possible effects on transcriptional regulation may perhaps influence the expression of the HLA-DQ molecule encoded by *DQA1*0102, DQB1*0602*. Further characterisation of this region will be needed to identify the loci that contribute to the genetic protection from type 1 diabetes conferred by *DRB1*15, DQB1*0602*. This may also help understand the mechanisms preceding the onset of the disease.

In conclusion, our data identify a region of potential interest and provide a marker that helps identify less protective *DQB1*0602* haplotypes. The ability to identify *DQB1*0602* haplotypes with attenuated protective effects is relevant to the design of prevention studies. Currently, first-degree relatives of type 1 diabetic patients who are screened for prevention studies and found to carry *DQB1*0602* are considered to be at extremely low risk and excluded from receiving treatment, although they are routinely offered follow-up. Typing for *DS6265* in this population should allow testing of whether this marker identifies those rare cases that may have attenuated protection and in turn increased risk of developing type 1 diabetes.

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