Association Analysis Between –308G/A and –238G/A TNF-Alpha Gene Promoter Polymorphisms and Insulin Resistance in Mexican Women With Gestational Diabetes Mellitus

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Background: Gestational diabetes mellitus (GDM) is characterized by insulin resistance. It has been described that tumor necrosis factor α (TNF-α) plays a key role in the pathogenesis of insulin resistance; moreover, increased levels of this proinflammatory cytokine have been reported in women with GDM. Therefore, this study was aimed to assess the presence of associations between the –308G/A and –238G/A polymorphisms and specific haplotypes of the TNF-α gene promoter region and insulin resistance in Mexican women with GDM.

Methods: This study included 51 women with GDM and 44 pregnant women with normal glucose tolerance. Measurements of anthropometric parameters and biochemical estimations were performed. We genotyped the TNF-α –308G/A and –238G/A polymorphisms using polymerase chain reaction–restriction fragment length polymorphism analysis.

Results: The genotype and allele frequencies of both polymorphisms did not differ significantly between the women with GDM and the controls. However, we found that the frequency of the AG haplotype was significantly increased in the patients with GDM compared with controls ($P = 0.019$; odds ratio, 4.11; 95% confidence interval, 1.31–12.85). In patients with GDM, we observed that insulin levels and homeostasis model assessment of insulin resistance were significantly higher in women bearing the G/G genotype than in carriers of the G/A and A/A genotypes of the –308G/A polymorphism ($P = 0.022$ and $P = 0.043$, respectively).

Conclusions: Our results suggest that the G/G genotype of the TNF-α –308G/A polymorphism increases insulin levels and insulin resistance in women with GDM and that the AG haplotype is a genetic risk factor for GDM in our study population.

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Gestational diabetes mellitus (GDM) is defined as any degree of glucose intolerance with onset or first recognition during pregnancy.[1] In the Mexican population, 3.2% to 8.0% of pregnant women have a diagnosis of GDM.[2] Gestational diabetes mellitus is characterized by peripheral insulin resistance,[3,4] which is not compensated by an increase in insulin secretion to maintain glucose homeostasis.[4,5] It has been suggested that inflammation is a central feature of the insulin resistance and evidence of inflammatory deregulation can be observed as early as the first trimester among pregnant women who later develop GDM.[6,7]

The tumor necrosis factor α (TNF-α), which is a proinflammatory cytokine, is synthesized and secreted from macrophages infiltrated in the adipose tissue and in the placenta.[8] Several studies have shown that TNF-α plays a key role in the pathogenesis of insulin resistance[9]; moreover, increased levels of TNF-α have been reported in women with GDM.[10–12] Hence, TNF-α may be involved in the pathogenesis of GDM.

Several TNF-α promoter polymorphisms have been identified and implicated in the regulation of TNF-α transcription. Single nucleotide polymorphisms (SNPs) located at positions –308 (rs1800629) and –238 (rs361525) of the promoter region of the TNF-α gene have been well studied. At both polymorphic sites, a guanine (G) is substituted by an adenine (A), and this change has been related to the modification of the transcription-binding site that affects the rate of transcription.[13,14] Because a close relationship between the pathophysiology of GDM and that of type 2 diabetes mellitus (T2DM) has been described,[4] it has also been of interest to investigate the genetic basis of GDM.[15] In particular, in recent years, the relationship between SNPs in cytokine has attracted a growing interest.[16–19] Based on this knowledge, we decided to analyze the genotype and allele distributions of the TNF-α –308G/A and –238G/A gene promoter polymorphisms in patients with GDM and in pregnant nondiabetic women, and to assess the existence of associations between insulin resistance and the presence of these polymorphisms or specific haplotypes.

MATERIALS AND METHODS

This study included 51 unrelated pregnant women with GDM and 44 unrelated pregnant women with normal glucose

tolerance, all them recruited during the third trimester of gestation. The age of all women ranged from 16 to 46 years, and women were Mexican mestizos living in western Mexico (Jalisco State). 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.[20] Written voluntary consent to participate in molecular genetics analyses was obtained from all women, and the study protocol was approved by the local ethics committee (#2000-03-02-033).

The patients with GDM were recruited from the high-risk pregnancy unit at the Obstetrics and Gynecology Hospital of the Instituto Mexicano del Seguro Social, under the following inclusion criteria: diagnosis of GDM according to the American Diabetes Association criteria, which included a 2-hour 75-g oral glucose tolerance test at 24 to 28 weeks of gestation, the cutoff values being 92 mg/dL (5.1 mmol/L) or more, fasting, 180 mg/dL (10.0 mmol/L) or more at 1 hour, and 153 mg/dL (8.5 mmol/L) or more at 2 hours[1]; and no personal or family history of type 2 diabetes mellitus or type 1 diabetes mellitus. Women with normoglycemic pregnancies had normal glucose tolerance in early and advanced pregnancy, no personal and family history of any disorder associated with glucose intolerance, and were selected randomly and referred to us during the labor period or after rupture of the amniotic membranes.

Exclusion criteria were the following: fetal disorder, multiple pregnancy, parity of more than four, in vitro fertilization treatment, arterial hypertension, or any other concomitant disease (chronic, acute, or infectious).

Age and anthropometric measurements, including height and current weight, were obtained. Body mass index was calculated as weight/height^2 (kg/m^2). Glucose concentration was determined using the glucose oxidase method, and insulin concentration was determined using a radioimmunoassay (Diagnostic Products Corp, Los Angeles, CA). To assess insulin resistance, homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated as follows: [fasting insulin (μU/mL)] × [fasting glucose (mmol/L)]/22.5. The HOMA-IR index is a mathematical model designed by Mathews et al.,[21] and validated later in GDM.[22] Serum levels of total cholesterol, high-density lipoprotein, and triglycerides were measured using an Abbott Aeroset autoanalyzer with original kits. Low-density lipoprotein levels were calculated using the Friedewald equation.

Genomic DNA was extracted from leukocytes from 5 mL of peripheral blood according to the Gustincich method.[23] The polymorphisms were identified using polymerase chain reaction (PCR)-restriction fragment length polymorphism.[24] 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 CTG CTC CGA TTC CG–3′. Polymerase chain reaction products were generated in a 10-μL reaction volume containing 50 ng of genomic DNA, 1× PCR buffer, 2 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP, 1 μmol/L of each primer, and 0.25 U of Taq DNA polymerase (Invitrogen Corporation, Carlsbad, CA). Cycling conditions consisted of an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds and a final elongation step at 72°C for 1 minute. Polymerase chain reaction products were digested with 2 U of *Nco*I restriction enzyme at 37°C, according to the manufacturer's instructions (New England BioLabs, Ipswich, MA). The –308G allele contains an *Nco*I restriction site not present in the –308A allele; thus, in the presence of the –308G allele, the PCR product (107 bp) is cut into 2 fragments of 80 and 27 bp in length. The –238G/A polymorphism was identified under the same PCR conditions mentioned earlier using the following primers: forward 5′-AGA AGA CCC CCC TCG GAA TC-3′ and reverse 5′-ATC TGG AGG AAG CGG TAG TG-3′. Polymerase chain reaction products were digested with 2 U of *Msp*I restriction enzyme at 37°C, according to the manufacturer's instructions (New England BioLabs), which yielded a 152-bp fragment if the –238A allele was present, and 2 fragments of 133 and 19 bp for the –238G allele. All enzyme digestion products were visualized on a 6% polyacrylamide gel stained with silver nitrate.

The haplotypes formed by the TNF-α –308G/A and –238G/A polymorphisms were inferred by considering –308G/A as the first position and –238G/A as the second position. This generated 4 haplotypes: GG (–308G, –238G), GA (–308G, –238A), AG (–308A, –238G), and AA (–308A, –238A).

Statistical Analyses

The genotypic and allelic distributions of both polymorphisms were determined by direct gene counting and are presented as simple frequencies. The χ^2 test, with Fisher correction when necessary, was applied with a significance level set at 5% ($P < 0.05$) to compare the allele, genotype, and haplotype frequencies between the patients with GDM and the control group and to assess Hardy-Weinberg equilibrium. Disease risk was estimated using the odds ratio with a 95% confidence interval. Anthropometric and biochemical parameters, alone or according to TNF-α genotype, were compared between the patients with GDM and the controls using the Student *t* test and, when necessary, the Mann-Whitney *U* test. The statistical program SPSS Statistics version 17.0 (SPSS Inc, Chicago, IL) was used for data analysis. We used the Arlequin software version 2007 (University of Berne, Switzerland) to infer haplotypes and to evaluate Hardy-Weinberg equilibrium.

RESULTS

The anthropometric and biochemical parameters of the patients with GDM and the control women are listed in Table 1. The women with GDM were older than the control women

TABLE 1. Anthropometrical and Biochemical Characteristics of Patients With GDM and Women With Normoglycemic Pregnancies

Characteristic	GDM (n = 51)	Controls (n = 44)	*P**
Age, yrs	33.0 ± 5.2	25.6 ± 5.8	<0.001
BMI, kg/m^2	29.6 ± 4.5	24.5 ± 4.4	<0.001
Insulin, μU/mL	14.8 ± 8.1	8.1 ± 7.5	<0.001
Fasting glycemia, mmol/L	6.14 ± 1.88	4.12 ± 0.78	<0.001
HOMA-IR	3.9 ± 2.3	1.6 ± 1.7	<0.001
Total cholesterol, mmol/L	5.27 ± 1.02	ND	ND
Triglycerides, mmol/L	1.95 ± 0.89	ND	ND
HDL cholesterol, mmol/L	1.07 ± 0.27	ND	ND
LDL cholesterol, mmol/L	3.09 ± 1.03	ND	ND
VLDL cholesterol, mmol/L	0.94 ± 0.42	ND	ND

Values are expressed as the mean ± SD.

*Comparison performed using the Student *t* test and the Mann-Whitney *U* test when necessary.

BMI indicates body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ND, not determined.

TABLE 2. Tumor Necrosis Factor α Polymorphic and Haplotypic Distribution in Patients With GDM and Controls

		Number (%) of Subjects or Chromosomes			
		GDM (n = 51 or 102)	**Controls (n = 44 or 88)**	***P****	**OR (95% CI)**
TNF−308					
Genotype	G/G	43 (84.3)	39 (88.6)	0.601	–
	G/A	7 (13.7)	5 (11.4)		
	A/A	1 (2.0)	0 (0)		
Allele	G	93 (91.2)	83 (94.3)	0.408	–
	A	9 (8.8)	5 (5.7)		
TNF−238					
Genotype	G/G	41 (80.4)	37 (84.1)	0.569	–
	G/A	9 (17.6)	7 (15.9)		
	A/A	1 (2.0)	0 (0)		
Allele	G	91 (89.2)	81 (92.0)	0.507	–
	A	11 (10.8)	7 (8.0)		
Haplotypes					
	GG	75 (73.6)	77 (87.6)	Reference	Reference
	GA	3 (2.9)	6 (6.8)	0.497	0.51 (0.12–2.13)
	AG	16 (15.7)	4 (4.5)	0.019	4.11 (1.31–12.85)
	AA	8 (7.8)	1 (1.1)	0.035	8.21 (1.00–67.27)

*Comparison performed using χ^2 test with the Fisher exact test when necessary.

AA indicates –308A and –238A alleles; AG, –308A and –238G alleles; CI, confidence interval; GA, –308G and –238A alleles; GG, –308G and –238G alleles; OR, odds ratio.

(33.0 ± 5.2 vs 25.6 ± 5.8 years). Body mass index, glucose and insulin blood levels, and HOMA-IR were also significantly higher in the patients with GDM than in the controls. The genotypic distributions of both polymorphisms were consistent with Hardy-Weinberg equilibrium ($P > 0.05$). There were no significant differences in genotype and allele frequencies for the TNF-α –308G/A and –238G/A polymorphisms between the patients with GDM and the women with normoglycemic pregnancies, as shown in Table 2.

The haplotype frequencies of the TNF-α –308G/A and –238G/A polymorphisms in the women with GDM and the pregnant women with normal glucose tolerance are also shown in Table 2. We found that the frequency of the AG and AA haplotypes was significantly increased in the patients with GDM compared with the controls ($P = 0.019$ and $P = 0.035$, respectively). In addition, the patients who carried haplotypes AG showed an increased risk of developing GDM compared with the controls (odds ratio, 4.11; 95% confidence interval, 1.31–12.85).

In the patients with GDM, anthropometric and biochemical parameters were analyzed according to the genotypes of the TNF-α –308G/A and –238G/A polymorphisms. For statistical purposes, the carriers of the –308A and –238A alleles in the heterozygous and homozygous states were treated together (Table 3). We observed that insulin levels and HOMA-IR were significantly higher in the women carrying the G/G genotype than in the carriers of the G/A and A/A genotypes of the TNF-α –308G/A polymorphism ($P = 0.022$ and $P = 0.043$,

TABLE 3. Anthropometric and Biochemical Traits in Patients With GDM According to TNF-α Genotypes for the –308 G/A and −238 G/A Polymorphisms

Characteristic	**–308 G/A**			**–238 G/A**		
	G/G (n = 43)	G/A + A/A (n = 8)	*P**	G/G (n = 41)	G/A + A/A (n = 10)	*P**
Age, yrs	32.93 ± 4.89	33.38 ± 7.35	0.866	32.76 ± 5.35	34 ± 5.01	0.433
BMI, kg/m^2	29.85 ± 4.43	28.19 ± 4.89	0.551	29.60 ± 4.36	29.55 ± 5.25	0.849
Insulin, μU/mL	15.76 ± 8.36	10.13 ± 3.62	0.022	15.25 ± 8.65	13.35 ± 4.88	0.695
Fasting glycemia, mmol/L	6.11 ± 1.85	6.30 ± 2.14	0.928	6.17 ± 2.07	6.00 ± 0.76	0.313
HOMA-IR	4.20 ± 2.37	2.64 ± 0.69	0.043	4.03 ± 2.39	3.63 ± 1.67	0.906
Total cholesterol, mmol/L	5.35 ± 0.99	4.74 ± 1.10	0.074	5.24 ± 1.00	5.35 ± 1.16	0.849
Triglycerides, mmol/L	1.99 ± 0.90	1.74 ± 0.86	0.473	1.97 ± 0.93	1.85 ± 0.74	0.906
HDL cholesterol, mmol/L	1.07 ± 0.29	1.05 ± 0.14	0.990	1.09 ± 0.28	0.98 ± 0.16	0.373
LDL cholesterol, mmol/L	3.15 ± 1.02	2.69 ± 1.06	0.090	3.04 ± 0.94	3.22 ± 1.35	0.953
VLDL cholesterol, mmol/L	0.97 ± 0.43	0.80 ± 0.39	0.344	0.93 ± 0.44	1.00 ± 0.39	0.553

Values are expressed as the mean ± standard deviation.

*Comparison performed using the Student *t* test and the Mann-Whitney *U* test when necessary.

respectively). There were no significant differences regarding clinical and biochemical parameters in the patients with GDM according to genotype of the TNF-α –238G/A polymorphism.

The anthropometric and biochemical parameters of the patients and the controls were also analyzed according to the haplotypes generated by the TNF-α –308G/A and –238G/A polymorphisms; no significant differences were found when the haplotype frequencies were compared between the 2 groups.

DISCUSSION

In the present study, we found that the –238G/A polymorphism of the TNF-α gene was not associated significantly with the gestational diabetes phenotype; however, it was not possible to correlate this result with those of other reports because to date, the relationship between the TNF-α –238G/A polymorphism and GDM has not been studied. In addition, we did not find an association between this polymorphic site of the TNF-α promoter and insulin resistance or other biochemical variables, as described in China and in the Danish population.[25,26]

The results of our study also revealed that the –308G/A polymorphism of the TNF-α gene was not significantly associated with the gestational diabetes phenotype, in accordance with that reported in other studies.[17–19] Nevertheless, it should be noted that the frequency of the –308A allele varies among ethnic groups[27]; therefore, caution is warranted when extending the findings of the present study to other populations.

Regarding the haplotype analysis, we found that the patients with GDM carrying the AG haplotype exhibited an increased risk of developing GDM compared with the controls. The present study is of particular importance because, to the best of our knowledge, this is the first report assessing the relationship between the haplotypes formed by the TNF-α –308G/A and –238G/A polymorphisms and gestational diabetes; studies exist regarding type 1[28] and type 2 diabetes,[24,29] but not with gestational diabetes. However, these results should be taken with caution because of the small number of subjects who exhibited these haplotypes.

We supposed that the –308A allele of the –308G/A polymorphism is associated with insulin resistance, as the –308A allele has been associated previously with high transcription of the gene[14] and insulin resistance in obesity.[30] Nevertheless, the association between the G/G genotype of the –308G/A polymorphism and high insulin levels and insulin resistance was a major, albeit unexpected, finding of our study. This finding is in contrast with a study conducted by Chang et al, who reported an association between the GDM phenotype and an insulin resistance state in relation with the AA + GA genotypes of the TNF-α –308 polymorphism.[16]

Other studies have described that individuals bearing the G/G genotype of the TNF-α –308 polymorphism have higher levels of this cytokine than carriers of the A allele,[31,32] which seems to support our results if we consider that the –308G allele affects the transcription of the gene and that this, in turn, may affect the production of the cytokine. Unfortunately, we did not have the opportunity to measure the serum levels of TNF-α and provide a correlation between genotype and levels of cytokine, as well as between the levels of cytokine and insulin resistance. Our findings are also supported by the controversial results found regarding the association between the TNF-α –308G/A polymorphism and insulin resistance in several populations. Some reports show that the –308A allele is associated with insulin resistance,[30] whereas other studies show no association[26,33]; Fontaine-Bisson et al. found an association only among obese subjects,[34] whereas Casano-Sancho et al. showed that the –308G/G homozygous state is associated with insulin resistance in children born at an appropriate gestational age,[35] which is similar to the results described here.

In conclusion, our results suggest that the G/G genotype of the TNF-α –308G/A polymorphism increases insulin levels and insulin resistance in women with GDM and, moreover, that the AG haplotype formed by the TNF-α –308G/A and –238G/A polymorphisms is a genetic risk factor for GDM in our population. However, because of the small number of subjects exhibited in the comparative groups, caution is warranted when extending these findings to other populations.

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