Genetic polymorphisms of the tumor necrosis factor and lymphotoxin α in type 2 diabetes

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Objective: to investigate the frequency of the single-base change polymorphic variants identified in tumor necrosis factor (TNF) gene (-308 G/A) and lymphotoxin α (LTA) (+252 G/A) in patients with type 2 diabetes (T2D).
Methods: a prospective study in a Mexican-mestizo population of 51 patients with T2D and 48 healthy subjects was carried out. We took a peripheral blood sample from each individual for identification of the polymorphic genotypes by polymerase chain reaction.
Results: the genotype distribution in T2D was: TNF alpha homozygous 0 %; TNFG/A heterozygous 20 %; TNFG homozygous 80 %.
Conclusions: in regards to the TNF -308 G/A genotype, we found a significant difference (p = 0.012) with a bigger frequency in the group of patients. The health controls showed a higher frequency of TNF -308 G/G genotype (p = 0.034).

Key words
diabetes mellitus, type 2
alleles
genotype
polymorphisms, single nucleotide
tumor necrosis factors

A disease related with genetic aspects is type 2 diabetes (T2D), which has an alarming prevalence in Mexican population. The prevalence for T2D is 14.42 % (7.3 million diabetics).[1] In addition, the majority of previously diagnosed subjects with diabetes are at levels of poor control.[2]

In relation to tumor necrosis factor (TNF) polymorphisms, there are controversies concerning the participation of these genes in the development of T2D, TNF,[3-5] as well as for lymphotoxin α (LTA).[6,7] There are studies that relate the TNF -308G/A gene polymorphism with T2D and with other complications, such as the two-times greater risk in women for developing coronary heart disease,[8] which is strongly associated with a risk for diabetes, but not with cardiovascular mortality at old age.[9]

Those studies stated that TNF genotype is linked with the common phenotype of T2D.[4] Although other authors mention that genotype is not involved with the pathogenesis of T2D. Patients with T2D who are carriers of the A/A homozygous genotype can be more susceptible to the complications of diabetes, such as atheroesclerosis[10], insulin resistance and abdominal fat accumulation.[11] There is a bigger predisposition for non-insulin-dependent patients to develop proliferative diabetic retinopathy.[7] There is also a relationship with obesity, high blood pressure, lipid and glucose serum levels in patients with T2D.[12] The insulin resistance and the metabolic syndrome[13] are related with the pathogenesis of intrauterine insuline resistance and the birth weight in Danish Caucasian population.[14] The excessive fat accumulation in women with T2D[15] is between the most prominent features associated to TNF genotype linked with common phenotype of T2D. On the other hand, the LTA +252G/A gene has been associated with diminution in insulin resistance.[16]

The TNF/LTA locus has been a long-standing T2D candidate gene. T2D and obesity have been hypothesized to have an inflammatory basis.[17,18] Insulin resistance is associated with the increased plasma levels of proinflammatory cytokines, such as TNF and IL6, and with interactions between TNF and NF-kβ that lead to an increase of oxidative stress.[19-21] Because TNF plays an important role in T2D, it would be useful to know the frequency of polymorphisms for T2D. No studies have examined this topic in Mexican-mestizo population and this is the aim of this paper.

Polimorfismos genéticos del factor de necrosis tumoral y la linfotoxina α en diabetes tipo 2

Objetivo: investigar la frecuencia de variantes polimórficas con el cambio de una sola base identificadas en los genes del *tumor necrosis factor* (TNF) (-308 gamma/alfa) y de la linfotoxina α (LTA) (+ 252 G/A) en pacientes con diabetes tipo 2.

Métodos: se llevó a cabo un estudio prospectivo en una población mestizo-mexicana de 51 pacientes con DT2 y 48 sujetos sanos. Se tomó una muestra de sangre periférica de cada individuo para la identificación de los genotipos polimórficos mediante la prueba de reacción en cadena de la polimerasa.

Resultados: la distribución de genotipos en los pacientes con diabetes tipo 2 fue la siguiente: TNFA homocigoto 0 %; TNFG/A heterocigoto 20 % y TNFG homocigoto 80 %.

Conclusiones: se encontró mayor frecuencia del genotipo TNF -308 G/A, con una diferencia significativa (p = 0.012). Los sujetos sanos tuvieron mayor frecuencia (p = 0.034) del genotipo de TNF -308G/G si se comparan con el grupo de pacientes.

Palabras clave
diabetes mellitus tipo 2
alelos
genotipos
polimorfismos de una sola base
factores de necrosis tumoral

Methods

A prospective study in healthy subjects and outpatients with T2D was conducted and followed along a year. We selected 48 healthy subjects, who were approved by the selection process established by the official mexican norm for donation of blood and its components,[22] and 51 patients with T2D. All subjects were above legal age, without autoimmune diseases, without human immunodeficiency virus (HIV), and without chronic diseases different from T2D at the time of admittance into the study.

T2D patients belonged to the Internal Medicine (Out-patient) Service, Hospital General "Gaudencio González Garza", Hospital de Infectología and Family Medicine Unit 6 of the *Instituto Mexicano del Seguro Social*. Healthy subjects were pick up at the *Banco Central de Sangre* and laboratory tests were performed at the Infectology Research Unit.

All patients were interviewed to gather information on general data. Additional information collected included time of evolution of the diagnosis of T2D and date of inclusion in the study. Also, a letter of informed consent was formulated and signed previously by study participants, since the test to be performed did not form part of the protocol of T2D studies.

Healthy subjects did not sign the letter of informed consent because study samples comprised the same as those taken for routine testing for the blood donation procedure; the interview was also not applied to the latter. A 5 mL peripheral blood sample was drawn into a Vacutainer™ tube (Becton, Dickinson, Franklin Lakes, NJ, US) with EDTA as anticoagulant to all subjects. We obtained peripheral-blood mononuclear cells by centrifugation with the Ficoll density gradient method.[23]

Healthy subjects included blood donors, both sexes, younger than 60 years, apparently healthy at the interview, clinical examination and self-exclusion questionnaire, with negative serology for immunological and molecular biology: hepatitis *B*, hepatitis *C*, HIV infection by *Trypanosoma cruzi* and *Treponema pallidum*. The group of T2D included patients with diabetes diagnosis according to the American Diabetes Association criteria.

DNA was isolated with guanidine thiocyanate and phenol utilizing 500 μL of the TRIzol reagent (Gibco BRL) according to the procedure described by Chomczynski;[24] the reagent was a monophase solution of guanidine thiocyanate and phenol. The DNA was resuspended in 50 μL of distilled water after precipitation with ethanol at 75 %. This solution was heated at 55 ºC for 20 min. Its absorbency relationship was

Table I General characteristics of the study groups

	Type 2 diabetes		Healthy	
Age (years)				
Median	60		35	
Range	36-79		19-57	
Time of evolution (years)				
Median	10		0	
Range	6 months-33 years		0	
Body mass index				
Median	30.45		27.55	
Range	25.75-35.15		20.83-35.56	
Major complications	Urinary tract infection, hypertension		None	
Gender	*n*	%	*n*	%
Male	15	31	27	53
Female	33	69	24	47

determined at 260/280 nm, and we took 5 μL for DNA amplification by polymerase chain reaction (PCR).

Restriction fragment length polymorphism (RFLP) genotypic analysis with Nco I: once the genomic DNA was extracted, we proceeded to the amplification of specific regions of each polymorphism. We amplified a 107 base-pair (bp) fragment (positions 327 to 220) of the TNF promoter by PCR of each sample with primers A1 (5′-AGG CAA TAG GTT TTG AGG GCC AT- 3′) and A2 (5′-TCC TCC CTG CTC CGA TTC CC- 3′).[25] The A1 primer was designed to incorporate the TNF polymorphic site in an Nco1 restriction sequence. PCR was performed in 2-mL tubes with (2 ng) of genomic DNA in 5 μL of solution, which served as a mold for the 50-μL reaction mixture. This reaction mixture contained the following chemicals: 20 pM of each primer (A1 and 2) in 1 μL of solution, and 25 μL of Promega™ Master Mix that contains polymerase Taq, reaction buffer, magnesium chloride, nucleosides, and 19 μL of nuclease-free water. Amplification was carried out in a Biometra™ thermocycler at the following times: one cycle at 95 ºC for 2 min, and 35 cycles at 94 ºC for 30 sec, at 58 ºC for 45 sec, at 72 ºC for 30 sec, and finally, for 7 min at 72 ºC. PCR products were digested overnight at 37 ºC with Nco1 at 4 international units (IU)/μL of reaction. Restriction patterns were visualized under ultraviolet (UV) light after electrophoresis (70 V for 45 min) in agarose gel at 3.5 % dyed with ethidium bromide. Digestion by Nco1 of the PCR-amplified DNA produced two fragments: one of 20 base pairs (bp); the other of 87 bp of the TNF 308G allele, and a sole 107-bp fragment of the TNF -308A allele. In heterozygous, three bands were observed: one of 107 bp, one of 87 bp, and a third of 20 bp.

LTA: we amplified a 782-bp fragment of genomic DNA containing the Nco1 polymorphic site; this was amplified by means of PCR. PCR was carried out in 0.2-mL tubes with (2 ng) of genomic DNA in 5 μL of solution, and the following chemicals served as template: 20 pM of each of the primers, including primer 1 (5′ CCG TGC TTC GTG CTT TGG ACT A 3′) and primer 2 (5′ AGA GGG GTG GAT GCT TGG GTT C 3′)[26] in 1 μL of solution, 25 μL of Promega™ Master Mix that contains polymerase Taq, reaction buffer, magnesium chloride, nucleosides, and 19 μL of nuclease-free water. We performed amplification in a Biometra™ thermocycler at the following times: first, a denaturation step was conducted for 3 min at 95 ºC, followed by 37 cycles of a 30-sec denaturation at 95 ºC, for 30-sec annealing at 57 ºC, and a 45-sec extension at 72 ºC. A final extension was performed for 6 min at 74 ºC. PCR product was digested with 10 IU/μL Nco1 of endonuclease during 4 h at 37 ºC. Restriction patterns were visualized under UV light after electrophoresis (70 V for 45 min) with 1.5 % agarose gel dyed with ethidium bromide. Digestion

Table II Allelic frequency by group of individuals

Group	Total	*n*	%	*n*	%	*p* value	OR	95 % CI
	Allele	-308G		-308A				
T2D	102	92	90	10	10	0.65	1.31	0.54-3.2
Healthy	96	84	88	12	12			
	Allele	+252G		+252A				
T2D	102	32	31	70	69	0.049	1.98	1.02-3.8
Healthy	96	18	19	78	81			

T2D = type 2 diabetes

Showing frequencies, percentages, *p* values, odds ratios (OR), and 95 % confidence intervals (95 % CI).

by Nco1 of PCR-amplified DNA produced the following fragments: the original of 782 bp (patients homozygous for the LTA +252A allele, lacking the Nco1 site); three fragments of 782, 586, and 196 bp in length (heterozygous patients), or two fragments of 586 and 196 bp in size (homozygous patients for the LTA +252G allele). For calculating sample size, we used general allele frequencies in healthy controls that were reported in previous studies.[25,26] The estimated difference for alleles TNF -308A and LTA +252A in patients with chronic TB is 25 %; we applied the stadigraph z formula,[27] with 38 patients for TNF and 48 patients for LTA per group (α, 0.05; β, 0.20). Because we determined the two genotypes (α and β) in the same patient, we concluded that with a 51-patient group with T2D, our necessary sample was complete. We added a group of 48 healthy subjects. The study was approved by Hospital de Infectología Research Committee. Descriptive statistics, utilizing central tendency and dispersion measurements as quantitative variables were used. To determine associations of the variables in the study groups, we utilized contingency tables and independent χ^2 for statistical analysis and the Wilcoxon sum of ranges (the Mann-Whitney U test). Statistical significance was considered with a $p \leq 0.05$. Odds ratio (OR) was calculated with the 95 % confidence interval (95 % CI).

Results

The general results of studied patients are presented in table I. We found a higher number of male in the healthy group ($p < 0.05$) due to a greater percentage of healthy male blood donors than female donors. In healthy subjects, median age found was 35 years ($p < 0.01$) due to that a selection process exists for healthy donors of blood and its components, in which the age limit ranges from 18 to a maximum of 65 years.

Allele frequency

We analyzed 102 alleles in the T2D group, of which 92 corresponded to allele TNF -308G and 10 to allele TNF -308A; we found no statistically significant differences compared with healthy subjects (table II). For the LTA gene, we studied 102 alleles in the T2D group, of which 32 (31 %) corresponded to allele LTA +252G, presenting a statistically significant difference (p, 0.049) with respect to the healthy group, and 70 (69 %) to allele LTA +252A (table II).

We studied a total of 51 genotypes of the TNF -308 promoter gene in T2D, with a general frequency of 41 (80 %) for the TNF -308G/G homozygous genotype, 10 (20 %) for the TNF -308G/A heterozygous genotype, and zero for the TNF -308A/A homozygous genotype (table III). The group fell within Hardy-Weinberg equilibrium, with non-significant values by χ^2 test for the genotype observed and expected for the polymorphism tested. We studied a total of 51 genotypes for the LTA +252G/A gene, with a general frequency of two (4 %) for the G/G homozygous genotype, 14 (29 %) for the G/A heterozygous genotype, and 32 (67 %) for the A/A homozygous genotype (table III). Statistical significance was not found. The general results for the TNF genotypes are shown in table IV. The group fell within Hardy-Weinberg equilibrium, with non-significant values by

Table III TNF and LTA genotype frequency by group of individuals

Group	Total	-308GG		-308GA		-308AA		+252GG		+252GA		+252AA	
	n	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
T2D	51	41	80	10	20	0	0	5	10	22	43	24	47
Healthy	48	41	85	2	5	5	10	2	4	14	29	32	67

n = number of genotypes, T2D = type 2 diabetes

χ^2 test for the genotype observed and expected for the polymorphism tested.

Discussion

In this study, the heterozygous genotype TNFA -308G/A (moderate TNF producer) presented with greater frequency in patients with T2D than in healthy subjects and it was statistically significant. On the other hand, the homozygous genotype of TNFA 308G/G (TNF hypoproducer) was more frequent in healthy subjects than in patients. The group of subjects with T2D was studied by evolution time, age, and gender, and no significant differences were found in terms of TNF/LTA genotype frequency. Likewise, we found no statistical significance for LTA +252G/A on comparison with the study groups.

It is pertinent to recall that this study was carried out only in Mexican population, a population that has not been reported with these single nucleotide polymorphisms (SNPs). The fact that several authors find or do not find a relationship with risk for T2D in these variants is due to the population type studied, because the disease possesses a very important ethnic factor. Heijmans *et al.*[9] report that a subject who is TNF -308A/A homozygous genotype (TNF hyperproducer) has a 4.6-times greater risk (95 % CI, 1.613.3) of presenting T2D than a subject with the TNF -308G/G homozygous genotype (TNF hypoproducer). In Taiwanese population, a relationship is not found for the TNF -308A/A genotype with the genesis of T2D, but a greater susceptibility to complications such as atherosclerosis had been found.[10] Similarly, it has been mentioned that the genotypes of the promoter of TNF and T2D are controversial because there are discrepancies among the different studies reported.[28] Ethnic differences play a very important role, because distribution of TNF-promoter polymorphisms is different among study subjects with diverse racial origin.[10,11] In our study, in the group of patients with T2D, we did not detect subjects with the TNF -308A/A genotype, without a resemblance in terms of frequencies in other similar studies carried out in patients with diabetes.

Table IV General results of the genotypes obtained from TNF genes

TNF -308 A/G genotypes	Type 2 diabetes		Healthy		*p* value	OR	95 % CI
	n = 51	%	*n* = 48	%			
-308GG	41	80	41	85	0.0345	0.2	0.04-10.9
-308GA	10	20	2	4			
-308GA	10	20	2	4	0.012	25	1.8-346
-308AA	0	0	5	10			
-308GG	41	80	41	85	0.057	11	0.58-205
-308AA	0	0	5	10			

Showing frequency, percentage, *p* value, odds ratios (OR), and 95 % confidence intervals (95 % CI).

However, in the group of patients with diabetes, we found an increased frequency in the heterozygous genotype of 20 % (10/51 patients) with a significant difference ($p = 0.012$), a number that is in agreement with the distribution found by Koch *et al*[29] in Germany in a group of patients with T2D, without reporting association with any genotype in particular. This differs from the results of Ishii *et al*.[12], who found in Japan only 3.3 % of patients with diabetes with the TNF -308G/A genotype, but who reported a tendency toward the TNF -308G/G genotype in patients with insulin resistance. Wybranska *et al.*[30] reported in Poland a greater frequency than that we found in our population of subjects with diabetes (46 % with the TNF -308G/A genotype,) as a risk factor for predisposing to insulin resistance, which leads us to think that this variable of the gene can be more frequent in subjects with T2D and is found to be ethnically conditioned. On the other hand, in Chilean and English patients, no association was found between T2D and TNF/LTA gene-region polymorphisms.[31,32]

The polymorphism frequencies reported in the literature include, in their great majority, solely the TNF gene, mainly in the promoter region. Concerning the LTA gene, it has only been reported as associated with Gram negative-related diseases (among these, sepsis and multiple organ failure). In T2D, there are few reports that associate it with the pathogeny of the disease. Only Kankova *et al.*[6] mention that they found a relationship of polymorphisms with low-density cholesterol and total cholesterol in the non-obese group of subjects. However, in our study, there was no significant difference with any LTA genotype.

In conclusion, in our study, the most frequently found genotype was TNFA -308G/A (moderate TNF producer) in the patients. These levels can be associated with insulin resistance, inflammation, and oxidative stress. A larger sample size is needed to study the association between gene polymorphisms and diseases. These results must be confirmed by further studies with larger sample sizes.

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