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Original Research

Association analysis of calpain 10 gene variants/haplotypes with gestational diabetes mellitus among Mexican women

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Abstract: Gestational diabetes mellitus (GDM) is a metabolically complex disease with major genetic determinants. GDM has been associated with insulin resistance and dysfunction of pancreatic beta cells, so the GDM candidate genes are those that encode proteins modulating the function and secretion of insulin, such as that for calpain 10 (*CAPN10*). This study aimed to assess whether single nucleotide polymorphism (SNP)-43, SNP-44, SNP-63, and the indel-19 variant, and specific haplotypes of the *CAPN10* gene were associated with gestational diabetes mellitus. We studied 116 patients with gestational diabetes mellitus and 83 women with normal glucose tolerance. Measurements of anthropometric and biochemical parameters were performed. SNP-43, SNP-44, and SNP-63 were identified by polymerase chain reaction (PCR)-restriction fragment length polymorphisms, while the indel-19 variant was detected by TaqMan qPCR assays. The allele, genotype, and haplotype frequencies of the four variants did not differ significantly between women with gestational diabetes mellitus and controls. However, in women with gestational diabetes mellitus, glucose levels were significantly higher bearing the 3R/3R genotype than in carriers of the 3R/2R genotype of the indel-19 variant ($p = 0.006$). In conclusion, the 3R/3R genotype of the indel-19 variant of the *CAPN-10* gene influenced increased glucose levels in these Mexican women with gestational diabetes mellitus.

Key words: *CAPN10* variants; Gestational diabetes; Haplotypes; Mexican women.

Introduction

Gestational diabetes mellitus (GDM) is defined as any degree of glucose intolerance with its onset or first recognition during pregnancy (1). In the Mexican population, 12.9% of pregnant women are diagnosed with GDM (2).

A close relationship between the pathophysiology of GDM and type 2 diabetes mellitus (T2DM) has been described (3,4). Similar to T2DM, GDM is a metabolic disorder characterized by insulin resistance and dysfunction of pancreatic β cells (4,5) and has a strong genetic background (5), so it is expected that genetic alterations associated with the development of T2DM are also involved in the development of GDM (6,7).

To investigate the genetic basis of GDM, several studies have focused on the study of genes encoding proteins that modulate the function and secretion of insulin (8), such as the *CAPN10* gene, which was the first candidate gene associated with the development of T2DM in a Mexican-American population (9). The *CAPN10* gene encodes for a Ca^{2+}-requiring non-lysosomal cysteine protease, calpain-10, that is expressed in tissues involved in the pathogenesis of T2DM, such as the pancreas, skeletal muscle, and adipocytes (10). Studies have suggested that calpain-10 itself plays a role in glucose transporter 4 translocation to the cell membrane, regulation of pancreatic glucose-induced insulin secretion, and pancreatic β-cell apoptosis (11,12). Those findings support the role of this gene in the development of T2DM; however, few studies have assessed its involvement in the development of GDM (6, 13-16). Therefore, the aim of this study was to evaluate the possible association between GDM and single nucleotide polymorphism (SNP)-43, SNP-44, SNP-63, and the indel-19 variant of the *CAPN10* gene, or its specific haplotypes.

Materials and Methods

Study subjects

The study included 116 unrelated patients with GDM and 83 unrelated women with normal glucose tolerance. All were recruited during the third trimester of gestation and the age range was 16–46 years. 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: no personal or family history of T2DM

or type 1 diabetes mellitus, and diagnosis of GDM according to the American Diabetes Association criteria, which include a 2-h 75-g oral glucose tolerance test at 24–28 weeks of gestation with cutoff values ≥92 mg/dL (5.1 mmol/L) fasting, ≥180 mg/dL (10.0 mmol/L) at 1 h, and ≥153 mg/dL (8.5 mmol/L) at 2 h (17). Control pregnant women with normoglycemia had normal glucose tolerance in early and advanced stages of pregnancy, and no personal or family history of any disorder associated with glucose intolerance. They were selected consecutively and referred to us during labor or after rupture of the amniotic membranes.

All participated women were Mexican mestizos living in western Mexico (Jalisco State). Written voluntary informed consent to participate in genetic analyses was obtained from all of them and the study protocol was approved by the Local Research and Ethics Committee (#R-2013-1305-8).

Clinical and biochemical evaluation

Age and anthropometric measurements, including height and current weight, were obtained. The body mass index (BMI) was calculated as weight/height^2 (kg/m^2). Fasting glucose concentrations were determined using the glucose-oxidase method. In addition, in a subgroup of women with GDM (n = 48), who needed to remain hospitalized, insulin levels and lipid profiles were determined. Insulin concentrations were quantified using radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA, USA). Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) index was calculated as follows: [fasting insulin (μU/mL)] [fasting glucose (mmol/L)]/22.5. After a 12-hour fast serum levels of total cholesterol (TC), triglycerides (TG), and high-density lipoprotein (HDL) were measured using the Vitros Fusion 5.1 analyzer (Ortho-Clinical Diagnostics, Rochester, NY, USA). Low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) levels were calculated as follow: LDL= (TC-HDL)-TG/5 and VLDL= TG/5.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using a standard protocol (18). As previously described, the SNP-63 (rs5030952, g.241542703C>T, IVS13) was genotyped under PCR–restriction fragment length polymorphism (RFLP) conditions (19). SNP-43 (rs3792267, g.241531174G>A, IVS3) and SNP-44 (rs2975760, g.241531163T>C, IVS3) were also genotyped by PCR–RFLP analysis with some modifications. Briefly, because SNP-44 is located 11 bp upstream from SNP-43, we carried out a PCR-based assay to amplify the genomic region encompassing both SNP-43 and SNP-44 using primers previously designed to identify only SNP-43 (20). The amplified fragment of 212 bp was digested separately with 2U of *Nde*I restriction enzyme (New England BioLabs, Ipswich, MA, USA) at 37 °C for detecting SNP-43 and with 2U of *Tse*I (New England BioLabs, Ipswich, MA, USA at 65 °C for SNP-44. The A allele of SNP-43 contains an *Nde*I restriction site not present in the G allele; thus, in the presence of the A allele, the 212 bp PCR product is cut into two fragments of 191 and 21 bp in length. SNP-44 alleles were seen as 212 bp (T allele) and 176 + 36 bp fragments (C allele). All PCR and enzyme-digested products were separated by electrophoresis into 6% polyacrylamide gels and visualized using silver nitrate staining. The indel-19 variant (three 32-bp repeats/two 32-bp, rs3842570, g.241534293_241534294ins, IVS6) was detected by TaqMan qPCR assay using the LightCycler FastStart Essential DNA Probes Master Kit in LightCycler® 96 System (Roche Diagnostic GmbH, Mannheim, Germany) according to the conditions specified by the manufacturer.

The haplotypes formed by the four *CAPN10* variants were inferred considering the following order of alleles (from centromere to telomere): SNP-44 (allele 1 = T, allele 2 = C), SNP-43 (allele 1 = G, allele 2 = A), indel-19

Table 1. Anthropometric and biochemical characteristics of patients with GDM and controls.

	GDM n = 68	Controls n = 83	p value*
Age (years)	32.13 ± 5.19	30.63 ± 5.77	0.250
Weight (kg)	81.54 ± 14.18	74.94 ± 13.15	0.259
Height (m)	1.61 ± 0.704	1.60 ± 0.74	0.694
BMI (kg/m^2)	31.56 ± 4.52	29.89 ± 5.49	0.538
Glucose (mg/dL)	105.13 ± 31.96	72.51 ± 11.51	**0.003**
	Subgroup GDM n = 48		
Age (years)	32.9 ± 5.22		
BMI (kg/m^2)	29.48 ± 4.22		
Glucose (mg/dL)	111.83 ± 34.43		
Insulin (μU/mL)	14.4 ± 6.97		
HOMA	3.86 ± 2.02		
TC (mg/dL)	204.31 ± 40.75		
TG (mg/dL)	174.96 ± 81.15		
LDL (mg/dL)	119.95 ± 40.83		
HDL (mg/dL)	41.63 ± 10.70		
VLDL (mg/dL)	36.67 ± 16.97		

GDM, Gestational diabetes mellitus; BMI, body mass index; HOMA, homeostatic model assessment of insulin resistance; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein. *Comparisons were done using the Mann–Whitney nonparametric U test.

variant (allele 1 = two 32-bp repeats, allele 2 = three 32 bp repeats), and SNP-63 (allele 1 = C, allele 2 = T).

Statistical analysis

Allele frequencies of the four *CAPN10* variants were determined by direct counting from observed genotype frequencies. Chi-squared tests, with Fisher's correction when necessary, were applied to verify agreement with Hardy–Weinberg equilibrium and to compare the allele, genotype and haplotype frequencies between patients with GDM and controls. Disease risk associated with the *CAPN10* variants was estimated using odds ratios with 95% confidence interval. Anthropometric and biochemical parameters were compared between patients with GDM and controls using the Mann–Whitney nonparametric *U* test. In a subgroup of patients with GDM, anthropometric and biochemical variables were compared according to the distribution of different *CAPN10* genotypes using analysis of variance (ANOVA). Maximum-likelihood haplotypes in patients with GDM and control women were inferred using the Arlequin software v.3.1 (University of Bern, Switzerland). The data analyses were conducted using the statistical software package IBM SPSS v. 21.0 (IBM Corp., Armonk, NY, USA). Results were regarded as significant when $p < 0.05$.

Results

The anthropometric and biochemical parameters of the patients with GDM and control women are listed in Table 1. Fasting glucose levels were significantly higher in patients with GDM than in controls ($p = 0.003$).

The genotypic distributions of the four *CAPN10* variants were consistent with Hardy–Weinberg equilibrium.

Table 2. Genotype and allele distribution of the four *CAPN10* variants in patients with GDM and controls.

Genotype and allele		GDM n = 116 n (%)	Controls n = 83 n (%)	p*	OR	95% CI
SNP-43	G/G	63 (54.3)	41 (49.4)	Reference		
	G/A	42 (36.2)	39 (47)	0.235	0.701	0.390-1.261
	A/A	11 (9.5)	3 (3.6)	0.247	2.386	0.627-9.075
	G/A vs A/A	---	---	0.083	3.405	0.884-13.120
	A/A +G/A vs G/G	53 (45.7)	42 (50.6)	0.494	0.821	0.467-1.444
	G/A+G/G vs A/A	105 (90.5)	80 (96.4)	0.110	2.794	0.754-10.347
	G/G+ A/A vs G/A	74 (63.8)	44 (53)	0.127	0.640	0.361-1.137
	G	168 (72.4)	121 (72.9)	Reference		
	A	64 (27.6)	45 (27.1)	0.916	1.024	0.655-1.602
SNP-44	T/T	93 (80.2)	64 (77.1)	Reference		
	T/C	22(18.9)	18 (21.7)	0.628	0.841	0.418-1.693
	C/C	1 (0.9)	1 (1.2)	1.0	0.688	0.042–11.24
	T/C vs CC	---	---	1.0	0.818	0.480-14.017
	C/C+T/C vs T/T	23 (19.8)	19 (22.9)	0.602	0.833	0.420-1.654
	T/C+T/T vs C/C	115 (99.1)	82 (98.8)	1.0	0.713	0.044-11.56
	T/T+C/C vs T/C	94 (81.1)	65 (78.3)	0.637	0.845	0.420-1.669
	T	208 (89.7)	146 (88)	Reference		
	C	24 (10.3)	20 (12)	0.593	0.842	0.449-1.582
SNP-63	C/C	71 (61.2)	60 (72.3)	Reference		
	C/T	40 (34.5)	20 (24.1)	0.105	1.690	0.894-3.197
	T/T	5 (4.3)	3 (3.6)	0.729	1.408	0.323–6.138
	C/T vs T/T	---	---	1.0	0.833	0.181-3.843
	T/T+C/T vs C/C	45 (38.8)	23 (27.7)	0.104	1.653	0.900-3.039
	C/T+C/C vs T/T	111 (95.7)	80 (96.4)	1.0	1.201	0.279-5.172
	C/C+T/T vs C/T	76 (65.5)	63 (75.9)	0.115	1.658	0.881-3.120
	C	182 (78.4)	140 (84.3)	Reference		
	T	50 (21.6)	26 (15.7)	0.141	1.479	0.877-2.495
Indel-19	2R/2R	22 (18.9)	8 (9.6)	Reference		
	2R/3R	53 (45.7)	41 (49.4)	0.823	1.072	0.582-1.974
	3R/3R	41 (35.4)	34 (41)	0.078	2.280	0.901-5.769
	2R/3R vs 3R/3R	---	---	0.823	1.072	0.582-1.974
	2R/3R+3R/3R vs 2R/2R	94 (81.1)	75 (90.4)	0.075	2.194	0.925-5.207
	2R/3R+2R/2R vs 3R/3R	75 (64.6)	49 (59)	0.420	1.269	0.711-2.267
	2R/2R+3R/3R vs 2R/3R	63 (54.3)	42 (50.6)	0.605	0.862	0.490-1.515
	2R	97 (41.9)	57 (34.3)	Reference		
	3R	135 (58.1)	109 (65.7)	0.131	0.728	0.481-1.100

GDM, Gestational diabetes mellitus; *Comparisons were done using Fisher's exact (genotypes) and Chi-squared (alleles) test.

Table 3. Distribution of genotypes according to anthropometric and biochemical variables in a subgroup of patients with GDM.

	Genotype											
	SNP-44			SNP-43			Indel-19			SNP-63		
	T/T	T/C	C/C	G/G	G/A	A/A	3R/3R	3R/2R	2R/2R	C/C	C/T	T/T
BMI	29.7 ± 4.3	28.7 ± 3.9	0	29.0 ± 4.4	29.7 ± 3.4	32.0 ± 5.5	29.8 ± 5.3	30.4 ± 3.7	27.5 ± 2.1	30.2 ± 4.5	28.4 ± 3.9	28.3 ± 1.6
Insulin	14.6 ± 7.6	13.5 ± 2.7	0	14.1 ± 6.6	13.7 ± 5.6	19.6 ± 13.0	14.1 ± 7.5	15.6 ± 8.4	12.9 ± 2.1	14.2 ± 7.5	15.3 ± 6.6	11.8 ± 1.1
Glucose	115.3 ± 37.3	98.7 ± 14.8	0	110.8 ± 3	115.0 ± 47.3	107.5 ± 16.1	**131.0 ± 46.4***	**95.3 ± 13.9***	105.8 ± 12.1	117.5 ± 41.7	102.3 ± 12.5	102.3 ± 15.3
HOMA	4.0 ± 2.2	3.3 ± 0.9	0	3.7 ± 1.8	3.8 ± 2.1	5.3 ± 3.5	4.3 ± 2.4	3.7 ± 2.2	3.4 ± 1	4.0 ± 2.3	3.9 ± 1.7	3.0 ± 0.2
TC	203.7 ± 38.7	206.6 ± 50.2	0	209.3 ± 4	195.4 ± 44.3	201.5 ± 23.4	202.3 ± 37.5	205.2 ± 44.1	206.5 ± 44.3	205.2 ± 39.4	196.0 ± 42.4	237.3 ± 42.0
TG	186.1 ± 81.8	132.6 ± 66.2	0	171.2 ± 75	159.4 ± 87.3	260.5 ± 66.3	174.3 ± 79.9	187.8 ± 84.6	155 ± 80.8	182.6 ± 84.1	156.3 ± 71.9	191.7 ± 110.2
LDL	120.0 ± 41.9	119.6 ± 40.8	0	120.9 ± 37	115.6 ± 47.1	129.3 ± 49.4	121.5 ± 41.1	120.0 ± 43.7	117.3 ± 39.1	121.8 ± 42.5	112.8 ± 39.5	137.3 ± 34.0
HDL	40.7 ± 9.6	45.1 ± 14.2	0	42.2 ± 10.5	40.3 ± 12.7	42.3 ± 3.4	41.3 ± 10.9	40.5 ± 11.1	44.0 ± 10.5	40.8 ± 11.1	43.0 ± 10.7	42.7 ± 9.1
VLDL	38.7 ± 16.6	28.8 ± 16.8	0	36.5 ± 16.4	32.9 ± 17.5	52.3 ± 13.2	34.7 ± 16.3	39.5 ± 17.0	35.6 ± 18.9	37.5 ± 17.3	34.3 ± 16.2	40.0 ± 22.9

GDM, Gestational diabetes mellitus; BMI, body mass index; HOMA, homeostatic model assessment of insulin resistance; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein. *p = 0.006 (ANOVA).

Table 4. Estimated haplotype frequencies in the patients with GDM and controls.

	GDM 2n = 232		**Control 2n = 166**		
Haplotypes	**n**	**Frequency**	**n**	**Frequency**	**p***
1121	100	0.43	81	0.49	0.262
1221	37	0.16	27	0.16	1.000
1112	31	0.13	16	0.10	0.284
1111	19	0.08	9	0.05	0.329
2111	12	0.05	12	0.07	0.418
1212	12	0.05	6	0.04	0.627
1211	10	0.04	6	0.04	0.799
2112	6	0.03	3	0.02	0.780
2211	5	0.02	5	0.03	0.746
1222			1	0.01	–

GDM, Gestational diabetes mellitus; *Comparisons were done with the Chi-squared test.

Genotype and allele frequencies for the four *CAPN10* variants in patients with GDM and women with normoglycemic pregnancies are shown in the Table 2.

There were no significant differences in genotype frequencies for the four *CAPN10* variants between patients and controls under the co-dominant model of allelic interaction (1/1 *vs* 1/2, 1/1 *vs* 2/2 and 1/2 *vs* 2/2), or under other genetic models: dominant (1/2 + 2/2 *vs* 1/1), recessive (1/2 +1/1 *vs* 2/2) and over-dominant (1/1 + 2/2 *vs* 1/2). In addition, statistical significance was no found in the allele distribution.

In the subgroup of 48 patients with GDM, anthropometric and biochemical parameters were analyzed according to the genotypes of the four *CAPN10* variants (Table 3). Glucose levels were significantly higher in patients with GDM carrying the homozygous genotype 3R/3R only when compared with carriers of the heterozygous genotype 3R/2R of the indel-19 variant ($p = 0.006$).

The haplotype frequencies of the four *CAPN10* variants in women with GDM and controls are shown in Table 4. From 16 inferred haplotypes, only nine were observed in the patients with GDM and 10 in the control group. The 1222 haplotype was observed only in the control group. The distributions of haplotypes did not differ significantly between patients with GDM and controls.

Discussion

Although the specific biological effects of the four intronic *CAPN10* variants studied here are not known, they have been reported to show associations with different phenotypes involving insulin resistance (12, 21, 22). To our knowledge, this is the first report regarding the possible participation of these four genetic *CAPN10* variants and their haplotype in the development of GDM in a Mexican population.

We found that SNP-43, SNP-44, SNP-63, and the indel-19 variant of *CAPN10* were not associated significantly with the GDM phenotype. This finding is in agreement with Neuhaus et al (16) and Shaat et al (13) who evaluated the same variants in German women, and the SNP-43 and SNP-44 in Swiss women, respectively, and did not find any association, and also with the study of Khan et al (15) who evaluated only SNP-44 and did not observe an association of this variant with GDM in Asian Indian women. In contrast, in a study conducted in Austria found that the C/C genotype of SNP-63 (C>T) was associated with the GDM phenotype (6), while in another study carried out in China the association was with the G/G genotype of SNP-43 (G>A) comparing GDM patients and control group and also with the 2R allele of the indel-19 variant, but comparing jointly the patient with GDM and patients with impaired glucose tolerance (IGT) with a control group (14). Recently, Cui et al (23) published a meta-analysis that included five previous studies on the association between *CAPN10* variants and GDM (6,13-16) and reported that the association with GDM lies in the SNP-63 in the heterozygous model (C/T vs C/C).

We also found that in a subgroup of patients with GDM there was a differential distribution of the homozygous 3R/3R genotype compared with the heterozygous 3R/2R genotype of the indel-19 variant in regard to glucose levels, suggesting that the increased glucose level in this subgroup of patients is determined by this genotype. This finding is somewhat similar to that reported by Wu et al (24) who concluded that the indel-19 variant was associated with a disorder in glucose metabolism in pregnant women, but in the presence of two 32-bp repeats (the 2R allele).

Three studies have assessed the association between haplotypes of the *CAPN10* gene and GDM: one conducted in Austria (6), other in Germany (16) and another in China (14). In the present study, we found that the most frequent haplotype was 1121. Although Neuhaus et al (16) analyzed the same four variants, they performed haplotype analysis with only three variants (SNP-43, indel-19, and SNP-63), and also found that haplotype 121 was the most frequent. Leipold et al (6) only analyzed SNP-43, indel-19, and SNP-63, and they also found that haplotype 121 was the most common. Consistent with the studies carried out in woman from Austria (6) and Germany (16), we found no association between *CAPN10* haplotypes and GDM. This finding contrasts with that reported by Luo et al (14) who analyzed the SNP-43, indel-19 variant, and SNP-63 and reported an association between the haplotype 112 and GDM in Chinese women.

Our results suggest that the Mexican population has very particular genetic characteristics that differentiate it from other populations worldwide. In western Mexico (including state of Jalisco, where the women studied reside), the proportion of admixture is represented by 53.2% Europeans (Spaniard colonizers), 30.8% ancient Amerindians, and 15.9% African origin (25). Hence ethnic differences between study subjects appear to be one cause of the association discrepancies.

In conclusion, we found that SNP-43, SNP-44, SNP-63, and the indel-19 variant of the *CAPN10* gene do not participate either individually or as haplotypes in the development of GDM in this group of Mexican patients. However, the 3R/3R genotype of the indel-19 variant seems to have influenced the increased glucose levels in a subgroup of patients with GDM. Nevertheless, because of the small number of subjects in the studied groups, caution is needed in generalizing these findings to other populations.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

Anna Gabriela Castro-Martínez. Acquisition, analysis and interpretation of data and drafting of the article. José Sánchez-Corona. Critical revision for important intellectual content and final approval of the version to be published. Adriana Patricia Vázquez-Vargas. Acquisition of data for the work. Alejandra Guadalupe García-Zapién. Acquisition of data for the work and critical revision for important intellectual content. Andres

López-Quintero. Acquisition of data for the work and critical revision for important intellectual content. Héctor Javier Villalpando-Velazco. Acquisition of data for the work and critical revision for important intellectual content. Silvia Esperanza Flores-Martínez. Conception and design of the work, edition of the manuscript for intellectual content, and final approval of the version to be published.

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