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RESEARCH ARTICLE

Expression of mitofusin 2 in placentae of women with gestational diabetes mellitus

BINGJIN CHEN[1], YAJUAN GE[2]*, HONGLIN WANG[1], HAIHONG ZHU[3], JINYU XU[4], ZHENGHONG WU[1] and SIYE TANG[2]

[1] *Department of Obstetrics and Gynecology, and* [2] *Department of Clinical Laboratory Medicine, Shanghai Fifth People's Hospital of Fudan University, Shanghai 200240, People's Republic of China*
[3] *Department of Obstetrics, Shanghai Baijia Maternity Hospital, Shanghai 201103, People's Republic of China*
[4] *Department of Outpatient, Shanghai Fifth People's Hospital of Fudan University, Shanghai 200240, People's Republic of China*
*For correspondence. E-mail: JudyYG8@163.com.

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Abstract. Gestational diabetes mellitus (GDM) represents a common carbohydrate metabolism disorder during pregnancy. The objective of this study was to evaluate the expression levels of mitofusin 2 (MFN2) expression in placentae of GDM patients compared to that in the placental tissues from normal uncomplicated pregnancies. A total of 70 subjects were enrolled from September 2014 to June 2016, including 42 patients with GDM (the GDM group) and 28 normal uncomplicated pregnancies (the control group). Immunohistochemical staining and qRT-PCR were used for the detection of the expression levels and distribution of MFN2 in the placentae of GDM patients and normal controls. Kolmogorov–Smirnov test was used for statistical analysis. $P < 0.05$ and $P < 0.01$ were used for assessing statistical significance. The baseline characteristics were comparable in both groups. The 1-h and 2-h postprandial glucose levels (PPG) were 7.94 ± 1.26 versus 6.88 ± 0.51 mmol/L and 7.01 ± 1.34 versus 6.14 ± 0.63 mmol/L, respectively, for the GDM group and the control group ($P < 0.05$). The relative expression levels of MFN2 mRNA were 0.982 ± 1.242 for GDM and 1.257 ± 0.815 for control, respectively, with significant between group difference ($P < 0.01$). Immunohistochemical staining analysis showed that MFN2 was mostly distributed in the cytoplasm of syncytiotrophoblasts under optical microscopy. Additionally, about 50% of samples of the GDM group were within the intensity of moderate staining of MFN2 and more than 50% of patients in the control group were within the intensity of strong staining of MFN2. The expression levels of MFN2 in GDM placentae was significantly lower compared to that of placentae from normal uncomplicated pregnancies.

Keywords. gestational diabetes mellitus; placenta; mitofusin 2; immunohistochemical staining.

Introduction

Gestational diabetes mellitus (GDM) is defined as carbohydrate intolerance or hyperglycaemia of variable severity with the onset or first recognition during pregnancy (Rani and BeGuM 2016). It can be diagnosed in either first or subsequent trimesters with the screening time recommended at the end of second trimester of 24–28 weeks' gestation (Rani and BeGuM 2016; Tieu *et al.* 2017). The offspring of women with GDM are prone to adverse side effects such as stillbirth, macrosomia, shoulder dystocia, neonatal hypoglycaemia, pregnancy hypertension and type 2 diabetes (T2D) (Rosenstein *et al.* 2012). A timely intervention can not only reduce the risk of perinatal complications of early pregnancy loss and onset of GDM, but also improve the postpartum maternal health status and health-related quality of life (Crowther *et al.* 2005; Rojas *et al.* 2014). However, the precise mechanisms underlying GDM are still not completely understood.

The accumulating evidence has supported that GDM and T2D may share a common aetiology and pathological path (Weijers and Bekedam 2007; Lehnen *et al.* 2013). Increasing food supply to the intrauterine foetus triggers a diabetogenic status (moderate peripheral insulin

Bingjin Chen, Yajuan Ge and Honglin Wang contributed equally to this study.

resistance) for the mother during late pregnancy (Weijers and Bekedam 2007; Lehnen *et al.* 2013). GDM is thus developed with temporal maternal islet β-cell function incapable of adapting to this increased insulin demand of late pregnancy, which usually recovers after delivery. Several genetic variants including genes for islet β-cell function have been observed relevant to GDM (Lehnen *et al.* 2013). In addition, other factors particularly epigenetic programming of the metabolism prior to and/or during pregnancy seem to play an indispensable role (Weijers and Bekedam 2007; Lehnen *et al.* 2013). Bouchard and colleagues proved that the epigenetic modification of ADIPOQ DNA methylation profile was associated with maternal glucose status and maternal circulating adiponectin concentration (Bouchard *et al.* 2012). A similar epigenetic mechanism of DNA methylation of promoter of PPARGC1A, a master regulator of mitochondrial genes involved in energy metabolism and ATP production that drives glucose-stimulating insulin secretion of human islet, was observed in association with both T2D and GDM (Fernandez-Morera *et al.* 2010).

As a highly conserved GTPase embedded in the outer layer of mitochondria membrane, mitofusin-2 (MFN2) is essential for mitochondrial fusion to maintain its normal structure and function (Weijers and Bekedam 2007; Pang *et al.* 2011). A recent report demonstrated that low levels of MFN2 may be correlated with mitochondrial damage and apoptosis in the placental villi of unexplained miscarriage (Pang *et al.* 2013). The difference of MFN2 expression between normal pregnant women and certain pathological gestation such as a missed abortion has been documented (Pang *et al.* 2011). However, little robust evidence is available on the relationship between MFN2 and GDM especially in the Chinese population. The objective of this study was to detect the possible alteration of MFN2 expression in placental tissues of GDM as a pathology mechanism in comparison with normal nondiabetic gestational control. The findings may present a new therapeutic target for GDM patients.

Participants and methods

Study population and diagnosis

A total of 70 pregnant women were recruited and studied between September 2014 to June 2016, who were admitted for delivery in the Fifth People's Hospital of Fudan University in Shanghai, including 42 pregnant women with GDM and 28 normal control within same term excluding glucose metabolism disorders and other confirmed obstetric complications. The inclusion criteria were as follows: healthy without pregestational history of diabetes, aged 20–35 years, and singleton pregnancy. The exclusion criteria were: pregnancy-induced hypertension, cardiac diseases, severe hepatic and renal diseases, other endocrinology diseases, haematological diseases, premature rupture of foetal membrane, medical history of recent infection, alcoholism, smoking, drug addiction and history of insulin therapy. This study was reviewed and approved by the ethics committee of the Fifth Hospital affiliated to Fudan University in Shanghai, with informed consent signed and dated by all subjects.

We followed the diagnostic criteria of GDM recommended by the International Association of the Diabetes and Pregnancy Study Groups (IADPSG) 2010 criteria (Sacks *et al.* 2012). The clinical diagnosis can be established with one of below criteria met in 75 g OGTT (oral glucose tolerance test) at gestational 24–28 weeks: (i) fasting plasma glucose (FPG) $\geq$5.1 mmol/L; (ii) 1-h postprandial glucose (PPG) $\geq$10.0 mmol/L; (iii) 2-h PPG $\geq$8.5 mmol/L.

Sampling of placental tissue

A sample of placental tissue of 0.5 × 0.5 × 2 cm was cut off from the maternal side within 5 min postdelivery of placenta, avoiding organizing, calcified and haemorrhagic lesions to be collected. The specimens were flushed clean with cold neutral saline and then placed in EP sterile tube without contamination by RNA enzymes. They were preserved in a refrigerator at −80°C for future analysis.

RNA extraction

The RNA extraction process was started from the homogenization of placental specimens then prepared for further centrifugation, extraction and sedimentation. The total RNA was extracted from the frozen placental sample tissue using TRIZOL (9109, RNAiso Plus, Dalian TaKaRa Bio-engineering, China) reagent according to manufacturer's instructions. The purity and quantity of RNA was evaluated with 0.1% diethylpyrocarbonate-treated water (DEPC-H_2O) as blank. The concentration and quality of RNA from placental specimens were measured using 2 μL RNA solution by microplate spectrophotometric methods (Infinite M100 PRO, TECAN, Männedorf) while referring to the results of agarose gel electrophoresis.

Determination of MFN2 gene expression in placental tissues

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was used to estimate MFN2 expression in GDM placentas compared to the control. Total RNA extracted using trizol reagent described above was subjected to reverse transcription using a kit (RR036A, 5xprimeScriptRT Master MIX, Dalian Takara, China) under the conditions of 37°C for 15 min then 85°C for 5 s. qPCR was performed with real-time fluorescent quantitative PCR (Viia7, ABI, Carlsbad, USA) using SYBR GREEN PCR kit (1504490,

ABI, Carlsbad, USA) and the primer pairs, GAPDH, 5′-TGACAACTTTGGTATCGTGGAAGG-3′ (forward), and 5′-AGGCAGGGATGATGTTCTGGAGAG-3′ (reverse), and MFN2, 5′-GAACTGGACCCCGTTACCAC-3′ (forward), and 5′-TTGATCACGGTGCTCTTCCC-3′ (reverse). The reaction was carried out under the following laboratory conditions: 50°C for 3 min, 95°C for 3 min, followed by 40 cycles at 95°C for 10 s then 60°C for 30 s, melt curve 60°C to 95°C increment 0.5°C for 10 s (plate read). The comparative threshold cycle (CT) method was used for relative quantification. All specimen reactions were performed in triplicate, with data averaged across the mRNA levels of each specimen and then normalized to GAPDH.

Assay of electrophoresis

Agarose gel preparation was performed as follows: 2.0 g agarose (111860, Shanghai Nanxi Bio-technological) was heated for dissolving in 100 mL 1x TAE. The nucleic acid dye was added until the agarose solution was cooled down to around 50–60°C. The mixture was blended and poured slowly into a trough plate. Subsequently, the mixture was cooled and condensed for 30 min and then electrophoresis was run for 30 min with electric voltage 120 V (DYY-6C, Beijing Liuyi Instrument Plant). Images were taken for the display of electrophoresis assay results.

Immunohistochemical staining

After routine fixation with paraformaldehyde and dehydration with stepwise alcohol and dimethylbenzene, the specimens of placental tissue processed were embedded in paraffin and cut as 4-μm-thick serial sections followed by baking at 60°C for 30 min and then stored at room temperature. After rebaking at 62°C for 1 h, these sections were subjected to stepwise deparaffination, rehydration, blocking of endogenous peroxidase, antibody incubation with rabbit anti-MFNs Ab (20161101, Jiang Nan Jie) and subsequent goat anti-rabbit IgG HRP (H&L) (111-035-045, Jackson Immunoresearch laboratory, China), 3, 3′-diaminobenzidine colouration (DAB, ZLI-9018, Beijing Zhongshan Origene Bio-technology, China) and haematoxylin counterstaining (BA-4097, Zhuhai Beisuo Biotechnology, China). Finally these sliced pieces were subjected to dehydration, transparency, mounting and sealing for identification. Specimens of both groups were processed under exactly identical laboratory conditions.

The results of immunohistochemical staining were evaluated and scored using below method: the amounts of positively stained cells in five independent high visions (400) were calculated under optical microscopy. With semiquantitative method, staining intensity (intensity, I) can be divided into four classes of no stain as 0 score, weak stain as 1 score, moderate stain as 2 score and strong staining as 3 score. The percentage of positively stained cells (percentage, P) was calculated. The equation for calculating immunohistochemical score (histological score, H) was $H = \Sigma I \times P$.

Statistical analysis

All the data were described as mean ± SEM. The software for statistical analysis was SPSS22.0. The software for graph drawing was Graphpad Prism 5 (Graphpad Software, San Diego, USA). Kolmogorov–Smirnov test was used for testing on both statistical normality and statistical significance. $P < 0.05$ and $P < 0.01$ was the criteria used for statistical significance and great statistical significance. Informed consent was obtained from all subjects included in the investigation. The study involving human participants were in accordance with the ethical standards of the research committee. This article does not contain any studies involving animals.

Results

General baseline characteristics

The average age of subjects was 27.31 ± 4.38 years for control and 29.64 ± 4.81 years for GDM patients. The gestational weeks for delivery were 39.15 ± 1.14 for control and 38.23 ± 2.23 for GDM patients. These general baseline demographic characteristics were comparable in both groups (table 1).

FPGs, 1-h and 2-h PPGs of 75 g OGTT

The FPGs, 1-h and 2-h PPGs of 75 g OGTT were 5.33 ± 0.57 versus 4.19 ± 0.42 mmol/L, 7.94 ± 1.26 versus 6.88 ± 0.51 mmol/L and 7.01 ± 1.34 versus 6.14 ± 0.63 mmol/L for GDM group and normal uncomplicated pregnancy group, respectively. Except FPGs, between-group differences were significant in values of 1-h PPGs and 2-h PPGs ($P < 0.05$).

The expression of MFN2 mRNA in placental tissues

qRT-PCR data showed the relative expression levels of MFN2 mRNA in both groups were 0.982 ± 1.242 for GDM group and 1.257 ± 0.815 for normal uncomplicated pregnancy group, respectively. The relative expression level of MFN2 mRNA in GDM group was significantly lower compared to normal uncomplicated pregnancy group ($P < 0.01$, figure 1, a&b).

The expression and distribution of MFN2 proteins in placental tissues

Immunohistochemical staining analysis results showed MFN2 proteins were expressed in placental tissues of both

Table 1. Clinical characteristics of study subjects.

Group	Subject number	Age (y.o.)	Gravidity	Gestational weeks	FPG (mmol/L)	1-h PPG (mmol/L)	2 h PPG (mmol/L)
Control	28	27.31 ± 4.38	1.89 ± 0.77	39.15 ± 1.14	4.19 ± 0.42	6.88 ± 0.51	6.14 ± 0.63
GDM	42	29.64 ± 4.81	2.12 ± 0.89	38.23 ± 2.23	45.33 ± 0.57	7.94 ± 1.26	7.01 ± 1.34

GDM, gestational diabetes mellitus; y.o., years old; FPG, fasting plasma glucose; PPG, postprandial glucose; h, hour.

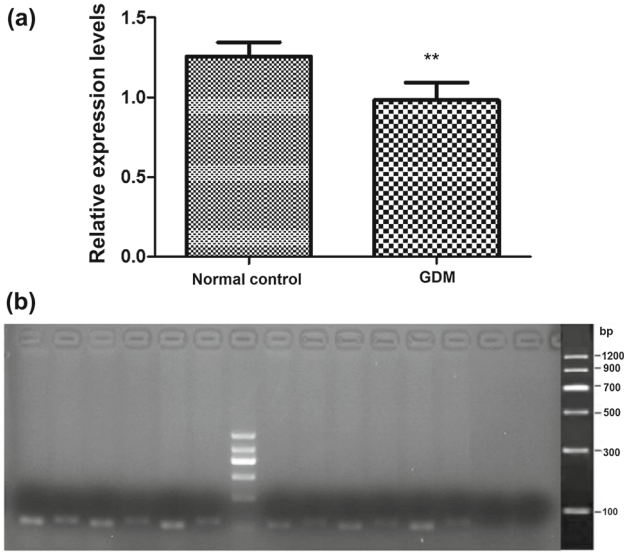

Figure 1. The relative expression levels of mitofusin-2 (MFN2) in gestational diabetes mellitus (GDM) versus normal control placentae. (a) MFN2 expression determined by qRT-PCR analysis. $^{**}P < 0.01$ versus normal control placentae. (b) The electrophoresis result image of qPCR products in GDM versus normal control placentae. From left to right the electrophoresis results in sequence were 52 (reference gene-MFN2)-23 (reference gene-MFN2)-97 (reference gene-MFN2)-marker-2 (reference gene-MFN2)-41 (reference gene-MFN2)-82 (reference gene-MFN2)-negative control (reference gene-MFN2). 52, 23 and 97 were normal placenta specimens. 2, 41 and 82 were GDM placenta specimens.

groups. Most of them existed in the cytoplasm of placental syncytiotrophoblasts while a few was deposited in the vascular endothelial cells as observed under an optical microscope (figure 2). The exact subject number for three intensity levels of immunohistochemical staining (score) were 11 versus 4 for weak staining ($H \leq 1$), 21 versus 9 for moderate staining ($1 < H \leq 2$), 10 versus 15 for strong staining ($H > 2$) in GDM and normal uncomplicated pregnancy group, respectively (table 2). About 50% of subjects in GDM group were within the intensity of moderate staining while more than 50% of subjects in normal uncomplicated pregnancy group were within the intensity of strong staining.

Discussion

This study is a pilot study on the relationship between MFN2 expression and GDM in Chinese women. By means of modern techniques of immunohistochemical staining and qRT-PCR, it was demonstrated that the distribution and expression of MFN2 mRNAs and proteins in placentae were much lower in the GDM group than that in normal uncomplicated pregnancy group. Under optical microscopy, the deposition of MFN2 was mostly in the cytoplasm of syncytiotrophoblasts, instead of cytotrophoblasts and mesenchymal cells of placental tissues.

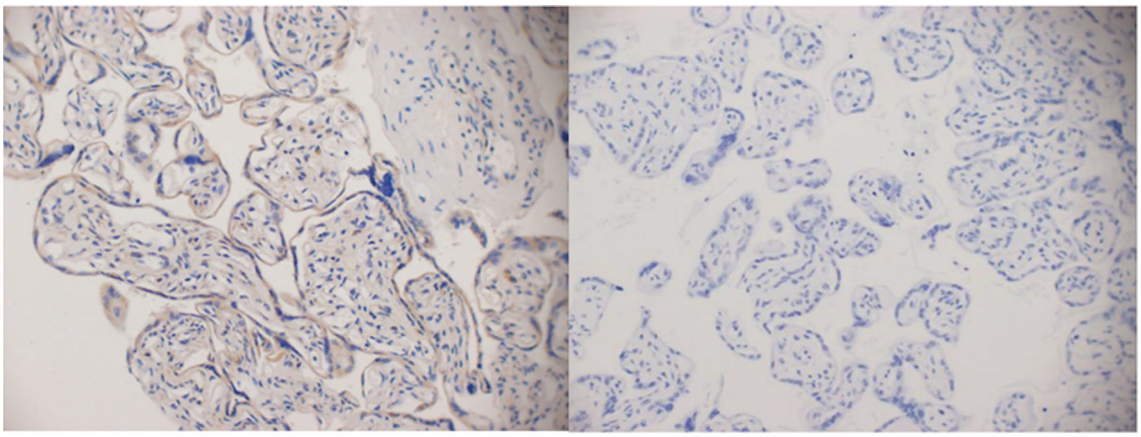

Figure 2. Immunohistochemical staining image of MFN2 expression and distribution in GDM versus negative control placentae under optical microscopy. MFN2 were expressed in both GDM and negative control placentae, which were mainly distributed in the cytoplasm of syncytiotrophoblasts with a little deposited in vascular endothelial cells under optical microscopy.

Table 2. Immunohistochemical staining scores on the expression of MFN2 in placental tissues.

Group	$H \leq 1$	$1 < H \leq 2$	$H > 2$
Control	4	9	15
GDM	11	21	10
		$P < 0.05$	

GDM, gestational diabetes mellitus; MFN2, mitofusin-2.

Several microarray and qRT-PCR studies have provided valuable information that GDM can modify the pattern of global placental transcriptome (Enquobahrie *et al.* 2009). These genes identified and confirmed differential expression in GDM placentae are involved in cell activation, immunological response, organ development and regulation of cell death (Enquobahrie *et al.* 2009). Gene ontology enrichment due to transcriptomic and methylomic differentials in GDM placentae is mainly manifested as over-representation of immune response pathways coordinated in the major histocompatibility complex (MHC) region, which coincides with the physiologically altered inflammation profiles or cytokine network during pregnancy and GDM (Binder *et al.* 2015; Wedekind and Belkacemi 2016). It was also investigated that compared to nondiabetic control placentae the markedly reduced apoptosis subsequent to the dysregulation or alterations of apoptotic and inflammatory genes including mitochondria pathway may promote larger placentae, with corresponding increased risk for delivering macrosomic newborns by mothers with GDM (Magee *et al.* 2014). Particularly, the obviously altered expression profiles of 5197 genes in blood and 243 genes in placenta are attributable to the pathogenesis of GDM in Chinese maternal population including freshly identified and previously unreported genes of VAV3, PTPN6, CD48 and IL15 with significantly different expression patterns, and two different pathways established as the GDM-associated 'Natural killer cell mediated cytotoxicity' in blood and 'Cytokine-cytokine receptor interaction' in placentae (Zhao *et al.* 2011). With the application of modern gene chip technique for pathology screening and analysis, the genotypes of single-nucleotide polymorphisms (SNPs) rs266729 for adiponectin (ADIPOQ), SNPs rs3802177 and rs13266634 for solute carrier family 30 (zinc transporter) showed significant differences between GDM and healthy pregnancy using the blood samples collected from Chinese women, further validated by DNA sequencing method (Liang *et al.* 2010).

MFN2 is critical for mitochondrial fusion, which in turn affects mitochondrial dynamics, distribution and functions such as mitochondrial oxidative metabolism. Mitochondrial biogenesis and oxidative capacity play important roles in the development of many diseases including T2D and GDM (Jahani-Asl *et al.* 2007; Liesa *et al.* 2009; Lappas *et al.* 2011). Hyperglycaemia or GDM exposure can be caused by the imbalanced ROS/antioxidants and systemic inflammation majorly produced in dysfunctional placental mitochondria, usually with MFN2 expression level as a marker. In recent studies, hyperglycaemia or GDM was found to be related to methylation of genes that are preferentially or predominantly involved in energy/glucose/adiposity metabolism and regulation (such as islet β-cell function and insulin resistance), affecting the feto-placental growth and development (Jahani-Asl *et al.* 2007; Liesa *et al.* 2009; Lappas *et al.* 2011; Ruchat *et al.* 2013; Kang *et al.* 2017). The consistent result between this study and other studies reported is that the decreased expression of MFN2 may be one of the new core mechanisms for GDM and subsequent short-term or long-term clinical outcomes. Therefore, new diagnostic therapeutic criteria and method can be researched and developed in near future for the purpose of preventing and treating GDM by targeting MFN2.

Although the data of this study is convincing with anticipated conclusion, two important defects of this study design are the small sample size and lack of short-term, medium-term and/or long-term postpartum follow-up on both mothers and their offspring, which suggests that further investigations are necessary. Considering the widespread expression of MFN2 in various human tissues and organs, its possible interplay with other genes, the existence of many modifying factors identified and potential, the study on MFN2 may not only be limited to GDM, but also can extend to other pathologies such as Alzheimer's disease, metabolism syndrome, obesity, T2D, cardiovascular diseases and cancer.

In conclusion, the significant decrease of MFN2 expression in GDM placentae may be one of the aetiologies or pathological mechanisms newly recognized for GDM in Chinese pregnant women compared to normal pregnancy control with multiple contributing factors.

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