

Original Article

Expression of TET2 in human placenta in gestational diabetes mellitus

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Received February 19, 2017; Accepted March 21, 2017; Epub May 1, 2017; Published May 15, 2017

Abstract: Purpose: The placenta is a vital organ that exchange nutrients in the maternal-fetal interface, and its appropriate development is necessary for pregnancy. Studies proved that changes in DNA methylation of several genes had been observed in GDM, but the mechanism has not been explored. TET2, as a member of the TET (ten-eleven translocation) family proteins, can catalyze the conversion of 5-methyl-cytosine (mC) to 5-hydroxymethyl-cytosine (hmC), and implements the DNA demethylation which was very necessary for gene transcription. However, the functions of TET2 in GDM have not been declared. The aim of this study was to explore whether TET2 played a role in the development of GDM. Methods: The protein and mRNA level of TET2 in 16 pairs of placentas from normal pregnant females and GDM were detected by western blotting and quantitative real-time polymerase chain reaction (RT-qPCR), respectively. The localization of TET2 in placentas was evaluated by immunohistochemistry. The expression of TET2 in trophoblast cell lines with high glucose in vitro was measured by western blotting. Results: TET2 was localized in cytoplasm of trophoblast cells. Its protein and mRNA expression were lower in GDM placentas compared with control. However, the expression of TET2 has no difference between normal and high glucose cultured in vitro. Conclusions: According to our results, we confirmed that TET2 was downregulated in GDM placenta. However, trophoblast cell lines cultured in high glucose in vitro is not a proper model to study the dysregulation of TET2 in GDM placenta.

Keywords: TET2, gestational diabetes mellitus, high glucose, trophoblast

Introduction

Gestational diabetes mellitus (GDM) is a type of diabetes or abnormal glucose tolerance during pregnancy (except those patients who was diagnosed diabetes before pregnancy). It was characterized by abnormal elevation of post-prandial blood glucose and hyperinsulinemia [1]. GDM is one of the major pregnancy complications and its prevalence is increasing [2, 3]. The current criteria of diagnosis on GDM was assessed after 75 g oral glucose tolerance test (OGTT) at 24-28 gestational weeks [4].

GDM is drawing more attention for its serious consequence to both mothers and fetus during pregnancy and its high prevalence [3, 5, 6]. Most previous researches were focused on the relationship of hyperinsulinemia and insulin resistance [7-9]. Increasing evidence suggests that genes and environment might play a criti-

cal role in the pathogenesis of many diseases. Lesseur et al. suggested that maternal metabolic status before and during pregnancy can alter placental DNA methylation profiles on birth and contribute to metabolic programming of obesity potentially. In this research, higher Leptin gene (LEP) methylation in GDM placentas was observed compared with the control [10]. Ruchat et al. found that GDM epigenetically affects genes that are predominantly involved in metabolic diseases pathway (including glucose-related disorders), in line with the hypothesis that GDM exposure increases the risk of metabolic disorders through epigenetic modifications [11].

Moreover, high glucose treatment or the hyperglycemia of diabetes mellitus increased nuclear protein O-linked β -N-acetylglucosamine glycosylation (O-GlcNAcylation) [12]. Previous studies provided compelling evidences of a close

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relationship among O-linked N-acetylglucosamine (O-GlcNAc) transferase (OGT), O-GlcNAcylation, and the DNA hydroxylase properties of the TET family proteins involved in the DNA demethylation on CpG islands. These studies indicated that TET family might regulate by high glucose and is associated with GDM [13-17]. As our previous study showed that TET2 is highly expressed in placenta than other TETs, we investigate the possible role of TET2 in GDM in this study.

Materials and methods

Patients and tissue samples

Human placental samples were collected from normal and GDM term pregnancies after elective caesarean section in the Shanghai First Maternity and Infant Hospital during December 2014 to June 2015. GDM was diagnosed using International Association of Diabetes and Pregnancy Study Group (IADPSG) criterion [18]. Eligibility criteria included all women delivering in the sample period, who were carrying a singleton fetus, and that have performed a 75 g OGTT between 24 to 28 weeks of gestation. We excluded women who delivered prior to 28 weeks of gestation, those with either known pre-pregnancy diabetes mellitus or overt diabetes diagnosed during pregnancy.

The information had been identifying after data collection. All patients provided written informed consent before the collection of placental biopsy samples. Placental tissues were obtained from a portion of normal ($n = 16$) and GDM patients ($n = 16$) and obtained immediately (< 30 min) after delivery by caesarean section. Small pieces (~ 0.5 cm³) were cut from the fetal part of the placentas under the aseptic conditions and washed briefly in sterile PBS to remove maternal blood contamination. All samples were frozen within 15 minutes of delivery and stored at -80°C for Western blotting analysis. Additional placental tissues were fixed at 4°C using 4% paraformaldehyde in 10 mM PBS within 24 hours and embedded them in paraffin for immunohistochemistry (IHC).

Cell culture

The trophoblast cell line HTR-8/SVneo was cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Gibco,

California, USA) and JEG3 in α -MEM (Gibco, California, USA), containing 10% FBS (Gibco, California, USA), 100 unit/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (Gibco, California, USA). For the hyperglycemia mimicking condition cell culture, the cells were cultured in the DMEM/F-12 or α -MEM medium with final concentration of 5 mmol/L (the normal glycemic control), 15 mmol/L and 25 mmol/L (the hyperglycemic group) D-glucose (Merck, Darmstadt, Germany), and final concentration of 15 mmol/L and 25 mmol/L (the osmotic pressure matched to hyperglycemic control) mannitol (Sigma-Aldrich, St. Louis, MO, USA). All cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 .

RNA isolation and real-time PCR

Total RNA was isolated from placentas using TRIzol (Invitrogen, Carlsbad, CA) reagent. cDNA was synthesized using PrimeScript RT reagent kit (TaKaRa, Kusatsu, Japan) according to the manufacturer's recommendations. The mRNA expression of TET2 was quantified by a real time PCR with SYBR Green Premix Ex Taq (Tiangen, Beijing, China) according to the manufacturer's protocol. Real-time PCR was performed with the ABI StepOnePlus System (Applied Biosystems, Life Technologies, USA). The cycling parameters of reaction were as follows: 95°C for 15 min, 40 cycles of 95°C for 10 s and 65°C for 32 s. The primer sequences for TET2 were 5'-GGCTTTGGAATACTTGGGTT-3' (forward) and 5'-TGGACAAATCGGAACCTCTCC-3' (reverse). Relative levels of TET2 mRNA were normalized to β -actin. The primer sequences of β -actin were 5'-CCAACCGCGAGAAGATGA-3' (forward) and 5'-CCAGAGGCGTACAGGGATAG-3' (reverse). All reactions were carried out in triplicate. The products of this process were subjected to melting curve analysis and the relative mRNA level of TET2 was analyzed by the comparative threshold (Ct) cycle method ($2^{-\Delta\Delta\text{CT}}$).

Western blotting

Placental tissues and trophoblast cells lysate was extracted by lysis buffer (RIPA and 100 mM phenylmethyl sulfonyl fluoride [PMSF]) (Sigma-Aldrich, St. Louis, MO, USA), and the concentration of lysate was measured with the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). 20 μg tissue total protein extract or 10

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Table 1. Characteristics of women of normal control and gestational diabetes mellitus (GDM)

| Variable | NC | | GDM | | P value (unpaired t test) |
|---------------------------|----|----------------|-----|----------------|------------------------------|
| | n | Mean ± SEM | n | Mean ± SEM | |
| Median age (years) | 16 | 32.56 ± 3.48 | 16 | 32.00 ± 4.03 | 0.6814 |
| BMI (kg/m ²) | 13 | 21.96 ± 0.7884 | 16 | 23.26 ± 0.9225 | 0.3075 |
| Gestational weeks (w) | 16 | 39.37 ± 0.1679 | 16 | 39.21 ± 0.1403 | 0.4466 |
| Caesarean section (CS, %) | 16 | 100 | 16 | 100 | / |
| Baby birth weight (g) | 16 | 3527 ± 80.06 | 16 | 3457 ± 81.79 | 0.5490 |
| OGTT (mmol/L) | | | | | |
| Fasting | 16 | N/A | 16 | 4.44 ± 0.13 | / |
| 1 h | 16 | N/A | 16 | 10.32 ± 0.29 | / |
| 2 h | 16 | N/A | 16 | 8.77 ± 0.16 | / |

BMI, body mass index; OGTT, oral glucose tolerance test.

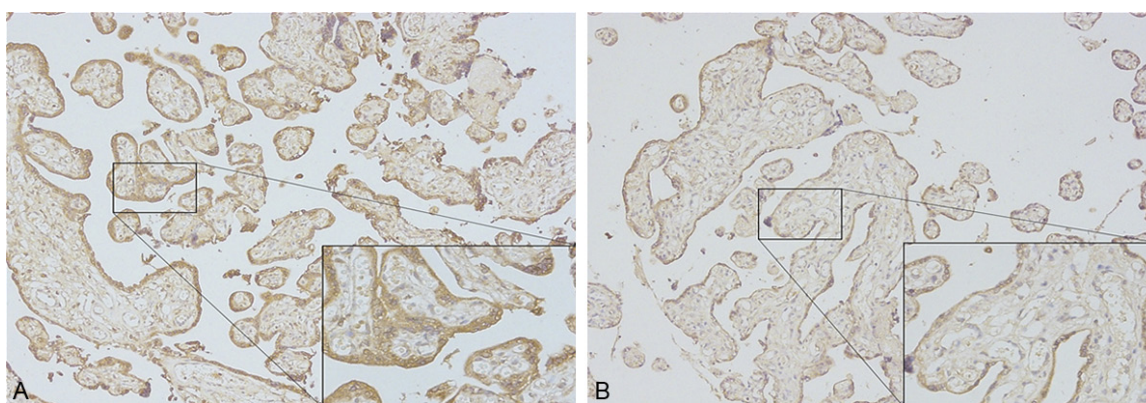


Figure 1. Immunohistochemistry of TET2 in placenta of normal control and gestational diabetes mellitus (GDM). TET2 was expressed in the cytoplasm of trophoblast cells of (A) normal and (B) GDM placenta. Compared with the control group, attenuated TET2 staining was shown in GDM.

µg cell total protein extract was subjected to 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to poly-vinylidenedifluoride membranes (Roche, Indianapolis, IN). After blocking with 5% skim milk in Tris buffered saline (TBS) with 0.1% Tween-20 (TBST) for 2 h at room temperature, the membranes were probed with primary antibodies directed against human TET2 (1:500; Santa Cruz, CA, USA) and β-actin (1:10000; Proteintech, Chicago, USA) overnight at 4°C, respectively. The membranes were then incubated with horseradish peroxidase streptavidin-linked second antibodies (SAB, Maryland, USA) for 1 h at room temperature and exposed to enhanced chemiluminescence reagent (ECL; Millipore, USA). Western blot data were standardized against β-actin and the relative expression of TET2 protein was analyzed using the Image-J imaging analysis software (NIH, Bethesda, MD) and presented as mean ± stan-

dard deviation. Western blot were performed three times for the quantification.

Immunohistochemistry

Immunohistochemistry staining was performed on sections of formalin-fixed and paraffin-embedded (FFPE) placental specimens from GDM patients and normal pregnancies. The placental tissue sections were cut, deparaffinized and dehydrated in deionized water. They were treated with 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity. The primary antibodies were detected using the anti-mouse/rabbit Poly-Horseradish-Peroxidase from the Immunohistochemistry Ready-to-use Detection kit-GTVision™ III Detection System/Mo&Rb (Gene Tech Biotechnology Co., Ltd., Shanghai, China). After rinsing and incubating with diaminobenzidine for 5 min, the sections were then washed in dis-

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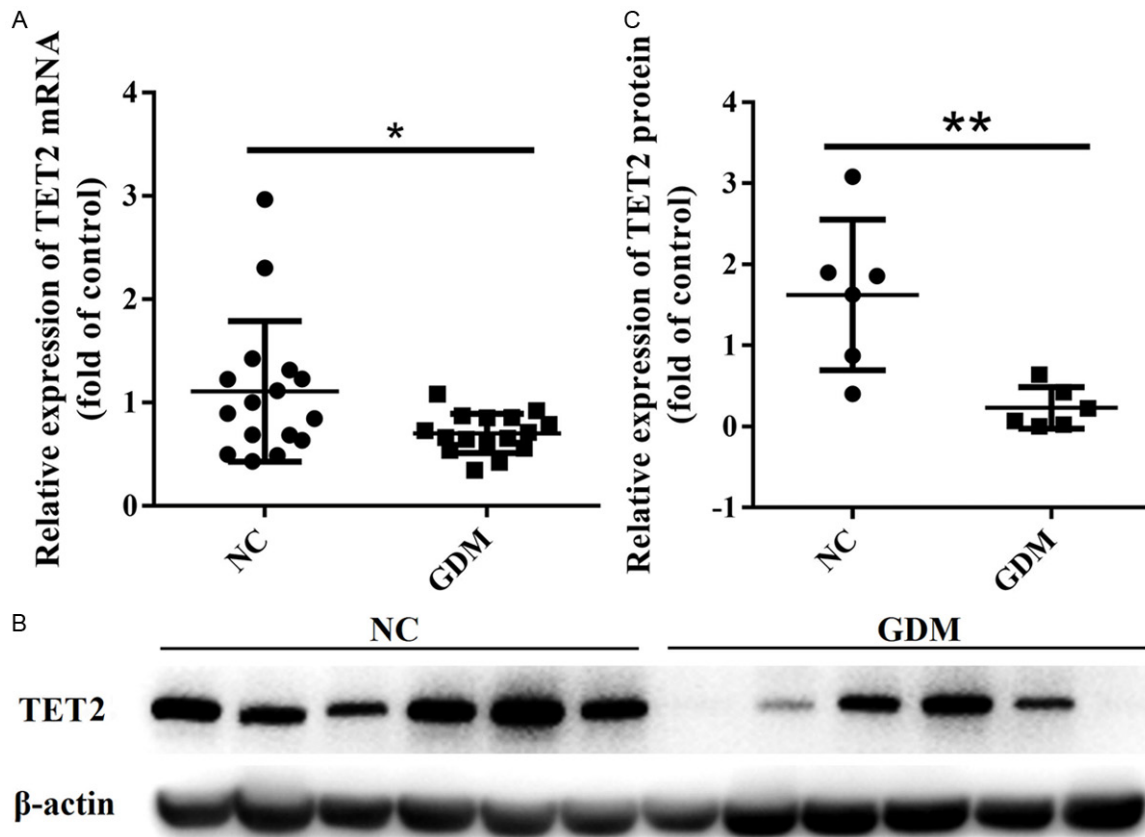


Figure 2. Expression of TET2 in normal control (n = 16) and GDM placenta (n = 16). Expression of TET2 (A) mRNA and (B and C) protein in placentae, which showed significantly decreases in GDM placenta than the control. Data are presented as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$ GDM versus normal control.

tilled water, counter-stained in hematoxylin, serially dehydrated and viewed under bright field microscopy.

Statistical analysis

All statistics were analyzed using the GraphPad Prism Program version 6.0 (GraphPad, San Diego, CA), applying unpaired Student's t-test for comparing expression of TET2 protein and mRNA levels between normal and GDM patients. Values were presented as the mean \pm standard error of the mean (S.E.M.). Difference between the two groups was considered significantly if $P < 0.05$ (* $P < 0.05$, ** $P < 0.01$).

Results

Demographic characteristics

The characteristics of normal and GDM pregnant female were shown in **Table 1**. There is no significant statistical difference on basic status, maternal age, BMI, gestational weeks and

baby birth weight ($P > 0.05$). According to the diagnose criterion, the GDM group had higher 1 h (10.32 ± 0.29 mmol/L, n = 16) and 2 h (8.77 ± 0.16 mmol/L, n = 16) post-oral glucose tolerance test (OGTT) glucose levels.

TET2 protein was present in human placenta

The localization of TET2 in placenta tissues was shown in **Figure 1**. Positive staining for TET2 was observed in the cytoplasm of trophoblast cells of the placentas in both normal control and GDM. Compared with the control group, attenuated TET2 staining was shown in GDM.

Both mRNA and protein levels of TET2 were lower in placentas from GDM patients

To investigate the expression levels of TET2 in placenta tissues, quantitative real-time PCR and western blotting analysis were performed. mRNA and protein levels of TET2 were down-regulated significantly in placentas from GDM compared with normal control, data was shown **Figure 2**.

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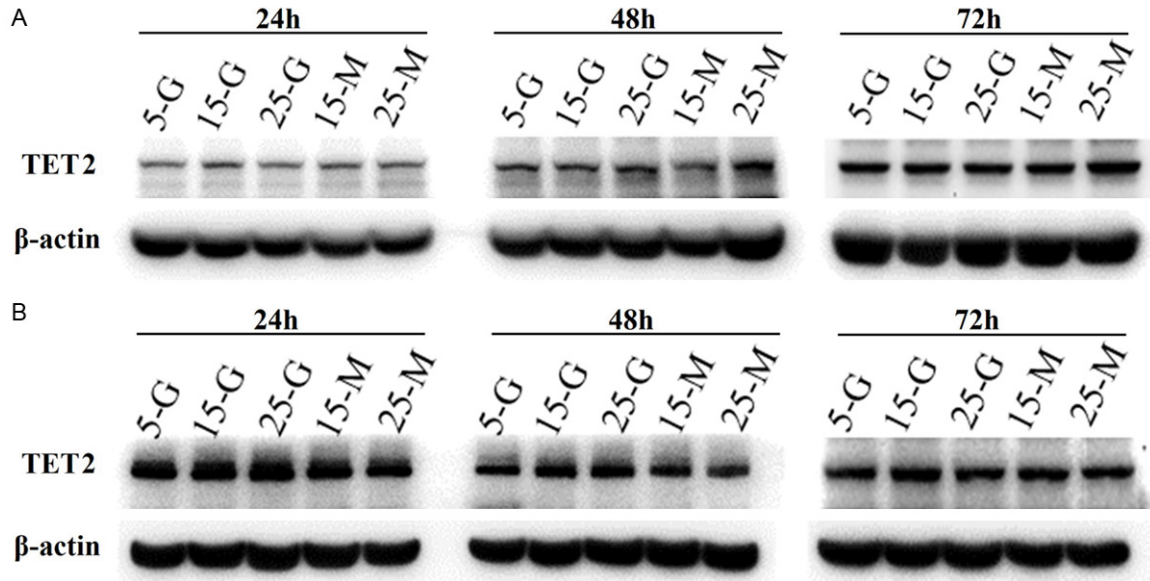


Figure 3. Hyperglycemia had no effect on the expression of TET2 in the trophoblast cell lines JEG3 and HTR-8/SVneo. The expression of TET2 in the trophoblast cell lines JEG3 (A) and HTR-8/SVneo (B) with the normal glucose and the high glucose group. (5-G, 5 mM glucose; 15-G, 15 mM glucose; 25-G, 25 mM glucose; 15-M, 15 mM mannitol; 25-M, 25 mM mannitol).

High glucose culture had no effect on the expression of TET2 in trophoblast cell lines JEG3 and HTR-8/SVneo

In order to explore the influence of high glucose on TET2 expression, the JEG3 and HTR-8/SVneo cells were cultured with different concentration of glucose. As shown in the **Figure 3A** and **3B**, the expression of TET2 in the trophoblast cell lines JEG3 and HTR-8/SVneo has no differences between the normal glucose group and the high glucose group, respectively.

Discussion

Nowadays, GDM becomes increasingly common with less pathogenesis and etiology researches. Previous studies found that GDM can cause the methylation of certain genes in placenta, which can suppress gene transcription and result in series of clinical diseases. TET2, a member of TET family proteins, possesses the characteristics of demethylation to control gene transcription.

In the present study, we found that TET2 expressed in placenta indeed and the expression level of TET2 decreased significantly in placentas from GDM compared with the normal pregnant ones. It illustrated that the lower

expression of TET2 in GDM placentas may be one reason for the higher level of DNA methylation of some genes in placenta. In order to elucidate the mechanism, we cultured the trophoblast cell lines in high glucose to simulate the hyperglycemia environment in vitro. We set the level as the normal glycemic control, and select 15 mmol/L and 25 mmol/L as the hyperglycemic group on account of the normal blood glucose level is 5 mmol/L. We also cultured cells in 5 mmol/L D-glucose and 15 mmol/L or 25 mmol/L mannitol as the osmotic matched hyperglycemic control. All groups of cells would be obtained total cell extract after incubating 24, 48 and 72 hours, respectively. Then the expression of TET2 was detected by western blotting. However, there was no significant difference in TET2 expression level among the three groups in every time point. In view of these results, there are some reasons and improvements speculated as follows. As we all known, GDM is one kind of multifactor diseases caused by the interaction between genetic factors and environmental factors [19]. It may not be able to well simulate the complicated environment of GDM in vivo when we simply cultured the trophoblast cell lines with high glucose in vitro. In the subsequent studies, we will commit to establish GDM mice model, and analyze the expression level of TET2 in model mice placenta.

Acknowledgements

This study were supported by the National Natural Science Foundation of China (No. 81501273 and No. 81401217) and Key Disciplines Group Construction Project of Pudong Health Bureau of Shanghai (No. PWZxq2014-02).

Disclosure of conflict of interest

None.

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