Saudi Journal of Biological Sciences (2015) 22, 243–248

King Saud University

Saudi Journal of Biological Sciences

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ORIGINAL ARTICLE

Screening of mitochondrial mutations and insertion–deletion polymorphism in gestational diabetes mellitus in the Asian Indian population

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Received 20 September 2014; revised 29 October 2014; accepted 2 November 2014
Available online 13 November 2014

KEYWORDS

GDM;
MELAS;
A3243G;
MERRF;
A8344G;
Mitochondria

Abstract In this study we scrutinized the association between the A8344G/A3243G mutations and a 9-bp deletion polymorphism with gestational diabetes mellitus (GDM) in an Asian Indian population. The A3243G mutation in the mitochondrial $tRNA^{Leu(UUR)}$ causes mitochondrial encephalopathy myopathy, lactic acidosis, and stroke-like episodes (MELAS), while the A8344G mutation in $tRNA^{Lys}$ causes myoclonus epilepsy with ragged red fibers (MERRF). We screened 140 pregnant women diagnosed with GDM and 140 non-GDM participants for these mutations by PCR-RFLP analysis. Both A3243G and A8344G were associated with GDM (A3243: OR-3.667, 95% CI = 1.001–13.43, $p = 0.03$; A8344G: OR-11.00, 95% CI = 0.6026–200.8, $p = 0.04$). Mitochondrial DNA mutations contribute to the development of GDM. Our results conclude that mitochondrial mutations are associated with the GDM women in our population. Thus it is important to screen other mitochondrial mutations in the GDM women.

1. Introduction

Gestational diabetes mellitus (GDM) is the most common metabolic disorder in pregnant women and is characterized by carbohydrate intolerance of variable severity with onset or first recognition during pregnancy regardless of glycemic status after delivery (Chen et al., 2000). GDM remains one of the most common clinical issues faced by obstetricians (Wang et al., 2013). The risk of developing GDM during

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.sjbs.2014.11.001

pregnancy can be affected by genetic mutations/polymorphisms in the mother and neonates (Petry et al., 2011). GDM can produce adverse neonatal outcomes including birth defects, neonatal hypoglycemia, macrosomia, cardiac dysfunction, and long-term consequences such as increased risk for obesity, arterial hypertension, and metabolic syndrome (Lee et al., 2008). In addition, GDM has a high predictive value for later development of type 2 diabetes mellitus (T2DM) in the mother. Although insulin resistance is universally observed in pregnant women with GDM, the cellular mechanisms underlying this type of insulin resistance are not well-understood (Wang et al., 2013).

In the past decade, many efforts have been made to identify pathogenic nuclear and mitochondrial mutations in GDM. Advanced genetic approaches such as genome-wide association studies (GWAS) have identified multiple susceptibility loci in the pathogenesis of T2DM, many of which, through linkage scans, candidate gene studies, and GWAS, have also been shown to have roles in GDM, consistent with the notion that both types of diabetes share a common pathophysiology. Mutations in mitochondrial DNA (mtDNA) have been associated with a wide spectrum of clinical abnormalities, including neuromuscular disorders, heart failure, diabetes, hearing and visual loss (Brandon et al., 2005; Jacobs, 2003; Larsson, 2002; Wallace, 2005). More than 50% of these mtDNA mutations are located in 22 mitochondrial tRNA genes, including $tRNA^{Leu(UUR)}$ (Li and Guan, 2010). mtDNA mutations have been described in association with various diseases, including T2DM (Li and Guan, 2010; Mathews and Berdanier, 1998; Duraisamy et al., 2010; Padma et al., 2010). The involvement of mtDNA in disease pathogenesis could contribute to biased maternal transmission of certain forms of diabetes.

Mitochondrial diseases are clinically heterogeneous group of disorders ranging from single-organ to severe multisystemic diseases (Chae et al., 2004). The mitochondrial $tRNA^{Leu(UUR)}$ gene is a hotspot for pathogenic mutations associated with mitochondrial diseases with various clinical features. In fact, 21 different point mutations in this gene have been reported. Among these, the A3243G mutation has been associated with various types of mitochondrial multisystem disorders, such as mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS), maternally inherited diabetes and deafness (MIDD), myoclonus epilepsy with ragged red fibers (MERRF), maternally inherited progressive external ophthalmoplegia (PEO), hypertrophic cardiomyopathy, and Leigh syndrome (Mkaouar-Rebai et al., 2007).

Cataloged mtDNA mutations include point mutations, deletions, and duplications, the majority of which affecting transcription and translation of mtDNA are the main etiological factors of most mitochondrial diseases (Viola et al., 2008; Shen et al., 2011). One such deletion polymorphism involves the loss of one copy of a 9-bp tandem repeat (CCCCCTCTA) in the intergenic region of cytochrome c oxidase II (COII)/mitochondrial TK2 thymidine kinase 2 (MTTK) (Wrischnik et al., 1987). The "CCCCCTCTA" repeat polymorphism present between the 8271 and 8279 nucleotides of mtDNA has been well investigated in phylogenic population studies. MERRF is a multisystem mitochondrial disorder defined by myoclonus, generalized epilepsy, ataxia, and ragged-red fibers (RRFs) in muscle biopsies (Hirano, 2010). In 1992, an A to G point mutation at nucleotide position (np) 3243 in $tRNA^{Leu(UUR)}$ was identified by a large pedigree analysis MIDD, which was later shown to be associated with diabetes in about 1.5% of the diabetic population worldwide. Subsequently, many other mutations in the $tRNA^{Leu(UUR)}$ gene and other mtDNA regions were reported in T2DM (Crispim et al., 2008).

Maternal influence on the predisposition to GDM has also been observed, since mitochondrial transmission is exclusively maternal and both β-cell dysfunction and decreased insulin sensitivity have been associated with mtDNA mutations; thus, defective mtDNA could be a candidate gene for GDM. However, the 9-bp deletion in combination with the A3243G and A8344G mutation has also been associated with several diseases including T2DM (Crispim et al., 2008). In this study, we aimed to explore whether A8344G/A3243G mutations and the 9-bp deletion influence the occurrence of GDM in Asian Indian population.

2. Materials and methodology

2.1. Study design

We recruited 280 pregnant women from two hospitals in Hyderabad, India. Women without previous diagnosis of glucose intolerance were routinely screened for GDM between 24 and 28 weeks of gestation by a 50-g glucose challenge test (GCT). This test was considered GCT negative (GCT−) if the plasma glucose concentration was less than 7.8 mmol/L 1 h after glucose intake; otherwise, the patient was diagnosed as GCT positive (GCT+). These patients were then given a 100-g oral glucose tolerance test (OGTT). Diagnosis of GDM was based on the criteria set by the American Diabetes Association (2010). Glucose thresholds were as follows: fasting, 95 mg/dL; 1 h, 180 mg/dL; 2 h, 165 mg/dL; and 3 h, 145 mg/dL. A diagnosis of GDM was made if two or more of the values met or exceeded the threshold. Normal glucose tolerance (NGT) or non-GDM (n = 140) was diagnosed when all plasma glucose values were below the threshold values. Based on the above criteria, 140 subjects with GDM and 140 non-GDM participants were recruited. The NGT subjects were classified as normal pregnancy controls or non-GDM (Khan et al., 2014).

2.2. Clinical and biochemical data

Clinical and biochemical data for all subjects were collected between 24 and 28 weeks of gestation. Clinical data included age and weight. A family history of T2DM was also recorded. Body mass index before gestation (pre-BMI) was calculated according to Quetelet's equation by using the weight divided by the square of height (kg/m^2). Biochemical data consisted of fasting blood sugar (FBS) and postprandial blood glucose (PPBG) levels, as well as the 50-g GCT and the 100-g OGTT results.

2.3. DNA extraction

Peripheral blood (2 mL) was collected in EDTA tubes. Nucleic acids were extracted from white blood cells by salting out extraction (Khan et al., 2015). DNA quantity and quality were assessed by Nano Drop and gel electrophoresis. DNA was stored at −80 °C. The genotyping was performed at the

Department of Genetics and Molecular Medicine (NABL accreditation laboratory), Kamineni Hospitals, Hyderabad, Telangana, India.

2.4. Analysis of the mitochondrial mutations

Genotyping for the A3242G and A8344G mutations was performed by polymerase chain reaction (PCR) in an Applied Biosystems thermal cycler. PCR was performed in a 50-μL volume containing 5 μL of 10× Tris buffer (cat#105878, Bangalore Genie, Bangalore, India), 4 μL of $MgCl_2$ (25 mM; cat#METB5 Bangalore Gene, Bangalore, India), 1 μL of each dNTP (10 mM; cat# Fermentas, Hannover, MD, USA), 1 μL of each primer (10 pmol; Bioserve Biotechnology, Hyderabad, India) (Table 1), and 2.5 U Taq polymerase (cat#MME27, Bangalore Gene, Bangalore, India) as described previously (Crispim et al., 2008).

A three-step PCR for A3242G was performed as described (Khan et al., 2015). The initial denaturation at 95 °C for 5 min was followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s for A3242G and 60 °C for 30 s for A8344G, extension at 72 °C for 45 s, and a final extension at 72 °C for 5 min. The amplified mutation products were digested with *Hae*III (A3243G; cat#ER 108, MBI Fermentas, Hannover, MD, USA), and *Ava*II (A8344G; cat#ER 0312, MBI Fermentas, Hannover, MD, USA) in a total volume of 20 μL for 2 h at 37 °C, then separated by electrophoresis on 12% polyacrylamide gel electrophoresis. Bands were analyzed and imaged by a gel documentation system (UVI tech documentation system, Cambridge, UK).

2.5. Statistical analysis

Statistical analysis to determine the odds ratios, Yates correction, and *p* values was performed using Openepi for Windows, version 2.3.1 (Openepi Software, United States). The frequencies of each group in cases and controls (non-GDM) were compared with Pearson χ^2/Fisher's exact test. A *p*-value less than 0.05 was considered significant. The *t*-tests were performed with SPSS software version 19.0 (Chicago, USA).

3. Results

3.1. Characteristics of study subjects

The clinical characteristics of GDM patients and non-GDM subjects are given in Table 2. GDM cases ($n = 140$) had an age range of 22–38 years, 31% of them were primigravida, while 69% were multigravida. The pre-pregnancy BMI range of the GDM cases was 19.8–35.6 kg/m^2 with a mean of 27.08 ± 3.93 kg/m^2 and in non-GDM cases the mean was 24.10 ± 3.55 kg/m^2 ($p = 0.31$). FBS values differ significantly between two groups i.e. cases and controls (non-GDM subjects) ($p = 0.004$). In the GDM group, 45% managed their diabetes with diet and exercise, while 55% of patients required 4–8 units of insulin for the remainder of the prenatal period. PPBG values were found to be significantly high in GDM cases compared with the non-GDM subjects ($p < .0001$).

3.2. Detection of the A3243G heteroplasmic mutation

The $tRNA^{Leu(UUR)}$ A3243G variant was assessed in all subjects. The variant was found in fourteen subjects: eleven in GDM cases and three in the non-GDM subjects. There was a significant difference in the frequency of the A3243G variants (Table 3).

3.3. Detection of the A8344G heteroplasmic mutation

A 200-bp product of $tRNA^{Lys}$ (A8344G) was digested with *Ava*II to yield a 134/66 bp fragment. The frequency of the A8344G mutation in $tRNA^{Lys}$ (MERRF) was significantly high in the cases when compared with non-GDM subjects. Five variants were identified in the GDM group. Thus, there was an association between this genotype and GDM after Yates correction (Table 3).

3.4. 9-bp repeat polymorphism

The COII/MTTK gene contains a 200-bp region with a double CCCCCTCTA repeat (i.e. CCCCCTCTA–CCCCCTCTA). Insertion of a third 9-bp repeat yields a 209-bp fragment and deletion of one of the repeats yields a 191-bp fragment. In this study, the deletion polymorphism was found in 4.3% of the GDM patients; the remainder of the GDM patients carried the double repeat. None carried the insertion genotype. None of the non-GDM subjects carried an insertion or deletion genotype; thus, all carried the double repeat (Table 4).

4. Discussion

We studied 280 GDM and non-GDM pregnant women in Asian Indian subjects to characterize the association between mitochondrial mutations [$tRNA^{Leu(UUR)}$ (A3243G) and the $tRNA^{Lys}$ gene (A8344G)/(CCCCCTCTA) polymorphism]. The clinical results showed a significant difference in age, weight, FBS, and PPBG ($p < 0.05$). The results recommended that there is a possible association and development of GDM in women of the Asian Indian population.

The 9-bp deletion was detected in 6% of the GDM patients. Mitochondrial mutations and GDM suggest that it may have some pathogenic roles in disease development (Chen et al., 2000). GDM is a heterogeneous group of disorders with long-term implications for the mother and fetus. Although the susceptibility to GDM is genetically determined, the role of mitochondrial mutations in the etiology of GDM is not

Table 1 Mitochondrial DNA mutations detected by the PCR-RFLP method.

Gene	Mutation	Amino acid change	Nature	Primers	Fragment	Annealing temp	Enzyme
$tRNA^{Leu(UUR)}$	A3243G	–	Heteroplasmic	L3029–3048/H3456–3437	501 bp	56 °C	*Hae*III
$tRNA^{Lys}$	A8344G	–	Heteroplasmic	L8278–8305/H8386–8336	200 bp	60 °C	*Ava*II

Table 2 Clinical characteristics of the study population.

S. No.	Profiles	GDM (n = 140)	non-GDM (n = 140)	p Value
1	Age (Years)	22–38 (29.1 ± 4.46)	17–34 (24.6 ± 3.55)	0.02
2	Weight (kg)	69.2 ± 10.43	51.2 ± 6.26	0.0001
3	BMI (kg/m^2)	27.0 ± 3.93	24.1 ± 3.55	0.31
4	Mean gestational age	24.9 ± 5.01	NA	NA
5	FBS (mg/dL)	121.9 ± 13.21	83.2 ± 10.37	0.004
6	PPBG (mg/dL)	158.8 ± 47.76	112.0 ± 39.70	0.0001
7	Family history	84 (60%)	56 (40%)	0.002
8	Insulin/diet (R_x)	77(55%)/63(45%)	NA	NA

NA = not analyzed/not applicable.

Table 3 Prevalence of mitochondrial mutations in GDM and non-GDM subjects.

Mutations	GDM (n = 140)	non-GDM (n = 140)	p Value	Odds ratio	95% CI
A3243G	7.8% (11/140)	2.1% (3/140)	0.03	3.667	(1.001–13.43)
A8344G	3.6% (5/140)	0 (0/140)	0.04*	11	(0.6026–200.8)

* Indicates Yates correction.

Table 4 Analysis of the 9-bp repeats.

Disease	One repeat (deletion)	Two repeats	Three repeats (insertion)
GDM (n = 140)	6 (4.3%)	134 (95.7%)	0 (0.0%)
non-GDM (n = 140)	0 (0.0%)	140 (100%)	0 (0.0%)

known. Several mitochondrial mutations have been associated with human diseases such as T2DM and GDM (Chen et al., 2000; Vijaya padma et al., 2010). T2DM and GDM share a common pathophysiology that is characterized by insulin resistance and pancreatic β-cell dysfunction (Bao et al., 2012; Matharoo et al., 2013).

We found an association between the tested mutations and GDM, trembling with the previous studies we also found a similar lack of association in Singaporean women (Chen et al., 2000). To our knowledge, this is the first molecular epidemiological study of the association between A3243G, A8344G, and the 9-bp deletion and GDM in an Asian Indian women.

Repetitive and insertion/deletion DNA sequences have been found in nuclear and mtDNA and are associated with various diseases (Komandur et al., 2011). A 9-bp double CCCCCTCTA repeat in the intergenic region of MTCO2 and MTTK is polymorphic in an Asian Indian population, appearing as a single repeat (deletion) or three repeats (insertion) in $<3\%$ of the population (Thangaraj et al., 2005). The 9-bp deletion is more frequent in individuals with mt diseases such as MELAS and MERRF (Lin and Beal, 2006).

Chen et al. (2000) were unable to detect the A3243G mutation in GDM patients. The mtDNA A3243G mutation does not confer a genetic risk for GDM in Singaporean women; however, five mtDNA mutations were identified in GDM patients, including T3398C, which might have a functional role in the development of GDM. Two novel mutations (C3254A and A3399T) are also worthy of study because the sites of these mutations appear important for mitochondrial function. The A3243G mutation was not detected in this prior study, suggesting it may not majorly contribute to mild forms of diabetes such as GDM, although it could lead to the development of overt diabetes mellitus by affecting the offspring mitochondrial function (Chen et al., 2000).

A3243G mutational studies were performed in pregnant Taiwanese women (Chou et al., 2004). In this study both the mother and fetus carried the MELAS-specific A3243G mutation; however, the mtDNA level of the amniotic fluid did not significantly differ from that of the postnatal peripheral blood and hair follicles. Chou et al., 2004 concluded that MELAS syndrome may help in identifying mitochondrial mutations during pregnancy.

No studies have explored the association between the 9-bp repeat insertion–deletion polymorphism and GDM. Ours is the first report of this association. We suggest the insertion/deletion polymorphism in the untranslated region of mtDNA could have a pathogenic role in the disease, whereas A8343G and A8344G mutations have a role in GDM.

We identified six GDM women who carried the deletion genotype. Of these, five had a family history of T2DM and all five required insulin therapy. One woman was on diet and had no family history. All the women diagnosed with GDM were overweight (BMI 25–30), in their mid-30 s, and had very high fasting glucose (>100 mg/dL) and were examined at a mean gestational age of 28 weeks. Table 5 lists the demographic data of the GDM women with the deletion genotype.

Duraisamy et al., 2010 performed a mitochondrial study of the A3243G variant in South Indian T2DM patients. The variant was identified in two patients with similar clinical characteristics. They concluded that these mitochondrial mutations indicate a genetic predisposition to DM in the Coimbatore South Indian population (Duraisamy et al., 2010). In our study, we found eleven mutations in the GDM cases and three

Table 5 Characteristics of the GDM cases with the single repeat variant.

S. No.	Age	BMI (kg/m^2)	FBS (mg/dL)	Family history	Gestational age	R_x
1	30	26.2	139	Positive	28	Insulin
2	28	29.6	184	Positive	26	Insulin
3	33	29.4	128	Negative	26	Diet
4	31	27.9	121	Positive	28	Insulin
5	25	26.5	175	Positive	26	Insulin
6	29	26.3	110	Positive	32	Insulin

in the non-GDM subjects. Earlier study by Malecki (2001) explored the A3243G variant in 129 T2DM subjects and 12 GDM cases in a Polish population. They concluded that A3243G has no role in diabetes in this population (Malecki, 2001). Another study of GDM by Allan et al. (1997) found no instances of the A3243G mutation in American women.

Lee and Wei (1997) explored the A3243G and A8344G mutations in Korean diabetics and found only one instance of A3243G in a diabetic subject. Otabe et al., 1994 reported a 0.9% frequency of A3243G in diabetic Japanese and no instances of A8344G. The A3243G mutation has been studied in various populations, none of which has shown a significant association with disease, although the mutation was identified in all tested population. A8344G was detected in our study, the results of which were consistent with all the previous studies on this subject.

5. Conclusion

Our results conclude that mitochondrial mutations are associated with the GDM women in our population. A3243G; A8344G and 9-bp repeat have a role in our study with the pregnant women. For further studies different ethnic populations are required.

Conflict of interest

None.

Acknowledgements

We are thankful to all the volunteers who have participated in this study. We are thankful to the Department of gynecology and obstetrics, Kamineni hospitals and Muslim maternity hospitals for helping with the samples. We are thankful to the Indian Council for Medical Research for the funding of this research (Sanction no. 5-3-8-39-2007; RHN).

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