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Leptin (*rs7799039*) and solute carrier family 30 zinc transporter (*rs13266634*) polymorphisms in Euro-Brazilian pregnant women with gestational diabetes

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Genet. Mol. Res. 16 (1): gmr16019515
Received November 3, 2016
Accepted January 30, 2017
Published March 30, 2017
DOI http://dx.doi.org/10.4238/gmr16019515

ABSTRACT. Leptin (LEP), a protein that plays a fundamental role in the metabolism of energy reserves, and the solute carrier family 30 A8 zinc transporter (SLC30A8) have been consistently associated with diabetes. Women with gestational diabetes are at moderate risk of developing diabetes type 1 and 2 after pregnancy, in addition to complications to the fetus. We investigated the association of the polymorphisms *rs7799039* (LEP) and *rs13266634* (SLC30A8) in a case-control study in Euro-Brazilians with gestational diabetes (GDM,

N = 134) and healthy pregnant women (control, N = 180). Real-time PCR with fluorescent probes (TaqMan system) was applied to genotyping. All polymorphisms were in Hardy-Weinberg equilibrium. The minor allele frequencies, for healthy and GDM, respectively, for the A-allele (LEP gene *rs7799039*) were 40.3% (95%CI = 35-45%) *vs* 36.6% (95%CI = 31-42%), P = 0.345; and for the T-allele (SLC30A8 gene *rs13266634*) were 27.8% (95%CI = 23-32%) *vs* 23.5% (95%CI = 18-29%), P = 0.227. Genotype comparisons for both polymorphisms showed no significant difference (P > 0.05). The polymorphisms *rs7799039* and *rs13266634* were not associated with GDM in the population studied (P > 0.05). The minor allele frequencies for both polymorphisms were similar to those of other Caucasian populations.

Key words: Gestational diabetes; Leptin; Genetic susceptibility; SNP; Solute carrier family 30 (zinc transporter) member 8; Mutation

INTRODUCTION

The number of adults with diabetes worldwide increased from 108 million in 1980 to about 422 million by the year 2014 [NCD Risk Factor Collaboration (NCD-RisC), 2016], and this number is estimated to increase to 592 million by 2035 (IDF - International Diabetes Federation, 2014).

Diabetes *mellitus* is a group of metabolic diseases that has a defect in insulin secretion, insulin action, or both, resulting in chronic hyperglycemia. Women with a history of gestational diabetes mellitus (GDM) are at increased risk of developing type 2 diabetes mellitus (T2D) compared to those whose pregnancy is normoglycemic (Kwak et al., 2013).

Glucose metabolism and insulin sensitivity always change in pregnant women who are usually able to meet the increased insulin demand, but in some cases, these requirements are not met, resulting in poor glycemic control (Liu et al., 2016).

GDM is usually diagnosed in the last half of pregnancy when insulin resistance increases progressively until birth. Risk factors for this disorder include obesity, advanced maternal age, and a family history of diabetes. Although GDM occurs frequently, its pathophysiology is not completely understood. Little is known about the gene expression profile in GDM. However, GDM is a common metabolic disorder affecting 1-14% of all pregnancies, and each year, the incidence of cases increases (Singh and Singh, 2015).

One of the most frequent types of genetic variation in the human genome is the single nucleotide polymorphism (SNP), which may contribute to differences between individuals in susceptibility to hereditary diseases. Polymorphisms in different genes have been associated with GDM (Huopio et al., 2013).

The LEP gene (Leptin, OMIM: 164160) is located on chromosome 7, in area 7q31.3, is 16,352 bp in length, and contains 3 exons. Leptin is a hormone secreted by adipocytes that promote a reduction in fuel uptake and an increase in energy expenditure when fat reserves are sufficient, thus controlling body weight and regulating energy balance (Jéquier and Tappy, 1999). Reduction in the leptin level reverses the process of thermogenesis, allowing fuel conservation (Jéquier and Tappy, 1999).

In addition to regulating appetite and metabolism, this adipokine is important in insulin

secretion (Romanowski et al., 2015). Insulin resistance is an important feature of gestational diabetes mellitus. Several adipokines, including leptin, are involved in the development of this resistance (Takhshid and Zare, 2015).

The hormone leptin was first identified in adipose tissue and is known to be expressed in the placenta and fetal tissues (Vaskú et al., 2006). Leptin is considered a fetal growth factor able to maintain energy and metabolic balance during pregnancy (Xu et al., 2014). Polymorphisms in the leptin gene have been associated with obesity, a risk factor for GDM and T2D. The polymorphism *rs7799039* is located in the promoter region of the LEP gene and can alter dynamic methylation, affecting LEP expression at the transcriptional level to increase or reduce leptin expression (Marcello et al., 2015).

The SLC30A8 gene (Solute Carrier Family 30 zinc transporter, OMIM 611145) is 226,442 bp in length and is located in the region 8q24.11 of chromosome 8 (Seman et al., 2015).

SLC30A8 is associated with the secretion of human insulin and encodes a specific zinc transporter (ZnT8) for β cells of the pancreas (Maruthur et al., 2015). The presence of zinc in the secretory insulin vesicles stabilizes the insulin hexamer, making it less susceptible to degradation (Dereke et al., 2016). This insulin packed into secretory vesicles becomes available for immediate release upon stimulation by glucose (Maruthur et al., 2015). The polymorphism *rs13266634* is a non-synonymous C → T mutation in exon 9 of the SLC30A8 gene, causing a non-conservative change of arginine (R) to tryptophan (W) at position 325 (R325W) (Dereke et al., 2016).

The polymorphisms *rs7799039* and *rs13266634* affecting the expression of the LEP and SLC30A8 genes, respectively, have been considered likely to be associated with diabetes risk. Insufficient production of leptin is usually related to human obesity and with altered glucose metabolism. The negative regulation of the zinc transporter is thought to destabilize insulin molecules (Seman et al., 2015).

The aim of this study was to determine if the polymorphisms *rs7799039* and *rs13266634* were associated with gestational diabetes in a case-control study with healthy and diabetic Euro-Brazilian pregnant women.

MATERIAL AND METHODS

Study subjects

A sample of 314 unrelated Euro-Brazilian pregnant women was classified as healthy (control, N = 180) or with gestational diabetes (GDM, N = 134) according to the Brazilian Diabetes Society (SBD, 2009). Briefly, GDM diagnosis was defined at 24-28 weeks of gestation by fasting glucose ≥6.1 mmol/L, and by a glucose level >7.8 mmol/L, 2-h after an oral glucose load of 75 g.

Clinical and anthropometric data were obtained from all patients. The study was approved by the University's Human Research Ethics Committee.

DNA extraction

Genomic DNA was obtained from peripheral blood leukocytes (buffy coat) by the salting-out method (Lahiri and Nurnberger, 1991). DNA samples with an A_{260}/A_{280} ratio between 1.6 and 1.9 were used, and the concentrations of all samples were normalized to 20 ng/μL.

TaqMan SNP Genotyping Assays® (Applied Biosystems, Foster City, CA, USA) utilizing fluorescent probes in the 7500 Fast Real-Time PCR System (Applied Biosystems) were used for genotyping. The TaqMan probe codes used were C_1328079_10 (*rs7799039*; LEP) and C_3578888_10 (*rs13266634*, SLC30A8). Briefly, the genotyping protocol involved the addition of 3 μL DNA (20 ng/μL), 3 μL TaqMan Genotyping Master Mix, 0.1 μL of each fluorescent probe and 1.9 μL water to each well of a 96-well plate. The cycling protocol was as recommended by the manufacturer (1 cycle: 95°C for 10 min, and 40 cycles: 95°C for 15 s and 60°C for 60 s). The Genotyping quality was 98% or higher in all samples measured by the software (7500 Fast SDS system software).

Biochemical markers

Biochemical parameters such as fasting glucose, glucose 2 h after 75 g oral glucose load, creatinine, and albumin were quantified using the Architect Ci 8200 automated system (Abbott Diagnostics) with reagents, calibrators, and controls from the equipment manufacturer. Glycated hemoglobin (HbA1c) was measured only in diabetic pregnant women by using a high-performance liquid chromatography (HPLC) machine equipped with a cation exchange column (Varian II, Bio-Rad).

Statistical analysis

Continuous variables with normal distribution, verified with Kolmogorov-Smirnov test, were compared with a two-tailed Student *t*-test for independent variables. The Mann-Whitney U-test was used for non-normal distribution variables. The chi-square test was used to compare categorical variables. Hardy-Weinberg equilibrium and allele comparisons were calculated with the DeFinetti program (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl).

Statistical analyses were performed using the Statistica for Windows version 8.0 software (StatSoft Inc., Tulsa, OK, USA). A P value lower than 5% ($P < 0.05$) was considered significant.

RESULTS

Patient characteristics are shown in Table 1. GDM patients were older (median age 32 *vs* 30 years) and heavier (BMI 32.6 *vs* 26.1 kg/m^2) compared with healthy pregnant controls. A history of family diabetes was high in the GDM group (66.7%) when compared to other Euro-descendent healthy pregnant women from Portugal (16%) (Ribeiro et al., 2015) and France (16%) (Miailhe et al., 2015). This information for the control group was not available.

Table 1. Anthropometric and laboratory data for healthy women and women with GDM.

Parameters	Control (N = 180)	GDM (N = 134)	P
Age (years)	30 (28-33)	32 (28-36)	0.034*
Weight (kg)	66 (58-73)	82 (71-93)	<0.001*
Height (m)	1.61 ± 0.06	1.60 ± 0.06	0.109
BMI (kg/m^2)	26.1 ± 5.1	32.6 ± 6.4	<0.001
Family history of Diabetes (%)	-	66.7	-
Fasting glucose (mM)	4.6 (4.4-4.9)	4.8 (4.0-5.4)	<0.001*
Glucose 2-h, 75 g (mM)	4.8 (4.4-5.7)	9.0 (8.3-9.7)	<0.001*
HbA1c (%)	-	5.8 (5.2-6.0)	-
Creatinine (μM)	70.7 (70.3-79.6)	61.9 (53.0-70.7)	<0.001*
Albumin (g/L)	43.0 (39-46)	34.0 (31-36)	<0.001*

Control, healthy pregnant women and GDM, women with gestational diabetes mellitus. Values are reported as means ± SD, median (interquartile range) or %. -, no information available. P value, Student *t*-test independent (two-sided), *Mann-Whitney U test.

Fasting glucose, and the glucose level 2 h after a 75-g glucose load, were high in the GDM group as expected ($P < 0.001$). Glycated hemoglobin, measured only in the GDM group (median 5.8%), suggested good glycemic control, and none of these patients were receiving insulin therapy. Serum creatinine and albumin were lower in the GDM than in the control group, but both markers were in the reference range.

Genotypes and allele frequencies of both polymorphisms showed no differences between the studied groups (Table 2). The polymorphisms were in Hardy-Weinberg equilibrium ($P > 0.05$) in both groups. For both polymorphisms, the genotype comparison in the modules dominant (frequent homozygous *vs* others) and recessive (rare homozygous *vs* others) did not show significance ($P > 0.05$).

Table 2. Genotype and allele frequencies for LEP and SLC30A8 gene polymorphisms in healthy (Control) women and women with GDM.

Gene/Polymorphisms		Control (N = 180)	GDM (N = 134)	P
LEP, rs7799039				0.627
	G/G	67 (37.2%)	57 (42.5%)	
	G/A	81 (45.0%)	56 (41.8%)	
	A/A	32 (17.8%)	21 (15.7%)	
MAF	A-allele (95%CI)	40.3% (35-45%)	36.6% (31-42%)	0.345*
SLC30A8, rs13266634				0.522
	C/C	99 (55.1%)	82 (61.2%)	
	C/T	62 (34.4%)	41 (30.6%)	
	T/T	19 (10.5%)	11 (8.2%)	
MAF	T-allele (95%CI)	27.8% (23-32%)	23.5% (18-29%)	0.227*

MAF, minor allele frequency. All polymorphisms were in Hardy-Weinberg equilibrium. P value, chi-squared test for genotype and *allele frequencies 95%CI = 95% confidence interval.

For Euro-Brazilian healthy pregnant women, the minor allele frequency for *rs7799039* (A-allele; LEP gene) was 40.3% (95%CI = 35-45%) and for *rs13266634* (T-allele; SLC30A8 gene) 27.8% (95%CI = 23-32%).

DISCUSSION

The number of cases of gestational diabetes mellitus in Brazil reaches 7% of pregnancies, similar to the rate in the United States (Schmidt et al., 2000). The prevalence varies in different populations and ethnic groups (Zhang et al., 2013).

In this study, diabetic pregnant women were older than healthy controls. Increasing age is known to correlate with the risk of developing GDM. In the sample analyzed, the median age for diabetic pregnant women was 32 years, in accordance with the literature, where there are reports that the prevalence of gestational diabetes increases with age, becoming more frequent in women from the age of 30 onward (Murgia et al., 2008).

Regarding weight and BMI, the group of diabetic women had significantly higher values than the control group. This was as expected given that being overweight and having obesity (BMI >30 kg/m^2) are factors that contribute to the development of GDM (Murgia et al., 2008; Takhshid and Zare, 2015).

A family history of diabetes also influences the development of GDM. Almost 67% of gestational diabetic patients in the study reported a family history of diabetes. In a study by Murgia et al. (2008), women with GDM also reported having first-degree relatives with diabetes, confirming that GDM and T2D have similar genetic character. Mothers with gestational diabetes have a higher incidence of T2D development, while their children are at greater risk of developing obesity (Huidobro et al., 2010).

The glycated hemoglobin is a biomarker of glycemic control (Arnold and Wang, 2014). Concentrations greater than 6.5% indicate inadequate glycemic control (SBD, 2009). Average concentrations of HbA1c for the GDM group of 5.8% suggest good glycemic control in these patients (Table 1).

The BMI, age, and HbA1c parameters of pregnant women in the groups in question were similar to those in pregnant women in groups of similar studies (Huopio et al., 2013; Takhshid and Zare, 2015).

There were significant differences in mean serum albumin and creatinine concentrations between the two groups. The GDM group showed lower concentrations of both markers compared to concentrations in the control group (Table 1). This reduction may be explained by increased protein loss in these patients and increased urine flow resulting from hyperglycemia, suggesting a reason for the decreased serum creatinine. Nevertheless, none of these biomarkers point to overt kidney failure or hypertension problems since they were in the reference range (Lim, 2014).

Leptin gene polymorphisms are associated with obesity, insulin resistance, and diabetes mellitus, as demonstrated in a study by (Romanowski et al., 2015). The association of polymorphisms in the leptin gene with GDM is controversial. Vaskú et al. (2006) showed an association of leptin polymorphism -2548G>A with preeclampsia in GDM, but Yang et al. (2016) failed to associate this polymorphism with GDM in a Chinese population.

The minor allele frequency (MAF) of the LEP gene polymorphism *rs7799039* (A-allele) for the control group (~40%) was similar to the frequency in Bulgaria (45%), Egypt (39.5%), and India (41.1%), as shown in Table 3. Caucasians from Finland and Germany showed slightly higher frequencies (50-58%). Different African populations showed dramatically different frequencies, such as 4% in one African study compared with 98.7% for Nigerians. A different Euro-Brazilian population, studied by Luperini et al. (2015), showed a frequency (41.1%) consistent with that observed in our study.

Studies using diabetic mice have shown that the gene expression level of SLC30A8 is repressed in the pancreas of animals that present this pathology, suggesting an involvement in diabetes (Seman et al., 2015).

The MAF of the SLC30A8 gene *rs13266634* (T-allele) for the control group (~28%) was similar to that found in Indian (23%), Mayan (25%), American (26.2%), and Polish (33%) populations. Orientals showed a higher frequency (~46%) and Africans a lower frequency (6-8%) as presented in Table 3. No studies were found for Brazilian populations.

Our findings should be confirmed with a larger sample size.

In summary, the studied polymorphisms (*rs7799039*; LEP and *rs13266634*; SLC30A8) were not associated with gestational diabetes in a Euro-Brazilian population.

Table 3. Comparison of minor allele frequencies of rs7799039 and rs13266634 with other populations.

Ethnic group	MAF (%) (95%CI)	References
	LEP/rs7799039 (A-allele)	
Euro-Brazilian	**40.3 (35-45)**	This work
Nigerian (Yoruban)	**98.7**	HapMap
Caucasian (Germany)	**58.3 (50-67)**	(Opgen-Rhein et al., 2010
Indian Obese	**53.9**	(Dasgupta et al., 2015)
Caucasian (Finland)	**50.0**	(Dougkas et al., 2013)
Bulgarian	45.0	(Nikolova et al., 2015)
Indian Lean	43.2	(Dasgupta et al., 2015)
Euro-Brazilian	41.1	(Luperini et al., 2015)
Egyptian	39.5	(Mehanna et al., 2016)
Asian (China)	**30.0 (23-37)**	(Wu et al., 2011)
African	**4.0**	(Okpechi et al., 2010)
	SLC30A8/rs13266634 (T-allele)	
Euro-Brazilian	**27.8 (23-32)**	This work
East Asian	**46.0**	(Lara-Riegos et al., 2015)
Han Chinese	**45.5**	(Zhang et al., 2015)
Asian (China)	**43.4 (38-49)**	(Zheng et al., 2012)
Caucasian (Poland)	33.0 (28-38)	(Kurzawski et al., 2012)
European	28.6	(Lara-Riegos et al., 2015)
American	26.2	(Lara-Riegos et al., 2015)
Mayan	25.0	(Lara-Riegos et al., 2015)
Indian	23.0	(Khan et al., 2015)
African	**8.3**	(Lara-Riegos et al., 2015)
Nigerian (Yoruban)	**6.1**	HapMap

MAF = minor allele frequency (%); 95%CI = confidence interval. HapMap (http://www.hapmap.org/). In bold: MAF outside the 95%CI obtained in our study.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by CNPq - Conselho Nacional de Desenvolvimento Científico e Tecnológico and Araucária Foundation.

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