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Tumor necrosis factor-alpha gene promoter −308G/A and −238G/A polymorphisms in Mexican patients with type 2 diabetes mellitus

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Abstract. The association between some Tumor necrosis factor-alpha (TNF-α) promoter polymorphisms and Type 2 diabetes mellitus (T2DM) remains controversial. Ethnic differences may play a role in these conflicting results. The aim of this study was to investigate the association between −308G/A and −238G/A polymorphisms located in the promoter region of the TNF-α gene and T2DM in Mexican mestizo patients. Nine hundred four individuals (259 patients with T2DM and 645 controls) were genotyped for the −308G/A and −238G/A polymorphisms by PCR–RFLP. We found that the −238A allele increased the risk of developing T2DM in Mexican patients (OR = 1.57, 95% CI: 1.07–2.29; $p = 0.018$). Moreover, we found that the frequency of the GA haplotype (created by the −308G and −238A alleles) was significantly increased in patients with T2DM when compared with controls (OR = 1.56, 95% CI: 1.05–2.31; $p = 0.026$). Our results suggest that the −238G/A polymorphism and a specific haplotype (GA) are genetic risk factors for the development of T2DM in Mexican population.

Keywords: Haplotype, Mexican patients, polymorphism, TNF-alpha, Type 2 diabetes mellitus

1. Introduction

Type 2 diabetes mellitus is a major worldwide public health problem. The International Diabetes Federation estimates that 285 million people have the disease in 2010, which represents 6.4% of the global adult population [1]. It has been suggested that inflammatory cytokines impair insulin signalling; therefore, genes that regulate cytokine responses are candidate genes for association with insulin resistance and T2DM [2, 3]. Tumor necrosis factor-alpha is a cytokine originally identified as the principal agent causing tumor necrosis in bacterially infected animals [4]. It was established that TNF-α is mainly synthesized and secreted from macrophages infiltrated in the adipose tissue and plays a key role in the pathogenesis of obesity and in the insulin-resistant state [5,6]. TNF-α can cause insulin resistance *in vitro* and *in vivo* and may affect carbohydrate metabolism by decreasing both insulin-stimulated

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autophosphorylation and tyrosine kinase activity of the insulin receptor in muscle and adipose tissue [7,8].

Two bi-allelic polymorphisms previously identified in the TNF-α promoter region have been considered to play an important pathogenic role in T2DM, insulin resistance and, obesity [9,10]. The first of these polymorphisms is located at nucleotide position -308 (rs1800629) and involves the substitution of a guanine with an adenine [11]. The second polymorphism also involves a G>A substitution, in this case at nucleotide position -238 (rs361525) [12]. Because it has been suggested that the -308G/A and -238G/A polymorphisms are associated with insulin resistance, obesity, and T2DM in different ethnic groups [9,10], the purpose of this study was to examine the association between these polymorphisms and T2DM in Mexican diabetic subjects.

2. Materials and methods

2.1. Subjects

This study included 645 unrelated healthy controls randomly selected among subjects aged above 35 years old with no personal or family history of T2DM or Type 1 diabetes mellitus who came to the Hospital for general health check-ups, as well as 259 unrelated patients with T2DM aged 35–75 years and a disease history of at least five years to avoid misclassifications. Patients with T2DM were recruited from the Endocrinology Services at Hospitals from Instituto Mexicano del Seguro Social. Diagnosis of T2DM was based on American Diabetes Association criteria [13]. All subjects were Mexican mestizos living in four states of western Mexico (Colima, Jalisco, Michoacán, and Nayarit). The definition of a Mexican mestizo, according to the National Institute of Anthropology, is a person who was born in the country and has a Spanish-derived last name, with family antecedents of Mexican ancestors at least back to the third generation [14]. Written informed consent was obtained from all study participants for the use of their blood samples for the investigation. The study protocol was approved by the Local Ethics Committee (#2000-03-02-033).

2.2. DNA isolation and genotyping

Genomic DNA was isolated from peripheral blood leukocytes using the standard protocol of Gustincich et al. [15] and stored in Tris-EDTA buffer pH 8.0 at -20°C until processing. The -308G/A and -238G/A polymorphic loci were detected by PCR–RFLP. For analysis of both polymorphisms, 100 ng of genomic DNA were amplified and alleles were detected using mutagenic primers containing a single base-pair mismatch adjacent to the polymorphic site to introduce a restriction site into the wild-type nucleotide sequence after amplification.

The genomic region encompassing the -308G/A polymorphism was amplified using the following primers: forward 5'–AGG CAA TAG GTT TTG AGG GCC AT–3' and reverse 5'–TCC TCC CTG CTC CGA TTC CG–3', and PCR conditions were as previously described [11]. PCR products were digested with *NcoI* restriction enzyme according to the manufacturer's instructions (New England BioLabs, Ipswich, MA, USA). The -308G allele contains an *NcoI* restriction site not present in the -308A allele; thus, in the presence of the -308G allele, the PCR product (107 bp) is cut into two fragments of 80 and 27 bp in length. To identify the -238G/A polymorphism, we used the following primers: forward 5'–AGA AGA CCC CCC TCG GAA TC–3' and reverse 5'–ATC TGG AGG AAG CGG TAG TG–3'. The -238A allele did not contain an *MspI* restriction site. PCR products were digested with *MspI* restriction enzyme according to the manufacturer's instructions (New England BioLabs, Ipswich, MA, USA), which yielded a 152 bp fragment if the variant was present (–238A) and two fragments of 133 and 19 bp for the wild-type allele (–238G) [16]. Enzyme digestion products were visualized on a 6% polyacrylamide gel stained with silver nitrate. To test for the presence of random distribution of the polymorphisms, Hardy-Weinberg equilibrium was tested with 1 degree of freedom (1 d.f.) for these markers in healthy controls.

The haplotypes formed by these two polymorphisms of the TNF-α gene were inferred by considering -308G/A as the first position and -238G/A as the second position. This generated four haplotypes: haplotype GG (-308G, -238G); haplotype GA (-308G, -238A); haplotype AG (-308A, -238G), and haplotype AA (-308A, -238A).

2.3. Statistical analysis

Genotypic and allelic distributions for both polymorphisms were determined by direct gene counting and are presented as simple frequencies. Chi-squared tests were used to evaluate the Hardy-Weinberg equilibrium and to compare the allele, genotype, and haplotype fre-

Table 1
Genotype and allele distribution of TNF-α -308G/A and -238G/A polymorphisms in patients with T2DM and controls

Polymorphism	Allele / genotype	Controls n (%)	T2DM n (%)	p-value	OR (95% CI)
-308 G/A	G	1215 (94.2)	481 (92.9)		Reference
	A	75 (5.8)	37 (7.1)	0.289	1.25 (0.83–1.87)
	G/G	573 (88.8)	225 (86.9)		Reference
	G/A	69 (10.7)	31 (12.0)	0.558	1.14 (0.73–1.80)
	A/A	3 (0.5)	3 (1.1)	0.238	2.55 (0.51–12.71)
–238 G/A	G	1213 (94.0)	471 (90.9)		Reference
	A	77 (6.0)	47 (9.1)	**0.018***	**1.57 (1.07– 2.29)**
	G/G	571 (88.5)	220 (84.9)		Reference
	G/A	71 (11.0)	31 (12.0)	0.585	1.13 (0.72–1.78)
	A/A	3 (0.5)	8 (3.1)	**0.003****	**6.92 (1.82–26.33)**
Recessive Model	G/G + G/A	642 (99.5)	251 (96.9)		Reference
	A/A	3 (0.5)	8 (3.1)	**0.003****	**6.82 (1.79–25.92)**

T2DM: Patients with type 2 diabetes mellitus. The first number is the number of patients or controls (genotype) and chromosomes (allele). The numbers in parentheses are the percentages. *Significant difference using Chi-squared test. **Significant difference using Fisher's exact test.

quencies between patients with T2DM and control subjects. Fisher's exact test was employed if the number in any cell of the contingency table was less than five. A commercially available statistical software package was used for data analysis (IBM SPSS STATISTICS 18, Inc. Chicago, IL, USA). To infer haplotypes for unrelated patients with T2DM and healthy individuals, we used the Arlequin software [17]. Results were regarded as significant when $p < 0.05$. Odd ratios (ORs) with 95% Confidence intervals (CIs) were calculated.

3. Results

Genotype frequencies for the two polymorphisms among control subjects were confirmed to be in Hardy-Weinberg equilibrium ($p > 0.05$). Genotype and allele frequencies of TNF-α -308G/A and -238G/A polymorphisms in patients with T2DM and control subjects are shown in Table 1. Patients with T2DM and controls presented similar allele and genotype frequencies for the -308G/A polymorphism. Allele frequency analysis demonstrated that the -238A allele was more frequent in patients with T2DM than controls ($p = 0.018$). Patients bearing the -238A allele exhibited an increased risk of developing T2DM (OR $=$ 1.57, 95% CI: 1.07–2.29). Genotype distribution for the -238G/A polymorphism did show statistical difference between the two study groups ($p = 0.004$) (p value from 3×2 contingency table). Moreover, we observed an increased frequency of A/A genotype in patients when compared with controls ($p = 0.003$). Odds ratio shows that individuals bearing the A/A genotype have a risk of 6.92 for developing T2DM (95% CI: 1.82–26.32) assuming a co-dominant model (G/G vs. AA). This risk was constant (OR $= 6.82$, 95% CI: 1.79–25.92; $p = 0.003$) under a recessive model (G/G + G/A vs. A/A).

Haplotype frequencies of TNF-α -308G/A and -238G/A promoter polymorphisms in patients with T2DM and controls were inferred utilizing Arlequin software, and results of the haplotype analysis are depicted in Table 2. In the population under study, we observed the four possible haplotypes formed from TNF-α -308G/A and -238G/A polymorphisms. The GG haplotype was the most frequent in both study groups. We found that frequency of the GA haplotype was significantly increased in patients with T2DM with respect to controls ($p = 0.026$). Patients carrying the GA haplotype exhibited a risk of 1.56 for developing T2DM (95% CI: 1.05–2.31).

In addition to the above analysis, we also compared genotype and allele frequencies of the -308G/A and -238G/A polymorphisms observed in our study population with those described in other studies conducted in populations from America (Chilean and Caucasian American), Africa (Tunisian), Asia (Chinese, Japanese, Taiwanese, and Indian) and Europe (Finnish, Greek, Dutch, and British). Table 3 shows the results of the comparative analysis of allele frequencies in these populations.

4. Discussion

Substitution of G with A at nucleotide positions -308 and -238 in the TNF-α promoter region

Table 2
Estimated haplotype counts in patients with T2DM and controls

Haplotype	Controls $n = 1290$ (%)	T2DM $n = 518$ (%)	p-value	OR (95% CI)
GG	1143 (88.6)	438 (84.5)		Reference
GA	72 (5.6)	43 (8.4)	**0.026***	**1.56 (1.05–2.31)**
AG	71 (5.5)	33 (6.4)	0.375	1.21 (0.79–1.86)
AA	4 (0.3)	4 (0.7)	0.160	2.61 (0.65–10.48)

T2DM: Patients with type 2 diabetes mellitus. GG: −308G and −238G alleles. GA: −308G and −238A alleles. AG: −308A and −238G alleles. AA: −308A and −238A alleles. *Significant difference using Chi-squared test.

Table 3
Comparison of TNF-α −308G/A and TNF-α −238G/A allele frequencies with other populations with T2DM

Continent	Study population	Control /T2DM	TNF-α −308G/A				TNF-α −238G/A			
			Allele G		Allele A		Allele G		Allele A	
			Control	T2DM	Control	T2DM	Control	T2DM	Control	T2DM
	Present study*	**1290/518**	**0.942**	**0.929**	**0.058**	**0.071**	**0.940**	**0.909**	**0.060**	**0.091**
America	Chilean [26]	106/60	0.925	0.950	0.075	0.050	0.868	0.933	0.132	0.067
	American [21]	114/276	0.895	0.880	0.105	0.120^c	0.974	0.968	0.026	0.032^a
Africa	Tunisian [28]	299/195	0.831	0.853	0.169^c	0.147^c	NA	NA	NA	NA
	Chinese [23]	404/678	0.923	0.911	0.077	0.089^a	NA	NA	NA	NA
Asia	Japanese [22]	284/264	0.990	0.989	0.010^b	0.011^b	NA	NA	NA	NA
	Taiwanese [24]	374/514	0.941	0.916	0.059	0.084	0.992	0.987	0.008^c	0.013^c
	Indian [29]	202/198	0.490	0.538	0.510^c	0.462^c	NA	NA	NA	NA
	Finnish [33]	568/790	0.812	0.832	0.188^c	0.168^c	0.965	0.961	0.035^a	0.039^a
Europe	Greek [25]	78/64	0.910	0.953	0.090	0.047	NA	NA	NA	NA
	Dutch [31]	1154/158	0.819	0.785	0.181^c	0.215^c	NA	NA	NA	NA
	British [27]	344/560	0.800	0.787	0.200^c	0.213^c	0.943	0.917	0.057	0.083
	British [27]	890/274	0.804	0.800	0.196^c	0.200^c	0.926	0.927	0.074	0.072

T2DM: Patients with type 2 diabetes mellitus. *Reference group, $^a p < 0.05$, $^b p < 0.01$, $^c p < 0.001$. Comparison made using chi-square statistical test. NA: Not available.

enhances transcription of this cytokine in cultured cells [18,19], although other studies suggest otherwise [20]. However has also been previously suggested that increased expression of TNF-α in adipocytes may influence insulin sensitivity [5]. Because insulin resistance is one of the major critical factors in the pathogenesis of T2DM, in the present study we evaluated whether TNF-α polymorphisms are associated with the occurrence of T2DM.

In accordance with previous studies on T2DM [21–30], we found no association between TNF-α −308G/A polymorphism and T2DM. Although Heijmans et al. reported that this polymorphism was associated with T2DM in Dutch people, their patients were aged 85 years and over, which suggests that age contributed to this result, because adverse effects of mild alterations in TNF-α expression become apparent only in old age [31].

There have been relatively few studies of the relationship between the −238G/A variant and T2DM. Zeggini et al. [27] reported a borderline significant association between this polymorphism and T2DM. In accordance with these findings, although one limitation of the present study comprised that we had no opportunity to measure serum levels of TNF-α and metabolic parameters and in this way could provide a correlation between genotype and levels of cytokine, as well as metabolic parameters, we found that genotype and allele frequencies of the −238G/A polymorphism were different between patients and controls and that the −238A allele increased the risk of developing T2DM in our patients, thus suggesting a potential association of this polymorphism with T2DM. Our results also are in agreement with those described in obese individuals with T2DM, in whom the –238G/A polymorphism alters circulating free fatty acids and insulin resistance [32], both of which are T2DM-associated metabolic features. In contrast to these findings, the TNF-α −238G/A polymorphism was not associated with T2DM in Finnish [33] and Danish [34] populations. Recently, Feng et al. [10] published a meta-analysis in which reported a lack of association between −238G/A polymorphism and T2DM. However, in one of the populations analyzed by these authors, the

British population [27], there is a trend toward association with T2DM [10,27]. Interestingly, our study and performed in the British population had similar sample sizes under analysis. In addition, Feng et al. [10] also suggest that extended haplotype analysis is needed to explore the contribution of the TNF-α genotype, such as the study that we have conducted.

Two haplotype studies that included, among other polymorphic sites, the -308G/A and -238G/A variant, have been carried out; in one, no common haplotypes were found [32], and in the other, the ACCGGC haplotype harbouring the major alleles of -308G/A and -238G/A polymorphisms was reported as associated with T2DM [30]. Another haplotype study of the -308G/A and -238G/A TNF-α polymorphisms was carried out in healthy Danish subjects; in this study, the authors only reported that three haplotypes were observed (GG, GA, and AG) [34]. We found that the GA haplotype carrying the minor allele of the -238G/A polymorphism, allele that was also found to be independently associated with T2DM in our studied patients, is a genetic risk factor for the development of T2DM; therefore, the present study is of particular importance, because, to our knowledge, this is the first report of an association of a specific haplotype defined by -308G/A and -238G/A TNF-α gene polymorphisms with T2DM in Mexican patients.

Allele frequencies of the -308G/A polymorphism in our patients with T2DM were significantly different from those found in Caucasian-American individuals with T2DM, may be due to the different degrees of admixture in the two populations. With respect to Asian populations, we found differences with Japanese and Indian populations. Finnish, Dutch and British populations also showed significant differences when compared with the frequencies observed in our study, which may be because of little or no documented migration, particularly of Finnish or Dutch people, to Mexico. The -238G/A polymorphism showed a similar pattern to that of the -308G/A polymorphism, because we found significant differences when we compared the allele frequencies observed in our Mexican patients with T2DM with those reported in Caucasian-American subjects and with populations from Asia (Taiwanese and Indian) and Europe (Finnish). Our results suggest that the Mexican mestizo population has very particular genetic characteristics that differentiate it from other populations worldwide.

Many studies have focused on the association between TNF-α and T2DM; however, these have led to contradictory results, and the association between TNF-α and T2DM remains controversial. Ethnic differences between study subjects appears to contribute to these discrepancies, because a differential distribution of TNF-α -308G/A and -238G/A polymorphisms has been observed in different populations. However, because T2DM is a polygenic disease, the genes involved in the development of the phenotype (susceptibility genes) exert only a partial effect; thus, the effect of a single gene is not sufficient to cause T2DM. Only the additive effect of these genes, in certain combinations, confers genetic susceptibility; therefore, more extensive studies are needed to characterize precisely the role of genetic factors in the development of T2DM.

In summary, we have shown that the -238G/A polymorphism of the TNF-α gene and a specific haplotype (GA) are associated with T2DM in Mexican population. Moreover, our results also suggest that there is a strong heterogeneity in the frequencies of TNF-α polymorphisms among the different T2DM incidence areas.

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