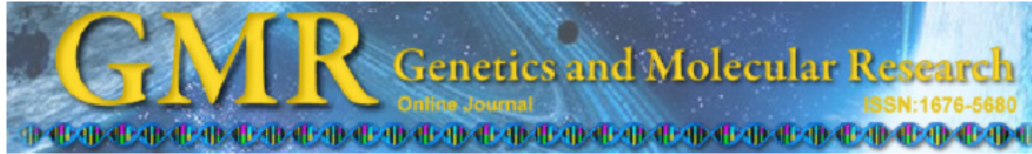

Low prevalence of glucokinase gene mutations in gestational diabetic patients with good glycemic control

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ABSTRACT. Glucokinase (GCK) plays a key role in glucose homeostasis. Gestational diabetes mellitus increases the risk of gestational complications in pregnant women and fetuses. We screened for mutations in coding and flanking regions of the *GCK* gene in pregnant women with or without gestational diabetes in a Brazilian population. A sample of 200 pregnant women classified as healthy (control, N = 100) or with gestational diabetes (N = 100) was analyzed for mutations in the *GCK* gene. All gestational diabetes mellitus patients had good glycemic control maintained by diet alone and no complications during pregnancy. Mutations were detected by single-strand conformation polymorphism and DNA sequencing. Thirteen of the 200 subjects had *GCK* gene

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mutations. The mutations detected were in intron 3 (c.43331A>G, new), intron 6 (c.47702T>C, rs2268574), intron 9 (c.48935C>T, rs2908274), and exon 10 (c.49620G>A, rs13306388). None of these *GCK* mutations were found to be significantly associated with gestational diabetes mellitus. In summary, we report a low frequency of *GCK* mutations in a pregnant Brazilian population and describe a new intronic variation (c.43331A>G, intron 3). We conclude that mutations in GCK introns and in non-translatable regions of the *GCK* gene do not affect glycemic control and are not correlated with gestational diabetes mellitus.

Key words: Gestational diabetes; Glucokinase; GCK; SNPs; Mutations; Genetic susceptibility

INTRODUCTION

Glucokinase (GCK, HK-IV, HK-D, or ATP:D-hexose 6-phosphotransferase), EC 2.7.1.1 (Printz et al., 1993; Jetton et al., 1994; Matschinsky et al., 2006) was first identified in 1962 in rat liver (Iynedjian, 2009). GCK is a monomeric enzyme with dual function in glucose homeostasis (Matschinsky and Ellerman, 1968). GCK phosphorylates glucose in the C6 hydroxyl group and acts as a D-glucose sensor for insulin release in pancreatic beta cells (Matschinsky, 2009).

The affinity of glucokinase toward glucose (K_m, 5-7 mM) is about 20 times lower than that for other hexokinases (K_m, 20-130 µM) and is not inhibited by D-glucose-6-phosphate like the other hexokinase isoforms I, II, and III (Ruzzo et al., 1998).

In the nucleus of hepatocytes and pancreatic beta cells, GCK is bound to the glucokinase regulatory protein (GCKRP; 68 kDa) when blood glucose is in physiological concentrations (5-7 mM). Under this condition, GCK is in the super-open form and inactive. As the glucose levels increase in the blood and pancreatic beta cells, GCK is released from GCKRP and changes to the closed active form. In the active form, GCK promotes insulin release from beta cells, stimulating glucose phosphorylation (de la Iglesia et al., 1999; Arden et al., 2004).

The human *GCK* gene is located in the short arm of chromosome 7 (7p15.3-p15.1); it encodes a protein with 465 amino acids (Printz et al., 1993; Gloyn, 2003; Tinto et al., 2008). This gene is about 52 kb long and has 10 exons according to the reference sequence on NCBI (NG_008847.1). Some authors report that the *GCK* gene might have 12 exons when all mature RNA transcript possibilities are considered (Jetton et al., 1994; Ellard et al., 2000; Gloyn, 2003; Tinto et al., 2008). *GCK* exon 1 could be described as exon 1a (pancreatic-controlled promoter) and exons 1b/1c as hepatic-controlled promoters (Magnuson and Shelton, 1989; Stoffel et al., 1992).

Gestational diabetes mellitus (GDM) is defined as any degree of glucose intolerance upon first detection or recognition during pregnancy (American Diabetes Association, 2011). This condition increases the risk for pregnant mothers and fetuses for gestational complications (Riskin-Mashiah et al., 2009). *GCK* gene polymorphisms are associated with monogenic diabetes maturity-onset diabetes of the young type 2 (MODY-2) and is the determinant of 5% of all cases of GDM in Caucasian populations (Ellard et al., 2000; Gloyn, 2003). Between 1992 and 2009, more than 600 *GCK* gene mutations were described in different populations (Osbak et al., 2009). *GCK* heterozygous mutations that reduce enzyme activity lead to MODY-2 diabetes, whereas homozygous mutations causing a complete loss of enzyme activity result

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in a rare and dangerous type of diabetes known as permanent neonatal diabetes mellitus, in which affected patients have very low birth weight, intrauterine growth retardation, and severe hyperglycemia (Gloyn, 2003; Greeley et al., 2010; Rubio-Cabezas et al., 2011).

In a European population, the *GCK* gene promoter polymorphism -30G>A was found to be associated with increased fasting glucose (Rose et al., 2005). This polymorphism was also associated with high levels of glycated hemoglobin and fasting plasma glucose (Rose et al., 2005; Weedon et al., 2005; Santos et al., 2010b).

The aim of this study was to screen mutations in the coding and flanking regions of the *GCK* gene of pregnant subjects of a Brazilian population with or without gestational diabetes.

MATERIAL AND METHODS

Subjects

A sample of 200 pregnant women was classified as healthy (control, N = 100) or having gestational diabetes (GDM, N = 100) according to American Diabetes Association criteria (American Diabetes Association, 2010). 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 the peripheral blood leukocytes (buffy coat) by the salting-out method (Lahiri and Nurberger, 1991). DNA samples with $A_{260/280}$ ratio between 1.6 and 1.9 were used, and the concentrations of all samples were normalized to 100 µg/mL.

Polymerase chain reaction (PCR)

The GenBank database sequence (NG_008847.1) of the pancreatic glucokinase isoform was used for designing the primers. Primers for amplifying the exons and non-coding exon boundaries 2, 5 + 6 (conjugated), 8, and 9 of the *GCK* gene have already been described (Tinto et al., 2008). The other primers were designed and analyzed *in silico* by using the tools Primer3Plus and Blastn. Primer sequences and their characteristics are shown in Table 1.

PCR was performed in a volume of 20 µL, with a final content of 20-100 ng DNA template, 10 pM each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1X *Taq* buffer (75 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)SO_4$, 0.01% Tween 20), and 1.0 U *Taq* DNA polymerase (Fermentas). The reaction cycle included 94°C for 60 s, 30-35 cycles of 94°C for 30 s, melting temperature for 30 s (see Table 1), extension (72°C) for 30 s, and an additional 10 min at 72°C for final extension.

PCR products were verified by 1.5% agarose gel electrophoresis in 1X TBE buffer.

PCR-single-strand conformation polymorphism (SSCP)

SSCP was performed as described previously (Souza et al., 2005). Briefly, 5 µL PCR product was added to 10 µL formamide loading solution (95% formamide; 2 mM ethylenediaminetetraacetic acid (EDTA); 0.05% bromophenol blue, and 0.05% xylene cyanol) and heated at 98°C for 10 min. The mixture was immediately cooled on ice and subjected to polyacrylamide

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Table 1. Primers and PCR-SSCP conditions used.

GCK Exons/Amplicons/Primers	Bases (bp, 5'→3')	Tm (°C)	PCR-SSCP conditions**		
			Gel (%)	Volts	Time (h)
Exon 1	515				
Amplicon GCKE1A1	337				
GCKE1F1 (20 bp)	5'-ggtcaccatgacaaccacag-3'	59.8	12	300	3.5
GCKE1R1 (20 bp)	5'-ttctggacccttcctcactg-3'	60.2			
Amplicon GCKE1A2	337				
GCKE1F2 (18 bp)	5'-cctgcctggagaacatcc-3'	59.1	12	300	3.5
GCKE1R2 (19 bp)	5'-gcaaacactcccagaatgc-3'	59.2			
Amplicon GCKE1A3	233				
GCKE1F3 (22 bp)	5'-gccttggatatttccacttcag-3'	60.0	12	300	3.5
GCKE1R3 (20 bp)	5'-ccttctcaaagagcctgtgc-3'	60.1			
Exon 2	163				
Amplicon GCKE2*	290				
GCKE2F1 (20 bp)	5'-tgcagatgcctggtgacagc-3'	66.0	10	190	3.5
GCKE2R1 (20 bp)	5'-cacagctgcttctggatgag-3'	59.7			
Exon 3	155				
Amplicon GCKE3	242				
GCKE3F1 (22 bp)	5'-tgaggctgacacacttctctct-3'	59.3	10	190	3.0
GCKE3R1 (18 bp)	5'-ggccctgagatcctgcat-3'	61.2			
Exon 4	120				
Amplicon GCKE4	251				
GCKE4F1 (20 bp)	5'-gtccctgaggaatagcttgg-3'	65.9	10	190	3.5
GCKE4R1 (20 bp)	5'-cccctcatctgccttctg-3'	60.5			
Exon 5 and 6	96 and 100				
Amplicon GCKE5e6*	505				
GCKE5e6F1 (20 bp)	5'-tctgagcctgtttcctcagc-3'	60.7	10	190	5.0
GCKE5e6R1 (20 bp)	5'-ggcccttgaagcctgttgta-3'	62.4			
Exon 7	184				
Amplicon GCKE7	286				
GCKE7F1 (20 bp)	5'-agtgcagctctcgctgacag-3'	62.1	10	190	4.0
GCKE7R1 (20 bp)	5'-catctgccgctgcaccagag-3'	67.8			
Exon 8	156				
Amplicon GCKE8*	400				
GCKE8F1 (20 bp)	5'-tggctcattaacgagggaaa-3'	60.6	10	210	5.0
GCKE8R1 (20 bp)	5'-ctgagaccaagtctgcagtg-3'	57.1			
Exon 9	234				
Amplicon GCKE9*	411				
GCKE9F1 (19 bp)	5'-cctccctggagaacgagag-3'	63.5	9	300	4.5
GCKE9R1 (20 bp)	5'-aatcttggagcttgggaacc-3'	60.4			
Exon 10	1010				
Amplicon GCKE10A1	347				
GCKE10F1 (20 bp)	5'-cagccctgcttctcttctgc-3'	62.7	10	300	2.0
GCKE10R1 (21 bp)	5'-ccgaaaaactgagggaagagg-3'	62.3			
Amplicon GCKE10A2	278				
GCKE10F2 (18 bp)	5'-cataccgctggggaacag-3'	60.1	13	300	2.5
GCKE10R2 (19 bp)	5'-cccacacaggatgagttcc-3'	58.9			
Amplicon GCKE10A3	289				
GCKE10F3 (20 bp)	5'-atcctgctgtggcctcactt-3'	62.2	13	300	3.5
GCKE10R3 (21 bp)	5'-ctctgtctcttgcacctgctg-3'	61.3			
Amplicon GCKE10A4	299				
GCKE10F4 (21 bp)	5'-gcttgtgattctgggatggac-3'	61.8	9	300	2.5
GCKE10R4 (21 bp)	5'-cccacagtcctaatgcacaga-3'	61.1			

*Primers described by Tinto et al. (2008). **PCR-SSCP conditions, polyacrylamide (29:1) gel concentration (%) for each amplicon, electrophoresis voltage and run time for BioRad TetraCell (100 x 100-mm plates). Tm = melting temperature (°C).

gel electrophoresis. Gels were prepared in 33 mM Tris-HCl, pH 3.66 (Table 1). All gels were run with 1X TBE buffer (89 mM Tris base; 89 mM boric acid, and 2 mM EDTA, pH 8.3). Gels were stained with ethidium bromide and visualized under UV (302 nm) light (UVP System).

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DNA sequencing

All variants were identified by PCR-SSCP, and about 20% of all the subjects were sequenced (ET terminator reagent - GE Healthcare; ABI 377 Applied Biosystems) according to manufacturer instructions.

The sequences were compared with the GenBank NG_008847.1. The electropherograms were analyzed by BioEdit v7.0.8.0 and CodonCode Aligner v3.7.1 softwares.

Biochemical markers

Biochemical parameters such as fasting glucose, lipid profile (total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and triglycerides), urea, creatinine, and uric acid were quantified using an Architect Ci 8200 automated system (Abbott Diagnostics) and reagents, calibrators, and controls from the equipment manufacturer. Glycosylated hemoglobin (HbA1C) was measured in diabetic pregnant women by using high-performance liquid chromatography equipped with a cation exchange column (Varian II, Biorad).

Statistical analysis

Continuous variables with normal distribution were compared with the Student *t*-test for independent variables. Triglyceride levels were logarithmically transformed before analysis. The Fisher two-tailed exact test was used to compare categorical variables. Statistical analyses were performed using the Statistica for Windows version 8.0 software (StatSoft Inc., Tulsa). P value lower than 0.05 ($P < 0.05$) was considered to be significant.

RESULTS

The characteristics of the subjects are shown in Table 2.

Table 2. Clinical characteristics and biomarker concentrations for the studied groups.

Characteristics	Control group (N = 100)	GDM group (N = 100)	P
Age (years)	24.9 ± 6.2	31.7 ± 6.3	<0.001
Euro-/Afro-Brazilians (%)	95/5	90/10	0.18
Body mass index (kg/m^2)	25.2 ± 4.2	33.5 ± 6.3	<0.001
Family history for diabetes (%)	5	69	<0.001*
Hypertension (%)	1.3	32	<0.001*
Total cholesterol (mM)	5.0 ± 1.2	5.8 ± 1.2	<0.001
HDL cholesterol (mM)	1.0 ± 0.2	1.4 ± 0.3	<0.001
LDL cholesterol (mM)	3.4 ± 1.0	3.3 ± 1.1	0.44
Triglycerides (mM)	1.3 ± 0.5	2.5 ± 0.8	<0.001**
Creatinine (μM)	74.2 ± 8.0	67.0 ± 7.9	<0.001
Fasting glucose (mM)	4.5 ± 0.4	5.2 ± 1.1	<0.001
HbA1C (%)	-	5.6 ± 0.8	-

Values are reported as means ± SD or %. P value = *t*-test for independent variables. *Fisher two-tailed exact test. **Calculated for log-transformed values. GDM = gestational diabetes mellitus.

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Diabetic patients were older, heavier, and had a high frequency of hypertension than the controls. No significant ethnic differences were observed between the Euro-Brazilian and Afro-Brazilian (P = 0.18) populations.

Total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides were higher in the GDM group, but the LDL cholesterol levels showed no significant differences between the groups studied.

Fasting glucose levels were statistically higher and glycated hemoglobin concentrations were within the reference range (4.0-6.0%) in the GDM group.

All GDM patients showed a good glycemic control maintained by diet alone and did not show complications during pregnancy.

Mutations were detected in 13 subjects (6.5% of total). All variations were found in the intronic (introns 3, 6, and 9 and 10) regions (Table 3).

Table 3. Mutations detected in the *GCK* gene in the studied population.

Mutation characteristics	Glucokinase gene region			
	Intron 3	Intron 6	Intron 9	Exon 10
Chromosome position[1]	c.43331A>G	c.47702T>C	c.48935C>T	c.49620G>A
dbSNP[2]	-	rs2268574	rs2908274	rs13306388
Presence in control/GDM	1/0	10/0	1/1	0/2
Genotype 11[3]	199	190	198	198
Genotype 12[3]	1	7	1	0
Genotype 22[3]	0	3	1	2
Rare allele frequency (%)	G-allele 0.3	C-allele 3.3	T-allele 0.8	A-allele 1.0
95%CI	(0-1)	(2-5)	(0-2)	(0-2)

[1]Chromosome position = SNP notation according NCBI NG_008847.1. [2]dbSNP = Data Base Reference SNP (http://www.ncbi.nlm.nih.gov/projects/SNP/). [3]Genotypes 11, 12 and 22 were usual homozygous, heterozygous and rare homozygous, respectively. 95%CI = 95% confidence interval.

The mutations c.47702T>C, c.48935C>T, and c.49620G>A were not in Hardy-Weinberg equilibrium (HWE) (P < 0.001). The mutation detected in intron 3, c.43331A>G, was in HWE (P = 0.97).

DISCUSSION

Gestational diabetes has a frequency of about 7% (Schmidt et al., 2000) in the Brazilian population, and 5-7% of all the cases were found to have a *GCK* mutation (Ben-Haroush et al., 2004; American Diabetes Association, 2010; Liang et al., 2010). New insights on gestational diabetes suggested that detection of affected subjects is important for determining the pregnancy outcome (Metzger et al., 2008).

Clinical characteristics and biochemical parameters (Table 2) of the studied groups were similar to those reported in other studies (Santos et al., 2010a). GDM patients were usually heavier since overweight is a risk factor associated with this pathology and type 2 diabetes (Rosenberg et al., 2005; Rivero et al., 2008).

The high levels of total cholesterol and triglycerides in GDM patients were observed previously (Meyers-Seifer and Vohr, 1996; Grundy et al., 2006; American Diabetes Association, 2010; Marcinkevage and Narayan, 2011) and are consistent with frequent association of GDM with lipid profile modifications (Knopp et al., 1980). The significantly higher levels of

HDL cholesterol observed in GDM patients compared to controls was not observed in other studies (Grundy et al., 2006; American Diabetes Association, 2010; Marcinkevage and Narayan, 2011) that reported low HDL cholesterol levels in GDM patients of different populations.

LDL cholesterol levels did not show significant differences between the studied groups, which were also consistent with the findings of other studies, although a reduction in LDL cholesterol fraction associated with GDM has been reported (Metzger et al., 1980; Hollingsworth and Grundy, 1982). Glucose levels and HbA1C from GDM indicate that the patients had good glycemic control.

The subjects also had healthy habits such as balanced meals and physical exercise, factors that increase HDL cholesterol and normalize LDL cholesterol and glycemia. An analysis of the biochemical parameters and clinical characteristics indicated that our GDM group was well controlled and substantially different from those reported in other studies.

In this study, we used PCR-SSCP, first proposed by Orita et al. (1989), under acidic conditions, as described by Souza et al. (2005). For this screening, we used small-size amplicons that allowed a sensitivity of about 90% with this technique (Hayashi, 1991). Several SSCP conditions were tested, such as addition of glycerol and polyethyleneglycol and adjusting pH and gel concentrations. The optimized procedure that showed the best mutation identification was obtained with polyacrylamide gels prepared in low pH, as described by Souza and colleagues (2005).

PCR-SSCP detected 13 samples (6.5%) with different electrophoresis patterns. Sequencing and analysis of these amplicons showed 4 different mutations (Table 3).

All detected mutations are transitions present in intron 3 (c.43331A>G), intron 6 (c.44702T>C, rs2268574), intron 9 (c.48935C>T, rs2908274), and the non-coding region of exon 10 (c.49620G>A, rs13306388). The mutation detected in intron 3 was the only that was in HWE. The relatively small sample size associated with low frequencies of the mutation could explain the HWE results. The dbSNP database showed a considerably higher frequency (>50%) for the rare allele of mutation rs2268574 in intron 6 than that observed in this study (3.3%). However, this database does not reflect the findings obtained in population studies, has a small sample size, and is composed of samples of different ethnicities, which could explain the differences observed. No published frequencies were found for the other mutations detected in this study. The rare mutation in intron 3 (c.43331A>G) was not described in any database. Overall, the low frequencies of the detected mutations did not show reliable association with GDM.

Ellard et al. (2000) found a high prevalence of *GCK* gene mutations (80%) in GDM patients, which could be explained by the selection of a small study group (N = 15) with several criteria that enriched the presence of mutations. Briefly, the criteria were 1) persisting fasting hyperglycemia outside pregnancy, 2) increment in fasting and 2-h plasma glucose, 3) insulin treatment during pregnancy, and 4) history of type 2 diabetes, gestational diabetes, or fasting hyperglycemia. Kousta and colleagues (2001) reported a prevalence of 12% *GCK* gene mutations in a multiethnic population; subjects in this study were also selected by criteria similar to those of Ellard et al. (2000).

Both the studies showed mutation prevalence in the *GCK* gene that was considerably higher than that reported in this study. These differences are probably due to the selection criteria of the GDM patients, since GDM alone was selected based on good glycemic control in this study.

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In summary, in this study, the frequency of *GCK* mutations in a pregnant Brazilian population has been reported, and a new intronic variation (c.43331A>G, intron 3) for this gene was described. The mutation in introns and non-translatable regions of the *GCK* gene did not affect glycemic control and was not correlated with the GDM condition in healthy and GDM women.

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