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Association of *TNF* promoter polymorphisms with type 1 diabetes in the South Croatian population

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ABSTRACT

Type 1 diabetes mellitus (T1DM) is an autoimmune disease characterized by the destruction of pancreatic β cells. Tumor necrosis factor (TNF) is a pleotropic cytokine with potent immunomodulatory and inflammatory activity. Association studies of *TNF* polymorphisms and type 1 diabetes (T1DM) frequently demonstrated *TNF* involvement with T1DM. Although *TNF* may play an important role in the pathogenesis of T1DM, the genetic association of *TNF* region with the disease has not been conclusive because of the strong linkage disequilibrium with *HLA* genes. In this study, we examined two *TNF* promoter variants (rs1800629 at position -308, and rs361525 at position -238) for T1DM association in 233 patients and 144 controls from the population of South Croatia. A higher frequency of *TNF* -308 A allele and also, a more frequent specific -308A -238G haplotype in T1DM patients were observed with a limited significance. However, we did not find strong evidence of association of *TNF* promoter polymorphisms with T1DM. In order to elucidate the true contribution of *TNF* to T1DM susceptibility in our population, more comprehensive studies with *HLA* adjustment in a larger sample are required.

Key terms: type 1 diabetes, tumor necrosis factor gene (*TNF*), polymorphism, genetic epidemiology, Croatia.

INTRODUCTION

Type 1 diabetes mellitus (T1DM) is a multifactorial autoimmune disorder characterized by T-cell mediated destruction of the pancreatic β-cells caused by multiple genetic and environmental influences (Todd 1995). Although human leukocyte antigen (*HLA*) located on chromosome 6p21 seems to be responsible for above 50% genetic risk of developing T1DM, there are other important loci associated with the disease that include an insulin-linked variable number of tandem repeats (*INS*-VNTR) located at the 5' regulatory region of the insulin gene (*IDDM2*), limphoid protein tyrosine phosphatase non-receptor type 22 (*PTPN22*), cytotoxic T-lymphocyte-associated antigen 4 (CTLA 4), the interleukin-2 receptor a chain (*IL2RA*), the interferon-induced helicase region (*IFIH1*), inositol 1,4,5-triphosphate receptor 3 gene (*ITPR3*)(Todd 1995, Rich et al. 2006, Smyth et al. 2006, Roach et al. 2006). The most recent genome-wide association studies (GWA) for T1DM identified sugar binding C-lecitin type gene (*KIAA350*) and another four regions 12q24, 12q13, 16p13 and 18p11 to be associated with T1DM (Hakonarson et al. 2007, Todd et al. 2007).

The multifactorial cytokine, tumor necrosis factor (TNF) is involved in the promotion of inflammatory responses and plays a critical role in the pathogenesis of inflammatory, autoimmune and malignant

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diseases (Bazzoni and Beuther 1996). Although the contribution of TNF to T1DM is not well established in humans, it has been shown in animal models that TNF can be cytotoxic for β-cells supported by both interleukin-1 and interferon-γ (Rabinovitch 1994, Rabinovitch and Suarez-Pinzon 1998). NOD mice that overexpress TNF in their β-cells are predisposed to diabetes (Vaux and Flavell 2000). Moreover, it has been reported that levels of circulating monocytes and T-cells type 1 proinflammatory cytokines are elevated in patients at the onset of diabetes (Hussain at al. 1996).

The *TNF* gene is located on chromosome 6 in the *HLA* region class III, 250 kb centromeric of the *HLA-B* and 850 kb telomeric of the class II *HLA-DR* genes in humans (Hamaguchi et al. 2000). Several *TNF* promoter polymorphisms have been identified and have been implicated in the regulation of TNF transcription (Kroeger et al. 1997 and Wilson et al. 1997). Single nucleotide polymorphisms (SNPs) at the positions -308 (rs1800629) and -238 (rs361525) of the promoter region of the *TNF* gene have been commonly studied. Both polymorphisms are G→A substitutions and changes they introduce can alter the transcription-binding site and affect the transcription rate (Kroeger et al. 1997). Indeed, -308 polymorphism affects gene expression with a rare A allele, resulting in higher *in vitro* TNF production (Bouma et al. 1996). As well, the rare *TNF* -238 A allele has been associated with high TNF production (Grove at al. 1997). Since both promoter polymorphisms have been associated with the transcriptional enhancement rate, it is possible that when acting *in cis,* these two markers show even stronger interaction (Kroeger et al. 1997 and Kaluza et al. 2000).

A relation between genetic variability of *TNF* and T1DM is widely suggested, but linkage disequilibrium (LD) between *TNF* and *HLA* alleles makes it difficult to evaluate the involvement of *TNF* alleles alone with T1DM susceptibility (Feugeas et al. 1997).

Kumar et al reported a significant increase of *TNF* -308 G/A and A/A genotypes in North Indian T1DM patients (Kumar et al. 2007). Das et al. suggested a significant association of *TNF* -308 A allele and G/A genotype with T1DM in North Indians, but did not observe such association with -238 SNP (Das et al. 2006). Krikovszky observed the same increase in the prevalence of *TNF* -308 A allele in diabetic patients in the Hungarian population (Krikovszky et al. 2002). In contrast, Deng et al. excluded -308 polymorphism to be a genetic element for susceptibility to T1DM in Chinese and Caucasian populations and reported that the associations of the *TNF* gene are due to a LD between *TNF* and DR3-DQB1*0201 haplotype (Deng et al. 1996). Noble et al. initially found association of *TNF* -308 and -238 polymorphisms with T1DM, but after adjusting the data for LD with DRB1-DQB1 and B18-DR3 haplotypes, the association lost its significance (Noble et al. 2006). As well, Deja et al. reported that association between *TNF* -308 A allele is dependent of *HLA*-DRB1 and DQB1 alleles in the Polish south-west population (Deja et al. 2006). As we can see, the association of *TNF* polymorphisms with T1DM was suggested to be due to its carrying specific DR-DQ haplotypes by some, but not all studies (Stayoussef et al. 2007).

Several studies observed an independent effect of *TNF* -308 polymorphism to T1DM (Das et al. 2006, Krikovszky et al. 2002). Kumar et al. suggested that the effect of *TNF* -308 A allele to susceptibility to T1DM is not due to LD with *HLA* alleles, but rather to its functional role in the destruction of pancreatic beta cells and its integrated effect with other cytokines (Kumar et al. 2007).

Croatians have a typical European maternal and paternal genetic landscape, with the exception of some mitochondrial DNA (mtDNA) and Y-chromosomal haplogroup that indicate connection to Central Asian populations. Moreover, structuring of the Y chromosome point to a Slavic component in Croatian men. Analysis of mtDNA and the non-recombining region of the Y chromosome shows predominance of "Paleolithic" mutations that are in concordance (70-80%) with the range of "Paleolithic genes" present in the European population gene

pool. Observed mitochondrial profile and the low percentage of "Neolithic mutations" in Croatia indicate a good agreement with neighboring populations. Higher frequency of "Neolithic haplogroups" found in the Y chromosome indicates that a large part of Croatia belongs to the Mediterranean region. Eastern Adriatic islands of Krk, Brač, Hvar and Korčula represent reproductive isolates of relatively small size, where genetic drift and founder effect have significant roles in shaping genetic diversity (Peričić M et al. 2005). The large islands Brač, Hvar and Korčula, some other small islands, and the southern part of the Croatian mainland represent South Croatia. The present study was undertaken to ascertain the association of *TNF* -308 and -238 promoter polymorphisms with T1DM in the South Croatian population.

MATERIALS AND METHODS

Participants

The study included 232 unrelated Caucasian patients (121 men and 111 women) from the Dalmatian region of South Croatia. The mean onset age of T1DM was 8.64.2. T1DM was diagnosed according to the World Health Organization criteria and all the patients required insulin for glycaemic control (Harris et al. 1985). The control group of 144 (67 men and 77 women) unrelated consecutive subjects was randomly recruited from individuals who came to the Split Clinical Hospital for general health check-ups. The mean age was 8.14.1. This study was approved by the ethics committee, and informed consent from patients and their parents was obtained prior to the blood sampling.

Gene polymorphism

Genomic DNA was extracted from peripheral blood leucocytes using the Perfect gDNA kit (Eppendorf, Hamburg, Germany). *TNF* –308 (rs1800629, G/A) and -238 (rs361525, G/A) promoter genotypes were identified by polymerase chain reaction, followed by restriction fragment length polymorphism (PCR/RFLP), according to previous reports (Wieser et al. 2002). The PCR amplified fragments (141 for -308 SNP and 151 bp for -238 SNP) were digested with *Nco*I and *Msp*I to detect G to A transition at the -308 and -238 promoter sites, respectively. The digested fragments were separated on 10% polyacrylamide gels and visualized by ethidium bromide staining. Results for *TNF* -238 SNP were quantitatively checked on Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). As a measure of quality control (QC), 30% of samples were re-genotyped in order to confirm previously established genotypes.

Statistical analysis

Prior to association analysis, QC of the obtained genotypes was performed. As a part of QC, Hardy-Weinberg equilibrium (HWE) was tested in healthy controls and minor allele frequencies (MAF) were compared with phase II HapMap (www.hapmap.org) MAF from the CEU population (The International HapMap Consortium 2003). Genotype and haplotype comparison between patients and controls were done by the chi-square test using Statistica 6.0 (StatSoft, Inc., Tulsa, OK). Haplotype analysis was made using the statistical program EHPLUS. This program assumes that alleles at different loci occur independently so haplotype frequencies are formed as the product of constituent allele frequencies (Zhao et al 2000). r^2 and D' measures of pairwise LD for two investigated SNPs were calculated using Haploview (Barrett et al. 2005). Power calculation was done using Quanto (Gauderman 2002). P-values less than 0.05 were considered nominally significant.

RESULTS

QC analysis established both SNPs to be in the HWE. MAF in healthy controls were concordant with phase II HapMap frequencies from the CEU population. Re-genotyped samples confirmed previously established genotypes.

Genotype counts and MAFs for *TNF* -308 and -238 SNPs in patients with T1DM and the control group are shown in Table 1. Genotype distribution for both *TNF* promoter variants did not differ between T1DM patients and controls. The *TNF* -308A minor allele was more frequent in the T1DM patients and showed a limited significance (P=0.076).

The results of a haplotype analysis are shown in Table 2. Specific (-308A -238G) haplotype was observed more often in the T1DM patients than in controls, but showed a borderline significance (P=0.076). Two investigated SNPs were not in LD with each other (r^2 =0, D'=0).

This study had 80% statistical power to detect (at a=0.05) an effect of [OR] =1.62 for -308 SNP assuming an additive model and was underpowered to detect the same effect for -238 SNP.

DISCUSSION

This association study analysed a relation of two *TNF* gene promoter polymorphisms (-308, -238) with T1DM in a case-control sample from South Croatia. Specific haplotype (-308A -238G) was observed more often in T1DM patients than in controls with a borderline significance (P=0.076). Consistently, *TNF*-308 allele A was found to be more frequent in T1DM patients but also with limited significance (P=0.076). However, a genotype distribution of G-238A and G-308A *TNF* promoter variants did not differ between

TABLE 1

Genotype counts and minor allele frequencies (MAF) for *TNF* -308 and -238 promoter variants in patients with type1 diabetes mellitus and controls.

Genotype	Patients	Controls	P-value
***TNF* -308 (rs1800629)**	n=233 (%)	n=144 (%)	
GG	143 (61.4)	100 (69.4)	
GA	76 (32.6)	40 (27.8)	0.210
AA	13 (6)	4 (2.8)	
MAF (*A allele*)	0.219	0.167	0.076
***TNF* -238 (rs361525)**	n= 232 (%)	n=144 (%)	
GG	223 (96)	134 (93)	
GA	9 (4)	10 (7)	0.281
AA	0	0	
MAF (*A allele*)	0.019	0.036	0.162

TABLE 2

Number of observed *TNF* -308-238 haplotypes in patients with type1 diabetes mellitus and controls.

Haplotypes-308-238	T1DM patients (%)	Controls (%)	P-value
G-G	354 (76.3)	230 (79.8)	0.253
A-G	102 (22)	48 (16.7)	0.076
G-A	8 (1.7)	10 (3.5)	0.127

* A-A haplotype was not observed in any group (the probability of occurrence of A-A haplotype in patients was 1.9, in controls 1.7 and in both groups together 3.7)

patients and controls. In haplotype analysis we did not observe -308A -238A haploype. This haplotype is constituted of two rare *TNF* promoter alleles and the probability of its occurrence was 1.9 (9 haplotypes) in patients, 1.7 (8 haplotypes) in controls and 3.7 (28 haplotypes) in combined dataset (patients and controls). The Poisson distribution for finding 0 haplotypes in combined dataset produced a significant p-value (p=0.027). This implies that -308A -238A haplotype could produce some great impairment that could be lethal or semi-lethal to all individuals who carry this haplotype, and that could be a reason for not observing it in our combined dataset. However, a possibility of not observing -308A -238A haplotype in our dataset could also be due to a simple chance event.

The TNF gene is located inside the *HLA* III region that contains about 10 times more genes than other regions in the genome and is considered to be the densest region in the human genome (Nishimura et al. 2003). Beside *HLA* DR/DQ locus of the *HLA* region II, *HLA* class III region, particularly around the *TNF* gene, has been regarded as a susceptible locus for T1DM (Nishimura et al. 2003). In our study we observe the same trend of increase of *TNF* -308 A allele frequency in T1DM patients, suggesting a small effect of this allele on susceptibility to T1DM.

Some reports show that the association between alleles at the *TNF* locus and T1DM can be attributed to LD with the susceptible DQB1-DRB1 haplotypes, instead of an independent effect (Feugeas et al 1997, Koeleman et al. 2000, Deja et al. 2006). However, several other studies reported an independent effect of the *TNF* gene with T1DM (Das et al.2006, Krikovszky et al. 2002 and Kumar et al. 2007).

Different studies presented above have shown different *TNF* impact on T1DM susceptibility, probably due to differences in surrounding environmental factors and in LD relations between *TNF* and *HLA* genes. LD differences are most probably caused by genetic admixture, isolation or some other historical facts. The population of South Croatia partly consists of reproductive isolates located on a few Adriatic islands. The phylogeography of mtDNA and Y chromosome variants of South Croatians present typical European maternal and paternal genetic landscapes, with certain connections to Central Asian populations, the Mediterranean region and a clearly evident Slavic component in the paternal gene pool (Peričić M et al. 2005). Since *HLA* loci have not been typed in our sample, we cannot measure the LD between *HLA* and *TNF* loci. However, our population shares a great similarity in genetic background to other European populations, and we assume that *HLA-TNF* LD relations are like those in other European populations. Nevertheless, specific environmental factors, lifestyle and the possible existence of specific population-based polymorphisms can alter LD relations and influence the *TNF* gene effect on susceptibility to T1DM. Therefore, although we cannot determine if the effects of *TNF* SNPs are primarily or secondarily to *HLA* loci, we believe these are valuable population specific results.

As well as LD between *TNF* and *HLA* genes, interaction of the *TNF* gene with other pro- and anti- inflammatory cytokine genes plays an integrated role in destruction of pancreatic beta cells (Koeleman et al. 2000, Kumar et al. 2007). Also, *TNF* promoter -308 and -238 polymorphic sites have been shown to enhance the rate of transcription of *TNF* gene (Kroeger et al. 1997). The functional effect of *TNF* promoter SNPs on TNF expression, immunomodulatory effects of TNF and its role in the destruction of beta cells support the idea of independent TNF influence in susceptibility to T1DM.

In conclusion, this study is the first to investigate two *TNF* gene promoter polymorphisms (-308 and -238) in a case-control sample from South Croatia. This study observed, with a limited significance, a higher frequency of *TNF* -308 A allele and a specific (-308A -238G) haplotype in T1DM patients. However, we did not find strong evidence of association of *TNF* promoter polymorphisms with T1DM. It is important to evaluate these findings in additional investigations with a larger sample that will be sufficiently powered to

detect a true association. Extended haplotype analysis across the region, and adjustment for *HLA* alleles, are needed to elucidate the contribution of *TNF* genotypes and haplotypes to T1DM susceptibility.

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