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Research Communication

Is the Association Between TNF-α-308 A Allele and DMT1 Independent of HLA-DRB1, DQB1 Alleles?

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The aim of the study was to assess chosen factors of genetic susceptibility to DMT1: DRB1, DQB1, and TNF-α polymorphisms-308 (G/A) in children with DMT1 and their up-to-now healthy siblings. Then we tested whether the association between TNF-α genes and DMT1 is independent of HLA. 87 diabetic children, their 78 siblings, and 85 persons from healthy control group were followed up. The highest risk of DMT1 was connected with alleles: DRB1*0401 (OR = 3.39; CI: 1.55–7.41), DRB1*0301 (OR = 2.72; CI: 1.48–5.01), DQB1*0201 (OR = 4.04; CI: 2.17–7.52), DQB1*0302 (OR = 5.08; CI: 2.54–10.14), and TNF-α-308 A allele (OR = 2.59; CI: 1.23–5.44). Moreover linkage disequilibrium for TNF-α-308 A allele with DRB1*0301 and DQB1*0201 was observed in both diabetic children and their siblings. Diabetic children and their siblings present similar genetic risk factors for DMT1. The association between TNF-α-308 A allele and DMT1 is dependent of HLA-DRB1 and DQB1 alleles.

INTRODUCTION

Diabetes mellitus type 1 (DMT1) is believed to result from selective elimination of pancreatic β cells caused by progressive inflammatory autoimmune reaction. It is currently accepted that genetic susceptibility interacting with unknown environmental factors is responsible for the autoimmune basis of DMT1 [1, 2]. Both genome screens and studies searching candidate genes have confirmed that DMT1 is a heterogeneous polygenic disorder, with about 20 of loci contributing to the susceptibility to disease [3–5]. It is believed that the most important genes, responsible for above 50% genetic risk of developing diabetes, are located in HLA region on chromosome 6p21 [6]. The first region of HLA connected with susceptibility to DMT1 (HLA class I B8 and B15) was found thirty years ago [7]. The positive association of certain HLA class II alleles with DMT1—DQ8/DR4 and DQ2/DR3 in most Caucasian populations has been very well documented [1, 6, 8]. The tumor necrosis factor α (TNF-α) is known as proinflammatory cytokine implicated in the pathogenesis of autoimmune and infectious diseases. The genes of TNF-α are located in the region HLA class III (250 kb centromeric of the HLA-B and 850 kb telomeric of the class II HLA-DR genes in humans) [9, 10]. Although contribution of TNF-α to DMT1 is not well established in humans in multicenter studies, it has been shown in an animal model that TNF-α can be cytotoxic for β-cells supported by both interleukin-1 and interferon-γ [11, 12]. NOD mice that overexpress TNF-α in their β-cells are predisposed to diabetes [13]. Moreover it has been reported, that levels of circulating monocyte and T-cell type 1 proinflammatory cytokines are elevated in patients at the onset of diabetes [14]. It has been pointed in several studies [9, 10, 15–17], that TNF-α region contains a few polymorphisms (single nucleotide polymorphisms—SNP) which could be connected with a different cytokine secretion. Probably different places in the promotor region, in the first intron, in the 3'untranslated regions and microsatellites could determine different levels of TNF-α production [18–21]. The TNF-α promotor polymorphism-308 in the human involving the substitution of guanine (known as TNF1) by adenosine (known as TNF2) has been most often used in the associated studies. TNF2 allele seems to have a real functional significance because it is a much stronger transcriptional activator than the common allele TNF1 [9]. In view of gene location, it has been speculated that polymorphism of this locus might contribute to HLA association with DMT1. Previous studies in west European populations [22, 23] have indicated a strong association of

TNF2 allele with DMT1, however the authors of these papers suggest that these associations may originate from the typical for HLA linkage disequilibria (LD) between estimated alleles in the TNF locus and other alleles of HLA region. However studies concerning middle-east European populations are few and contradictory. It was shown in a Latvian study [24] that the microsatellites A5 and TNF2 are both independently associated with the disease.

The aim of this study is an assessment of chosen factors of genetic susceptibility to DMT1 such as HLA-DRB1, DQB1, and TNF-α polymorphism-308 (G/A) in children with diabetes and their up to now healthy siblings. Moreover we would like to estimate whether possible relationship of TNF-α-308 (G/A) is due to a primary association or mediated by LD with the susceptible DRB1 and DQB1 alleles.

MATERIAL AND METHODS

Subjects

A group of 87 diabetic children (52 boys and 35 girls) in median age of 10.19 years (quartile 7.52–12.56, age range 1.55–18.2) were randomly selected as the subject of this study. All patients were hospitalized in Department of Endocrinology and Diabetology in Katowice with a new onset of diabetes. The diagnosis of DMT1 was made according to WHO and ISPAD criteria [25]. An inclusion criterion for enrolment to the study was presence of at least one disease-associated autoantibody (GADA-65, IAA, or IA2A). The second group studied was a group of 78 of their related brother and/or sister (40 boys and 38 girls) in median age of 10.82 years (quartile 7.08–14.18, age range 0.69–19.99) without any symptoms of impaired glucose tolerance and normal fasting glucose level. Blood samples for genetic tests were taken once, both from diabetic children and their siblings with no diagnostic signs of diabetes. As the healthy control group, 85 subjects (44 men and 41 women, aged 18–35 years) without diabetes or any other autoimmune disease in family were enrolled.

BLOOD TESTS

Immunological test

Samples for disease-associated autoantibodies: glutamic acid decarboxylase antibodies (GADA-65), tyrosine phosphatase antibodies (IA2A) and insulin antibodies (IAA) were obtained from children with DMT1 before initiation of insulin treatment. Concentrations of autoantibodies were measured by radioimmune assay (RIA—CIS Bio International) in the Department of Isotope Diagnostics of the Medical University of Silesia in Katowice according to the protocol described in instructions of kits. In the GADA and IA2A assay tests, serum samples were first incubated with ^{125}I-labelled human GAD or human recombinant IA2, respectively. This was followed by addition of solid phase protein A to precipitate the labelled GAD-GAD antibodies or IA2-IA2 antibodies complexes. After centrifugation, the precipitate was counted for ^{125}I and the amount of radioactivity in the precipitate was proportional to the concentration of GADA or IA2A in the test sample. The presence of circulating IAA was estimated on a semiquantitative basis, by the determination of the binding of ^{125}I-Tyr-A 14 insulin to the serum fraction precipitated by the polyethylene glycol. After incubation and centrifugation radioactivity of supernatant was measured by counting in a gamma scintillation counter. All the tests were performed in duplicate. The measurements ranges were as follows: for IA2A 0–50 U/mL, for GADA 0–300 U/mL, and for IAA 0–100%. The results for GADA and IA2A above 0.75 U/mL and for IAA above 7% were taken as positive.

HLA tests

The genetic assays were performed in The Regional Blood Centre in Katowice. HLA class II alleles were typed using PCR-SSP method. Allele specific tests One Lambda (CYTGEN, USA) were used for HLA-DRB1* and DQB1* typing. Peripheral blood samples (EDTA) were the source of the genomic DNA. DNA was isolated by salting out method using 3–5 mL 6 M NaCl [26]. First leucocytes were resuspended in 5 mL SE buffer with adding 250 ul 20% SDS and 15 μL Proteinase (10 mg/μL). Incubation was carried out for 17 hours at 37°C. Next DNA extraction was performed using 12.5 mL absolute alcohol and DNA samples were stored at room temperature (30 min) to dry. The PCR mixture consisted of D-mix ready for use, Taq Polymerase (5 U/μL), and DNA (100 ng/μL). The PCR program was performed according to the protocol: 1 cycle: 96°C (130 sec) and 63°C (60 sec), next 9 cycles: 96°C (10 sec) and 63°C (60 sec), and finally 20 cycles: 96°C (10 sec), 59°C (50 sec), 72°C (30 sec). After gel electrophoresis of PCR products, UV transilluminator was used for visualization and completing an amplification pattern.

TNF-α polymorphism-308 (G/A) tests

The polymorphism in the promoter region of TNF-α polymorphism-308 (G/A) was identified using PCR-SSP method. Commercial One Lambda (CYTGEN, USA) kit was used [27]. DNA from human leucocytes was prepared by salting out method using 6 M NaCl [26]. DNA samples before PCR were resuspended in 10 mM Tris HLC, ph 8.0–9.0 at a concentration 100 ng/ul. The PCR reagents were as follows: DNA sample (19 ul), D-Mixture ready for use (180 ul), and Taq Polymerase (concentration 5 U/ul −1 ul). PCR cycling conditions were: 1 cycle 96°C (130 sec), and 63°C (60 sec), next 9 cycles: 96°C (10 sec) and 63°C (60 sec) and 20 cycles: 96°C (10 sec), 59°C (50 sec), and 72°C (30 sec). Finally PCR products were separated on agarose gel containing TBE buffer and visualized by UV transilluminator.

STATISTICAL ANALYSIS

The frequency of HLA alleles and polymorphism of TNF-α 308 (G/A) were estimated in each group with an expectation-maximization (EM) algorithm, as implemented in Arlequin 2.000 program [28]. The linkage disequilibrium between

alleles of estimated loci was determined by D statistic:

$$D = p_{ab} - p_a \cdot p_b \quad \text{for two compared alleles loci 1 and 2,} \tag{1}$$

where p_a means frequency of allele a on the first estimated locus, p_b means frequency of allele b on the second estimated locus, p_{ab} means frequency of pair ab.

To assess the risk associated with genetic factors the odds ratio (OR) with 95% confidence interval (CI) was calculated for all alleles and haplotypes according to the Woolf's formula. The final verification of risk factors for DMT1 was carried out after creating multiple logistic regression model. The model was built in stages. First univariate analysis was used to identify all discrete variables predictive for onset of diabetes. For each of them logistic regression equation was performed. Using algorithm step forward, the best classification model was searched. The variable of the highest predictive power was chosen as the first one. The remaining variables were added in the next step. The searching process was finished when none of the remaining variables significantly improved classification properties of the model. χ^2 statistic and qualification criterion C_p were applied for models' comparison.

RESULTS

Genetic typing of HLA-DRB1 and DQB1 on the low resolution level in diabetic children revealed predominance of the following alleles: DRB1*04 (33.33%), DRB1*03 (31.03%), DQB1*02 (39.65%), and DQB1*03 (37.93%). The haplotypes recognized as predisposing to the disease: DRB1*04 DQB1*03 (65.5%, 57 children) and/or DRB1*03 DQB1*02 (62%, 54 children) were found in the most ill children (91%, 79 children). There were no significant differences in the frequency of haplotype in relation to sex or age at the onset. Genetic typing of HLA-DRB1 and DQB1 on the low resolution level in siblings of diabetic children revealed predominance of the following alleles: DRB1*03 (23.72%), DRB1*04 (23.08%), DQB1*02 (30.77%), and DQB1*03 (33.33%). Similarly to ill siblings predominance of following haplotypes was observed: DRB1*03 DQB1*02 (47.4%, 37 children) and DRB1*04 DQB1*03 (44.8%, 35 children).

The comparative analysis of the distribution of DRB1 and DQB1 alleles was performed on the basis of families with pairs: ill child-healthy child. Diabetic children without siblings were excluded from this part of analysis. In the families with some healthy children, one of them was randomly selected. Finally the 44 pairs of children were compared with the 85 healthy subjects from the control group. The distribution of estimated alleles DRB1 and DQB1 in all groups is shown in Tables 1 and 2. Global test while comparing all groups showed that both diabetic children and their siblings significantly differed from the healthy control group ($P < .001$), with no difference between examined pairs ($P = .1039$). Alleles associated with the highest risk of DMT1 in the examined population within the DRB1 genes were as follows: 0401 (OR = 3.39; 1.55–7.41) and 0301 (OR = 2,72; 1.48–5.01), whereas within DQB1 genes: 0302 (OR = 5.08; 2.54–10.14) and 0201 (OR = 4.04; 2.17–7.52). The risk was significantly increased when alleles coexisted in both loci. The risk calculated for given haplotypes was as follows: DRB1*0401 DQB1*0302 (OR = 10.67; 3.49–32.67) and DRB1*0301 DQB1*0201 (OR = 4.73; 2.4–9.34). Moreover, the strong protection against diabetes was associated with the allele of DRB1*1501 (OR = 0.06; 0.01–0.44) and to a lesser extent with the allele of DQB1*0301 (OR = 0.1; 0.02–0.46).

Genetic variability of TNF-α-308 was analyzed in 44 pairs of diabetic child-sibling and 36 subjects randomly selected from the control group. The purpose of this analysis was to answer the question whether this polymorphism may be a risk factor for DMT1. The study revealed significant differences only between diabetic children and the healthy control group ($P = .013$). The presence of the allele TNF2 is associated with increased risk of DMT1 (36.36% versus 18.1%; OR = 2.59; 1.23 − 5.44). The distribution of TNF-α-308 polymorphisms in all groups is shown in Table 3.

The analysis of genetic samples confirms linkage disequilibrium (LD) for alleles DRB1 and DQB1 associated with predisposition to DMT1 in all groups, previously reported in literature [1, 6, 8]. For diabetic children value of D was as follows: for alleles DRB1*0401 and DQB1*0302 ($D = 0.14$; $\chi^2 = 46.04$; $P < .001$) and for alleles DRB1*0301 and DQB1*0201 ($D = 0.21$; $\chi^2 = 72.09$; $P < .001$). Moreover LD was noted for the TNF-α-308 A allele with DRB1*0301 allele ($D = 0.13$; $\chi^2 = 29.16$; $P < .001$) and DQB1*0201 allele ($D = 0.12$; $\chi^2 = 2.95$; $P < .001$) in diabetic children. The similar LD was observed in siblings for TNF2 with DRB1*0301 ($D = 0.13$; $\chi^2 = 49.76$; $P < .001$) and for TNF2 with DQB1*0201 ($D = 0.11$; $\chi^2 = 26.91$; $P < .001$).

The final assessment of genetic susceptibility to DMT1 in the examined group of diabetic children was based on the multiple logistic regression model. Firstly all the discrete variables predictive for onset of diabetes in the univariate analysis were taken into account as follows: the DRB1*04, DRB1*0301, DQB1*0302, DQB1*0201, DRB1*04 DBB1*0302, DRB1*0301 DQB1*0201, and the TNF2. Finally the following variables remained in the model: DRB1*04, DQB1*0302, and DRB1*0301 DQB1*0201. The presence of these alleles and haplotype determines correct classification of diabetic children in 90.1%. Allele TNF2 did not correct significantly classification properties of the model. The model is described in Table 4. The predictive model of onset of DMT1 could be described as follows:

$$P_C = \frac{e^{\beta}}{1 + e^{\beta}}, \tag{2}$$

where $\beta = -2.30 + 1.94 \cdot \text{DRB1*04} + 1.80 \cdot \text{DQB1*302} + 4.10 \cdot \text{DRB1*0301 DQB1*201}$.

DISCUSSION

This study is the first report of HLA: DRB1, DQB1, and TNF-α-308 association with DMT1 in the Polish south-west population (exactly in the Upper Silesia population). Like in most Caucasian populations studied so far [1, 6, 8, 29], haplotypes: DRB1*04 DQB1*0302 and DRB1*0301 DQB1*0201 showed the highest association with onset of DMT1 also in

Table 1: Estimated DRB1 alleles' frequencies in diabetic children, siblings, and healthy control group. $P < .001$: diabetic children versus healthy control group. $P < .001$: siblings versus healthy control group. P = NS: diabetic children versus siblings.

Allele DRB1	Diabetic children $n = 44$		Siblings $n = 44$		Healthy control group $n = 85$	
	Frequency (%)	SD	Frequency (%)	SD	Frequency (%)	SD
0101	5.68	2.48	9.09	3.08	8.82	2.18
0102	3.41	1.95	2.27	1.60	1.18	0.83
0301	32.95	5.04	22.73	4.49	15.29	2.77
0304	1.14	1.14	0.00	0.00	0.00	0.00
0401	20.45	4.33	14.77	3.80	7.06	1.97
0402	4.55	2.23	3.41	1.95	1.18	0.83
0404	3.41	1.95	3.41	1.95	0.59	0.59
0405	1.14	1.14	1.14	1.14	0.00	0.00
0408	1.14	1.14	1.14	1.14	0.00	0.00
0419	1.14	1.14	1.14	1.14	0.00	0.00
0701	9.09	3.08	9.09	3.08	13.53	2.63
0801	2.27	1.60	2.27	1.60	2.35	1.17
0901	1.14	1.14	0.00	0.00	2.94	1.30
1001	0.00	0.00	0.00	0.00	0.59	0.59
1101	1.14	1.14	2.27	1.60	5.29	1.72
1103	0.00	0.00	2.27	1.60	3.53	1.42
1123	0.00	0.00	2.27	1.60	0.00	0.00
1201	0.00	0.00	1.14	1.14	4.71	1.63
1301	0.00	0.00	2.27	1.60	7.06	1.97
1302	1.14	1.14	3.41	1.95	3.53	1.42
1303	0.00	0.00	1.14	1.14	0.00	0.00
1306	0.00	0.00	1.14	1.14	0.00	0.00
1401	1.14	1.14	0.00	0.00	4.12	1.53
1501	1.14	1.14	4.55	2.23	16.48	2.85
1502	0.00	0.00	1.14	1.14	0.00	0.00
1509	1.14	1.14	2.27	1.60	0.00	0.00
1601	6.82	2.70	5.68	2.48	0.59	0.59
1602	0.00	0.00	0.00	0.00	1.18	0.83

this study. Comparing our data with other studies performed in Poland [30, 31], some similarities and differences could be observed. Generally, distributions of DRB1 alleles and haplotypes connected with DMT1 in different subpopulations of our country are rather similar—the same haplotypes determine predisposition to and protection from the disease. However the frequencies of DQB1 alleles and associations with DMT1 differ in examined Polish subpopulations. In children from the central region of Poland [30] only allele DQB1*0302 determined high risk of DMT1 (OR = 5.59; CI: 3.26–9.58), that corresponds with the risk in our population (OR = 5.08; CI: 2.54–10.14) and the risk in the north-eastern region of Poland (RR = 19.8) [31]. The positive connection with DMT1 of DQB1*0201 was not observed in children from the central region of Poland, while it was confirmed in the north-western subpopulation of Poland (RR = 6.25) and in our study (OR = 4.04; CI: 2.17–7.52). At the other hand the highest protective effect of DQB1*0602 was not observed in our study as opposite to the study mentioned above. It was probably due to quite different distribution of this allele in the healthy control populations (the frequency of DQB1*0602 in the Silesian population is 8.2% versus 34.2% in the north-western subpopulation of Poland). Such big diversification of estimated alleles in the different regions of Poland is probably the result of historical and economical aspects, for example migration of Polish population because of borders changing and to the most urbanized region of Poland (Upper Silesia region).

Previous studies in European populations [23] and in the USA [32] indicated strong association between TNF-α-308 A allele (TNF2) and DMT1. In one of the first studies [23] a significant difference in allele frequency between patients and control group was observed ($P = .03$), but also very strong association of the uncommon TNF2 allele with the HLA-B8 and HLA-DR3 was noted. The relative risk of DMT1 (RR) in this study was calculated for HLA genes as follows: 3.1 for

Table 2: Estimated DQB1 alleles' frequencies in diabetic children, siblings, and healthy control group. $P < .001$: diabetic children versus healthy control group. $P < .001$: siblings versus healthy control group. P = NS: diabetic children versus siblings.

Allele DQB1	Diabetic children $n = 44$		Siblings $n = 44$		Healthy control group $n = 85$	
	Frequency (%)	SD	Frequency (%)	SD	Frequency (%)	SD
0201	37.50	5.19	28.41	4.84	12.94	2.58
0202	5.68	2.48	3.41	1.95	13.53	2.63
0301	2.27	1.59	10.23	3.25	18.82	3.01
0302	32.96	5.04	25.00	4.64	8.82	2.18
0303	0.00	0.00	0.00	0.00	4.71	1.63
0304	1.14	1.14	1.14	1.14	0.00	0.00
0402	0.00	0.00	0.00	0.00	0.59	0.59
0403	0.00	0.00	0.00	0.00	0.59	0.59
0501	12.50	3.55	14.77	3.80	8.24	2.12
0502	4.55	2.23	3.41	1.95	7.06	1.97
0503	0.00	0.00	0.00	0.00	3.53	1.42
0504	0.00	0.00	0.00	0.00	0.59	0.59
0601	0.00	0.00	3.41	1.95	4.71	1.63
0602	0.00	0.00	4.55	2.23	8.24	2.12
0603	2.27	1.59	2.27	1.60	4.71	1.63
0604	1.14	1.14	3.41	1.95	1.18	0.83
0609	0.00	0.00	0.00	0.00	0.59	0.59
0611	0.00	0.00	0.00	0.00	0.59	0.59
0619	0.00	0.00	0.00	0.00	0.59	0.59

Table 3: Estimated TNF-α-308 alleles' frequencies in diabetic children, siblings, and healthy control group. $P = .013$: diabetic children versus healthy control group. P = NS: siblings versus healthy control group. P = NS: diabetic children versus siblings.

Allele TNF-α	Diabetic children $n = 44$		Siblings $n = 44$		Healthy control group $n = 36$	
	Frequency (%)	SD	Frequency (%)	SD	Frequency (%)	SD
G	0.64	0.05	0.71	0.05	0.81	0.04
A	0.36	0.05	0.28	0.04	0.18	0.04

DR3 and 2.2 for TNF2. The connection between TNF2 and DMT1 was confirmed in other studies; moreover studies of different populations showed the diversification of distribution of TNF2 in general population. The frequency of TNF2 in Caucasian populations is estimated to be about 17–22% [22, 23], while in Asian countries it is found very rarely 1–3% [32]. It was also described in Moroccan population [33] that the presence of allele A on both chromosomes could bring surprising effect. Independent of LD with HLA class II, TNF haplotype-308 TNFA*2 + 252 TNFB*2 showed a significant protective effect (OR = 0.031) against the disease. Authors of this paper explained that this result could be detected because Morocco shows the highest frequency of the TNF2 allele yet reported (genotypes: 25.9% for the TNF*1, 2 and 19.8% for TNF*2, 2 in the general population).

In this study allele TNF2 responsible for the highest secretion was observed in 36.4% children with DMT1, while in control group it was found rarely—in 18.1% ($P = .013$). Therefore allele TNF2 could be recognized as a risk factor for DMT1 (OR = 2.59; 1.23–5.44). But this result should be interpreted with caution because of LD between TNF-α alleles and HLA alleles: DRB1 and DQB1. In a comparable analysis LD between TNF2 and alleles DRB1*0301 and also DQB1*0201 both in diabetic children and siblings were recognized. These results are yet another confirmation of results of the studies performed in other Caucasian populations that connection of TNF-α with DMT1 is dependent of HLA genes [22, 23, 32]. Obtained results are also the next proof for real genetic similarity of siblings. The significant differences in distribution of TNF2 were observed only when comparing diabetic children with control group; however there were no differences between diabetic children and siblings. It was shown in another study [34] that in healthy family members of children with DMT1 overproduction of proinflammatory cytokines existed. Because of the fact that many genetic factors and immunoregulatory abnormalities involved

Table 4: Parameters of logistic regression model.

	Parameter estimate	P value	OR (95% CI)
Intercept	−2.29	$P = .0002$	—
DRB1*04	1.93	$P = .0256$	6.94 (1.23–39.12)
DQB1*0302	1.80	$P = .0308$	6.05 (1.1–31.86)
DRB1*0301 DQB1*0201	4.10	$P < .0001$	60.12 (8.75–412.9)

in dibetogenesis are shared by healthy siblings, further studies should be performed to determine what factors are required to make the disease on this background.

CONCLUSION

Diabetic children and their healthy siblings present similar genetic risk factors for DMT1: allele DRB1, DQB1 and polymorphisms of TNF-α-308 (G/A). The association between TNF2 and DMT1 is dependent of HLA-DRB1 and DQB1 alleles.

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