

RESEARCH NOTE

Study of interleukin-10 promoter region polymorphisms (–1082A/G, –819T/C and –592A/C) in type 1 diabetes mellitus in Turkish population

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Introduction

Type 1 diabetes mellitus (T1DM) is an autoimmune disease characterized by progressive destruction of islet β -cells (Atkinson and MacLaren 1994). It apparently arises from T-cell dependent destruction of β -cells in genetically susceptible individuals (Eisenbarth 2005). The cytokines secreted by CD4⁺ T helper type 1 and 2 (Th1 and Th2) cells may play a crucial role in the pathogenesis of T1DM (Almawi *et al.* 1999). Cytokines can be classified into two groups: Th1 (proinflammatory) cytokines such as TNF- α and interleukin 1, and Th2 (anti-inflammatory) cytokines such as interleukin 10 and interleukin 4 (Keen 2002). Although the exact roles that Th1 and Th2 cells play in IDDM pathogenesis remain to be established, it was suggested that Th1 cytokines promote, whereas Th2 cytokines protect from the diabetes progression (Rapoport *et al.* 1993; Pennline *et al.* 1994). It is thought that the onset and progression of T1DM results from an altered balance between Th1 and Th2 cells (Almawi *et al.* 1999).

IL-10 is a pleiotropic Th2 cytokine that is usually considered to have a role in the downregulation of cell mediated and cytotoxic inflammatory responses, thus being a potent anti-inflammatory mediator. It has been suggested that Th2 induced component of anti- β cell immunity is mediated principally by IL-10 (Lee *et al.* 1994, 1996; Pakala *et al.* 1997). Approximately 75% of the variation in IL-10 production appears to be genetically determined (Westendorp *et al.* 1997). The gene encoding IL-10 has been mapped to chromosome 1q. Several polymorphic sites within the promoter region have been described, including two microsatellite polymorphisms and three biallelic polymorphisms at

positions: –1082, –819, and –592 from the transcription start site (Turner *et al.* 1997; Eskdale *et al.* 1998). Inter-individual differences in cytokine production appear to be related to allelic polymorphisms of cytokine genes (D'Alfonso *et al.* 2000). IL-10 promoter SNP genotype and haplotype frequencies appear to exhibit different distributions according to ethnicity (Moraes *et al.* 2003; Pyo *et al.* 2003; Urcelay *et al.* 2004). The genotype frequency of IL-10 polymorphisms has been found to be different in Caucasians and Asians (D'Alfonso *et al.* 2000; Mok *et al.* 1998). The distribution of alleles for these SNPs in Spanish population was, however, similar to other Caucasians (Urcelay *et al.* 2004).

We hypothesized that IL-10 gene polymorphism may participate in susceptibility to, or clinical presentation of T1DM in Turkish population as well.

Materials and methods

Subjects

We analysed two independent sample sets. The first comprised 117 randomly selected unrelated T1DM patients (62 men and 55 women, average age at onset 18.22 years old, range 16–75 years old, mean age 39 ± 16.62 years old from the endocrinology and metabolism clinic of Gazi University Hospital, Ankara, Turkey. The second comprised a local population of 116 unrelated healthy volunteers representing the same regional population pattern (50 men and 66 women, range 15–68 years old, mean age 37.01 ± 11.25 years old) with no family history of diabetes as controls. All subjects were studied after giving informed consent. The diagnosis of T1DM was based on patients' clinical features and laboratory data according to the criteria of the American Diabetes Association (ADA) (Expert Committee 2003). The study protocol was approved by the Gazi Faculty of Medicine Ethics Committee.

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Genotyping of *IL-10* polymorphisms

Genomic DNA was extracted from peripheral blood samples with a DNA extraction kit according to the manufacturer's instructions (Metis Biotechnology, Ankara, Turkey). Mismatched PCR-RFLP was used to genotype the polymorphic sites at *IL-10* -1082(A/G), *IL-10* -819(T/C) and *IL-10* -592(A/C). The polymerase chain reaction (PCR) was performed in 50 μ L of reaction volume containing 2.5 μ L of DNA, 0.5 μ M of each primer (forward: 5'-GACAACACTACTAAGGCTcCTTTGGGA-3' and reverse: 5'-GTGAGCAAACCTGAGGCACAGAAAT-3' for *IL-10* -1082(A/G) and -819T/C; forward: 5'-TGCAGACTACTCTTACCCACTTCC-3' and reverse: 5'-AATAATTGGGTCCC CCAAC-3' for *IL-10* -592(A/C) (Ma *et al.* 2005)), 200 μ M of deoxynucleoside triphosphate (dNTP), 5 μ L of 10 \times PCR buffer (75 mM Tris-HCl (pH 8.8), 200 mM (NH₄)₂SO₄, 0.1% Tween-20), 2.5 mM MgCl₂, 1 U of Taq DNA polymerase (Fermentas, Vilnius, Lithuania), and water to a total volume of 50 μ L. The primers in lower case letters denote the mismatched position that is required for subsequent restriction digestion for the differentiation of the polymorphisms. The cycling conditions were 5 min at 94°C, followed by 30 cycles consisting of 94°C for 15 s, annealing for 15 s (at 55°C) and 72°C for 30 s. The final elongation step was performed at 72°C for 5 min. For restriction enzyme digestion, 15 μ L of the PCR product was digested by 10 units of the required enzyme (*Bse*LI for *IL-10* -1082 (A/G), *S*spI for *IL-10* -819(T/C) and *Rsa*I for *IL-10*

-592(A/C)) in the presence of the accompanying buffer in a final volume of 17 μ L and incubated overnight at the temperature at which activity of the enzyme is optimal. After digestion, the polymorphism was visualized by separating the DNA fragments in a 4% agarose gel that was stained with ethidium bromide and illuminated by UV. To validate the mismatched PCR-RFLP results, direct sequencing (ABI, Foster City, USA) was performed on 20% of the samples.

Statistical analysis

Statistical analysis of genotype distribution and allele frequencies was performed by SPSS for Windows 15.0 (SPSS, Chicago, USA). Maximum-likelihood haplotype frequencies were estimated by applying the expectation-maximization (EM) algorithm implemented by the Arlequin 3.1 (Excoffier *et al.* 2005) and SNPStats (Sole *et al.* 2006) software packages. The frequencies of alleles, genotypes and haplotypes in different groups were compared using the chi-squared test (or Fisher's exact probability test) with contingency tables to obtain the *P* values, odds ratios (OR) and 95% confidence intervals (c.i.) were calculated. Findings were considered statistically significant at *P* < 0.05.

Results

No significant deviation of genotype frequencies from the Hardy-Weinberg equilibrium was noted in either the case or control groups (*P* > 0.05). Table 1 illustrates the

Table 1. The frequencies of *IL-10* gene promoter polymorphism in patients with type 1 diabetes and control subjects.

Genotypes	Controls (n = 116)(%)	Cases (n = 117)(%)	<i>P</i>	OR (95% c.i.)	<i>P</i>
-1082			0.060		
AA	48 (41.4)	55 (47)		1	
AG	47 (40.5)	53 (45.3)		0.98 (0.57-1.71)	0.955
GG	21 (18.1)	9 (7.7)		0.37 (0.16-0.89)	0.024
Alleles					
A	143 (61.6)	163 (79.7)		1	
G	89 (38.4)	71 (30.3)		0.70 (0.48-1.03)	0.068
-819			0.886		
CC	58 (50)	56 (47.9)		1	
CT	44 (37.9)	48 (41)		1.13 (0.65-1.96)	0.663
TT	14 (12.1)	13 (11.1)		0.96 (0.42-2.23)	0.927
Alleles					
C	160 (69)	160 (68.4)		1	
T	72 (31)	74 (31.6)		1.03 (0.69-1.52)	0.891
-592			0.772		
CC	56 (48.3)	52 (44.5)		1	
CA	48 (41.4)	50 (42.7)		1.12 (0.65-1.94)	0.681
AA	12 (10.3)	15 (12.8)		1.35 (0.58-3.14)	0.491
Alleles					
C	160 (69)	154 (66)		1	
A	72 (31)	80 (4)		1.15 (0.78-1.70)	0.468

OR, odds ratio; c.i., confidence interval.

Table 2. Haplotype frequencies in patient and control subjects.

Haplotype	Controls		Cases		OR (95% c.i.)	P
	Frequency	2n = 232	Frequency	2n = 234		
G C C	0.3836	90	0.3034	70	1	
A T A	0.3016	70	0.3119	72	1.32 (0.84-2.08)	0.227
A C C	0.2973	68	0.3503	82	1.55 (0.99-2.43)	0.055
A C A	0.0087	2	0.0300	8	5.14 (1.06-24.99)	0.045
A T C	0.0087	2	0.0044	2	1.29 (0.18-9.36)	1.000

OR, odds ratio; c.i., confidence interval.

frequencies of *IL-10* gene promoter polymorphism in patients with T1DM and control subjects. No significant difference in the genotype and allele frequencies of polymorphic sites at *IL-10* -819 (T/C) and *IL-10* -592 (A/C) was found between T1DM patients and healthy controls. At the *IL-10* -1082 (A/G) polymorphic site, GG genotype was significantly more prevalent in control group ($P = 0.024$), although no significant difference in the overall distribution of three genotypes was found between T1DM patients and healthy controls ($P = 0.060$). Frequencies of three common haplotypes, GCC, ATA and ACC were similar in patient and control groups (table 2). Furthermore, we assigned ACA and ATC haplotypes with lower frequencies (under 1%) in Turkish population.

Discussion

In our study, as mentioned above, no significant difference in the genotype and allele frequencies of *IL-10* -819 (T/C) and *IL-10* -592 (A/C) polymorphisms was found between T1DM patients and controls. At the *IL-10* -1082 (A/G) polymorphic site, the frequencies of GG genotype in cases and controls showed significant difference between these two groups. This genotype was more prevalent in control group. Thus we can conclude that the G allele is a protective allele and genotype 'GG' has a protective effect with a significant P value for a negative association with T1DM.

These polymorphisms might configure several different haplotypes, but we identified three common haplotypes, GCC (38.4%), ATA (30.2%) and ACC (29.7%), spanning the *IL-10* gene promoter region. Further, we assigned ACA and ATC haplotypes with lower frequencies (under 1%) in Turkish population. At the promoter region, the well-documented haplotype structure with only three haplotypes (GCC, ACC and ATA) was also demonstrated formerly in Turkish population and all three haplotypes were distributed relatively homogenously (Yilmaz *et al.* 2005). The evaluation of estimated frequencies of three common haplotypes structure (GCC, ACC and ATA) did not confirm any significant association of T1DM with different haplotypes in our study (table 2). However, haplotype ACA seems statistically more prevalent in T1DM patients, considering the rarity of this haplotype (found only in one subject of control group and in four T1DM patients). Yet, before speculating on its role in the susceptibility to the disease, the number of sub-

jects should be greatly increased and complementary studies with larger samples will be needed to substantiate the conclusion of the association of this haplotype with T1DM.

Tegoshi *et al.* (2002) did not observe a significant difference in *IL-10* -592 genotype and allele frequencies between control subjects and T1DM patients in the Japanese population. Studies conducted in France and Spain did not confirm any significant association of T1DM with different haplotypes or genotypes of *IL-10* promoter polymorphisms in Caucasians (Urcelay *et al.* 2004; Reynier *et al.* 2006). A genetic association between these three SNPs in the *IL-10* promoter region and T1DM has been reported in Japanese populations, showing that -819 and -592 polymorphisms were associated with age at onset of disease (Ide *et al.* 2002).

The discrepancies between our results and the previous studies may be explained by clinical heterogeneity. It has been proposed that variable production of Th2 cytokines including IL-10 may influence both the degree of β -cell destruction and the age of clinical onset (Ide *et al.* 2002). It is also possible that insufficient numbers of patients and/or controls may be the cause of this discrepancy. It is also possible, moreover, that several alternative disease pathways may be present in humans based on differing molecular mechanisms among ethnic groups.

Acknowledgements

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