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Type 1 diabetes genetic susceptibility encoded by HLA DQB1 genes in Romania

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Abstract

Most cases of type 1 diabetes (T1DM) are due to an immune-mediated destruction of the pancreatic beta cells, a process that is conditioned by multiple genes and environmental factors. The main susceptibility genes are represented by the class II *HLA-DRB1* and *DQB1* alleles. The aim of our study was to reconfirm the contribution of *HLA-DQB1* polymorphisms to T1DM genetic susceptibility for the Romanian population. For this, 219 Romanian T1DM families were genotyped at high resolution for HLA DQB1 using the PCR-SSOP method (Polymerase Chain Reaction - Sequence Specific Oligonucleotide Probes). Allele transmission to diabetics and unaffected siblings was studied using the Transmission Disequilibrium Test (TDT). We found an increased transmission of DQB1*02 (77.94% transmission, $p_{TDT} = 7.18 \times 10^{-11}$) and DQB1*0302 (80.95% transmission, $p_{TDT} = 2.25 \times 10^{-10}$) alleles to diabetics, indicating the diabetogenic effect of these alleles. Conversely, DQB1*0301, DQB1*0603, DQB1*0602, DQB1*0601 and DQB1*05 alleles are protective, being significantly less transmitted to diabetics. In conclusion, our results confirmed the strong effect of *HLA-DQB1* alleles on diabetes risk In Romania, with some characteristics which can contribute to the low incidence of T1DM in this country.

Keywords: Type 1 Diabetes - genetic susceptibility - HLA-DQB1 - Romania

Introduction

Type 1 diabetes mellitus (T1DM) is a common autoimmune disease that arises (at least in most of the cases) from the specific destruction of insulin secreting pancreatic β cells by autoreactive T lymphocytes [1]. The pathogenesis of T1DM is complex and multifactorial, involving the interaction between both genetic and environmental factors [2–4]. It is now known that T1DM is a genetically complex disease, caused by multiple susceptibility and protective alleles interacting with each other and with non-genetic factors [5,6]. The major susceptibility genes are encoded in the HLA region of the Major Histocompatibility Complex (MHC) on chromosome 6p21 [7–9].

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Other susceptibility loci have been identified in the 5' promoter region of the insulin gene (*INS*) on chromosome 11p15 [10] and the *CTLA-4* gene region on chromosome 2q33 [11,12]. Even though other regions of the genome have been linked with T1DM [13,14], none of these putative genes have been identified yet.

MHC genes represent the main susceptibility locus for T1DM, explaining up to 50% of the familial aggregation for this disease [6]. This association has been established for many years [15] and is observed in all ethnic groups [16]. The susceptibility for T1DM encoded by the MHC locus arises from the combined effects of the class II HLA DR and DQ alleles [9,16]. We previously reported the strong association between T1DM risk in the Romanian population and the diabetogenic *HLA-DRB1* and *DQB1* alleles [17,18]. The aim of our study was to expand the analysis of the effect of *HLA-DQB1* alleles on T1DM risk in Romania. For this we performed a high resolution *HLA-DQB1* typing of another 219 Romanian T1DM families.

Materials and Methods

Subjects

The study group included 219 simplex (one child affected) and multiplex (at least 2 children affected) Romanian T1DM families. The ascertainment of these families and collection of biological samples were made according to the same protocol as previously described [18,19]. Diabetic probands were selected as T1DM cases if they had an abrupt disease onset with ketoacidosis or required insulin treatment from the first month since diagnosis. Families were selected from Bucharest and other 11 counties. Most families were simplex (211) but there were also 4 multiplex families and 4 families with a parent/sibling pair affected. The family dataset comprised 759 individuals; 335 M (44.13%)/424 F (55.87%) of which 227 type 1 diabetic patients (102 M/125 F). There were 223 affected siblings, with the onset of disease between ages 1 to 30 years and a median age at onset of 12.77 ± 5.2 years. There were 532 unaffected individuals (parents and siblings), 233 M /299 F. All samples for DNA extraction were collected after informed consent. The protocol for DNA extraction was previously reported [18].

Polymorphisms

Class II HLA DQB1 alleles were typed at high resolution. We tested for the following alleles/antigens: DQB1*0201-0202 (HLA DQ2 antigen), DQB1*0301/0304 (HLA DQ7 antigen), DQB1*0302 (DQ8 antigen), DQB1*0303 (DQ9 antigen), DQB1*0401-0402 (DQ4 antigen), DQB1*0501-0503 (DQ5 antigen) and DQB1*0601, *0602, *0603, *0604 (DQ 6 antigen).

SSOP-PCR Genotyping

For HLA DQB1 genotyping we chose the Polymerase Chain Reaction - Sequence Specific Oligonucleotide Probes (PCR-SSOP) method. For this we used the Dynal RELI™ SSO HLA-DQB1 typing kit from Dynal® Biotech.

Briefly, the technique consists in three main steps. 1) PCR amplification of the target region (exon 2 of HLA DQB1 gene on chromosome 6p21). 2) Following PCR amplification, the corresponding amplicons are denatured to single stranded DNA's that will be added to a nylon membrane (strip) which contains an array of immobilized sequence-specific oligonucleotide probes (SSOP) corresponding to each tested DQB1 allele. The PCR amplicons will hybridise to those SSOP's that contain the complementary target sequence, sequence that will be specific for each DQB1 allele. 3) Finally, the amplicon-SSOP complex is visualised using a colour-metric reaction. This generates on the test nylon membranes (strips) a pattern of bands characteristic for each DQB1 allele. The interpretation of the results is made either manually or with a special Software (the Dynal RELI™ Pattern Matching Programme).

All protocols used were those of the producer and are available from Dynal® Biotech (www.tissue-typing.com). An example of the results on 2 reaction strips is given in Fig. 1.

Statistical analysis

HLA DQB1 alleles and genotypes frequencies were calculated and expressed as percentage of carriers of

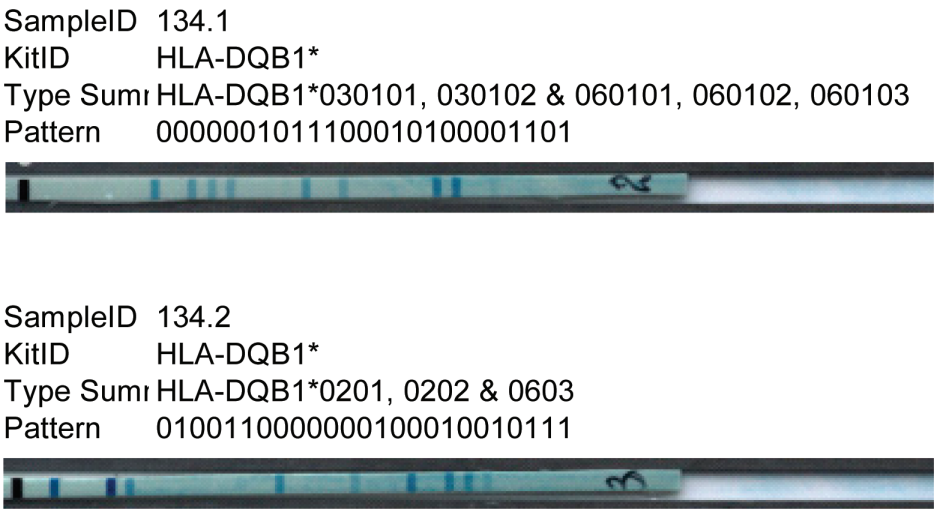

Fig. 1 An example of photo scan for the HLA DQB1 strips and their interpretation for 2 DNA samples.

each individual type. For the comparison of frequencies we used the Chi-square test with the Epi Info version 6.04d Software package. A p value of 0.05 or less was considered statistically significant. Genotyping data were also analysed using the Transmission Disequilibrium Test (TDT) [20]. The TDT tests the possibility that transmission of alleles from heterozygous parents to affected siblings is not the expected 50%. The TDT relative risk (RR) was calculated by dividing the number of times an allele was transmitted by the number not transmitted. TDT statistical analyses were performed with Stata® 8.1 (http://www.stata.com), making use of the *Genassoc* package (http://www-gene.cimr.cam.ac.uk/clayton/software/). Robust variance estimates were used for the calculation of p values, and 95% CI, in order to correct for non-independence of transmissions within families with more than one affected offspring. Genotyping data were tested for Hardy-Weinberg equilibrium.

Results

The frequencies of *HLA-DQB1* alleles, expressed as number/percentage of carriers (subjects positive for each type) are given in Table 1. HLA

Table 1. Frequency of HLA DQB1 alleles, expressed as number (percentage) of subjects positive for each allele.

HLA DQB1 alleles	Diabetics (227 subjects)	Unaffected (532 subjects)	p
DQB1*0201/0202	145 (63.88%)	247 (46.43%)	0.000011
DQB1*04	9 (3.96%)	22 (4.14%)	0.913
DQB1*05	80 (35.24%)	249 (46.80%)	0.00325
DQB1*0601-0604	23 (10.13%)	118 (22.18%)	0.000093
DQB1*0301	23 (10.13%)	143 (26.88%)	<0.000001
DQB1*0302	108 (47.58%)	166 (31.20%)	0.000017
DQB1*0303	4 (1.76%)	19 (3.57%)	0.183

Table 2. Transmission of *HLA-DQB1* alleles to affected siblings (TDT results).

DQB1 allele	T	NT	% T	pTDT
DQB1*0201/0202	106	30	77.94%	7.18×10^{-11}
DQB1*04	6	8	42.86%	0.59
DQB1*05	43	85	33.60%	0.0002
DQB1*0601	2	9	18.18%	0.034
DQB1*0602	4	10	28.57%	0.10
DQB1*0603	1	25	3.85%	2.5×10^{-6}
DQB1*0604	9	6	60%	0.44
DQB1*0301	11	68	13.92%	1.43×10^{-10}
DQB1*0302	85	20	80.95%	2.25×10^{-10}
DQB1*0303	3	9	25%	0.08

T - Transmitted, NT - Not transmitted, %T - Transmission percentage

DQB1*02 and DQB1*0302 alleles are significantly more frequent in diabetics compared to the unaffected group ($p = 1.1 \times 10^{-05}$ and $p = 1.7 \times 10^{-05}$ respectively), supporting the diabetogenic effect of HLA DQ2 and DQ8. The most diabetogenic genotype is DQB1*02/DQB1*0302 (24.23% in diabetics compared to 7.33% in unaffected, $p < 10^{-06}$) followed by DQB1*02 / DQB1*02 ($p = 1 \times 10^{-06}$). Conversely, HLA DQB1*06 and HLA DQB1*0301 alleles are significantly less frequent in diabetics ($p = 9.3 \times 10^{-05}$ and $p < 10^{-06}$ respectively), indicating the protective effect of HLA DQ6 and DQ7 molecules. Also less frequent in diabetics are HLA DQB1*05 alleles ($p = 0.00325$) while DQB1*04 and DQB1*0303 alleles are equally represented in the two subgroups.

The results of the TDT analysis of the genotyping data for the 219 Romanian T1DM families are given in Table 1 and Table 2 and come as a confirmation of the frequency data. We observed a significantly increased transmission of the diabetogenic DQB1*02 (77.94% transmission, $p_{TDT} = 7.18 \times 10^{-11}$) and DQB1*0302 (80.95% transmission, $p_{TDT} = 2.25 \times 10^{-10}$) alleles to diabetics. In the same time, DQB1*0301 and DQB1*0603 alleles were significantly under-transmitted to diabetics (13.92% transmission, $p_{TDT} = 1.43 \times 10^{-10}$ and, respectively, 3.85% transmission, $p_{TDT} = 2.5 \times 10^{-06}$). Also protective (less transmitted to diabetics) are the alleles *HLA-DQB1* *0601, *0602 and *05.

As it can be seen in Table 3, all alleles were transmitted to unaffected Romanian siblings at a percentage not significantly different than the expected 50%, indicating that the associations suggested by the transmissions to diabetic offspring are real.

Discussion

The association between the HLA genes and T1DM was first documented in the mid 1970s by studies that observed that the Class I HLA B8, B15 and B18 alleles were more frequent in diabetics [7,21]. Subsequently typing for class II HLA alleles in diabetics showed a more significant association between HLA DR alleles and

Table 3. Transmission of *HLA-DQB1* alleles to unaffected siblings (TDT results).

DQB1 allele	T	NT	% T	pTDT
DQB1*0201/0202	42	49	46.15%	0.46
DQB1*04	5	6	45.45%	0.76
DQB1*05	57	43	57%	0.16
DQB1*0601	5	5	50%	1
DQB1*0602	4	6	40%	0.52
DQB1*0603	17	8	68%	0.07
DQB1*0604	2	8	20%	0.31
DQB1*0301	23	25	47.92%	0.77
DQB1*0302	33	37	47.14%	0.63
DQB1*0303	6	6	50%	1

T - Transmitted, NT - Not transmitted, %T - Transmission percentage

T1DM [22, 23]. Indeed, more than 95% of Caucasian T1DM patients carry the HLA DR3 or DR4 antigens compared to 45–55% of control subjects [15]. Subsequently the importance of class II *HLA-DQB1* alleles was established [8,24], these genes being actually considered as the most diabetogenic [6]. However, the presence of particular alleles at the *DRB1* locus significantly influences the *DQB1* encoded risk for T1DM [6, 9, 25].

Although less polymorphic than the *HLA-DRB1* locus, the DQ region contains extensive genetic diversity. The HLA-DQ region consists of two expressed loci, *DQA1* and *DQB1*, and two pseudogenes, *DQA2* and *DQB2*. The most polymorphic locus is *DQB1* which has 44 currently recognized alleles that are divided into five subgroups based upon shared sequence motifs: DQB1*02, *03, *04, *05, and *06. The number of alleles currently recognized within each subgroup are 3, 11, 2, 5, and 21, respectively [26]. The haplotypes associated with the highest risk for T1DM in Caucasians are DRB1*04 - DQA1*0301 - DQB1*0302 (encoding for the antigen DQ8) and DRB1*03 -DQA1*0501 -DQB1*02 (encoding for the antigen DQ2) [6, 16, 27]. The best known protective allele is DQB1*0602 [6], followed by DQB1*0603 [28] and DQB1*0301 [27].

In 2001, we reported the same strong effect of the *HLA-DQB1* locus on T1DM risk in Romania (with some particularities) by analysing a sample of 204 Romanian families [18]. Our data suggested that particularities regarding the prevalence of diabetogenic and protective *DQB1* alleles [18] could partially explain the very low incidence of the disease in Romania, currently estimated at 3-4 cases/100000/year in children and adolescents [29]. In the present study we aimed to reconfirm our previous data by expanding the HLA DQB1 genotyping to another 219 Romanian T1DM families. On this second independent Romanian dataset, we proved again the strong diabetogenic effect of DQB1*02 and DQB1*0302 alleles, both comparing their frequencies in diabetics and their unaffected relatives and by TDT analysis. With transmission percentages to diabetics of 78% and 81% respectively, these alleles are equally predisposing in Romanians as in higher incidence populations such as Sardinia, UK and USA (transmission 79% and 84% in a combined dataset [27]).

From the 227 T1DM patients included in this study, 198 (87.23%) carry either or both of these two diabetogenic alleles, percentage lower than that (> 95%) reported in other Caucasian populations with a higher T1DM incidence [6,15].

The most diabetogenic genotype is DQB1*02/DQB1*0302 (coding for DQ2/DQ8 molecules and almost always linked with DR3/DR4) with a frequency of 24.23% in diabetics and 7.33% in unaffected individuals ($p < 10^{-06}$). Interestingly this is lower than in other Caucasian populations (for example 35% in USA [6]). The analysis of the distribution of the heterozygous DQ2/DQ8 genotype according to the age at disease onset revealed that it is more frequent (34%) in diabetics with disease onset between ages 5 to 9 years compared to patients with disease onset in infancy or early childhood (20% for the age group 0 - 4 years) or in adulthood (8.69% for those with diabetes onset after the age of 20). It is worthy to note that the prevalence of DQ2/DQ8 heterozygous genotype in patients with disease onset at an age younger than 5 years is significantly lower in Romania (20%) than in USA (50% in one report [6]), contributing probably to the very low incidence of T1DM for this age group in Romania.

On this second Romanian dataset we confirmed the strong protective effect of DQB1*0301 (13.92% transmission to diabetics) and DQB1*0603 (3.85% transmission to diabetics) as we previously reported on the first 204 families [18]. Also protective are DQB1*0602 (less significant than for the first Romanian dataset on which it was the most protective allele), DQB1*0601 and, somehow surprisingly, DQB1*05 (33.6% transmission to diabetics, $p_{TDT} = 0.0002$). For the DQB1*05, the protective effect is conferred probably mainly by the DQB1*0503 subtype which, as suggested by previous studies on other populations [6,27,30], is very protective. We did not confirm the strong protection of DQB1*0303 (DQ9 antigen) previously reported by us, the effect of this allele being neutral on the second Romanian dataset. The global analysis of all 423 Romanian T1DM families (including 1515 subjects with 430 diabetic patients) showed that the most protective alleles for the Romanian population are DQB1*0603 (8.47% transmission to diabetics, $p_{TDT} = 1.78 \times 10^{-10}$) and DQB1*0301 (HLA antigen DQ7) with a 13.51% transmission to diabetics ($p_{TDT} = 3.23 \times 10^{-23}$). They are followed by DQB1*0303 (14.28% transmission to diabetic offspring) and DQB1*0602 (16.13% transmission).

Comparing the results of the first Romanian dataset (204 families collected in 1995, mean year of diabetes onset 1988) with those from this second dataset (219 families collected in 1999, mean year of diabetes onset 1993) we noticed that the percentage of T1DM patients that do not carry any of the main diabetogenic HLA alleles (DQB1*02 and DQB1*0302) increased from 9.43% to 12.77%. This is concordant with a decrease of their transmission to diabetic offspring, from 81% to 78% for DQB1*02 and from 86% to 81% for DQB1*0302. A possible interpretation for this observation is that in the last years the environment (diet, pollution, viral infections, etc.) became more permissive or diabetogenic prone, so subjects with less diabetogenic HLA genotypes are at risk for developing the disease. The hypothesis is supported by a similar report from Finland [31] and is consistent with the existence of protective factors in the environment as originally proposed by Todd and co-workers in 1991 [2].

In conclusion, we have confirmed the strong effect of *HLA-DQB1* alleles on diabetes risk for the Romanian population, some particularities regarding the distribution of the *DQB1* alleles/genotypes contributing to the low incidence of T1DM in this country. Our data also suggest an increasingly diabetogenic environment, with the result of T1DM occurrence in individuals with neutral or even protective HLA genotypes.

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