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Original Article

miR-9-5p plays an important role in gestational diabetes mellitus (GDM) progression by targeting HK-2

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Abstract: In this present study, we aimed to analyze the potential role of miR-9-5p in gestational diabetes mellitus (GDM). Expression levels of miR-9-5p in placental villous tissues and CTs cells from GDM women were determined by performing qRT-PCR. TargetScan was used to predict target genes of miR-9-5p and dual luciferase reporter assay was applied to reveal the prediction. To investigate the role of miR-9-5p in GDM, miR-9-5p was overexpressed in CTs cells from GDM women using miR-9-5p mimic. Expression of aerobic glycolysis related genes and mitochondrial complexes were then detected by qRT-PCR and Western blotting, respectively. We found miR-9-5p was significantly decreased in both placental villous tissues and CTs cells from GDM women. HK-2 is a direct target of miR-9-5p and can be negatively regulated by miR-9-5p in CTs cells. miR-9-5p upregulation significantly reduced expression of GLUT1, HK-2, PFK, and LDH at both mRNA and protein levels. Moreover, expression levels of mitochondrial complexes I, II, and III were markedly enhanced by miR-9-5p upregulation. In conclusion, miR-9-5p is involved in GDM progression through regulating aerobic glycolysis and mitochondrial complex expression by targeting HK-2.

Keywords: miR-9-5p, HK-2, GDM, aerobic glycolysis

Introduction

Gestational diabetes mellitus (GDM), characterized by damaged maternal glucose tolerance from the late second trimester of pregnancy onwards, is a common metabolic disease during pregnancy [1]. Hypernomic insulin resistance, decreased β-cell function, deficient β-cell compensation, or any combination of these can result in GDM [2]. Extensive research has indicated that GDM is associated with an increased risk of pregnancy-related maternal and neonatal morbidity [3]. Infants of pregnant women with GDM have been shown to suffer from complex diseases including obesity and metabolic and cardiovascular complications, in childhood and adulthood [4-6]. About 17-63% of GDM patients can develop type 2 diabetes mellitus 5-13 years after delivery. Due to use of different diagnostic criteria and other factors, incidence of GDM in different countries is different [7, 8]. At present, prevalence of GDM has been estimated at 3%~5.1% of pregnancies in China.

microRNAs (miRNAs), a class of small non-coding single-stranded RNAs (20~22 nucleotides in length), function in post-transcriptional regulation of gene expression via binding to the 3' untranslated region (UTR) of specific genes [9]. Studies have revealed that miRNA is involved in various cellular processes such as cell proliferation, apoptosis, and differentiation. Abnormal expression of miRNAs has been found in a variety of diseases including cancer, cardiovascular diseases, autoimmune diseases, and type 2 diabetes [9-12]. In recent years, numerous studies have indicated that miRNAs are involved in development of GDM [13-15].

miR-9 has been found to play critical roles in development and maturation of the nervous system in vertebrates [16] and in pathologies of the human brain [17, 18]. miR-9 also plays an important role in progression of tumors which are outside the nervous system [19, 20]. Moreover, studies have reported the protective role of miR-9-5p in lung, peritoneal, and skin

Table 1. Primer sequence for PCR

Sequence (5'-3')
HK2-Forward: AATGCTGGG AAACAA AGGT
HK2-Reverse: AGAGGA ATCCCTTCTTGG
GLUT1-Forward: GCCAGAAGGAGTCAGGTTCAA
GLUT1-Reverse: TCCTCGGAAAGGAGTTAGATCC
PFK-Forward: GCTGGGCGGCACTATCATT
PFK-Reverse: TCAGGTGCGAGTAGGTCCG
LDH-Forward: CACCTCGCTGGAGTACGGA
LDH-Reverse: CCATTCACATAGTGGCCCAAG
Complex I-Forward: GTGGTGCGTCCAGAGAGAC
Complex I-Reverse: GGCCTTCGCCATATCCTTTTC
Complex II-Forward: CGCGAGTTTGGGAATCTGAC
Complex II-Reverse: TAGTGAAGACGTGCGGATAGG
Complex III-Forward: ATCCGCACGTCTTCACTAAAG
Complex III-Reverse: TGGATCTCTCGAACTCTTCAGTC
miR-9-5p-Forward: GCGTCTTTGGTTATCTAGCTGTA
miR-9-5p-Reverse: GCGAGCACAGAATTAATACGAC
U6-Forward: 5'GCTTCGGCAGCACATATACTAAAAT3'
U6-Reverse: 5'CGCTTCACGAATTTGCGTGTCAT3'
GAPDH-Forward: 5'CTTTGGTATCGTGGAAGGACTC3'
GAPDH-Reverse: 5'GTAGAGGCAGGGATGATGTTCT3'

fibrosis [21, 22]. Previous studies have reported downregulation of miR-9 in GDM [14]. To the best of our knowledge, there is no information about miR-9-5p in GDM. Therefore, in this study, we aimed to investigate the potential role of miR-9-5p in GDM.

Materials and methods

Clinical specimen collection

A total of 10 paired placental villous tissues were collected from women with gestational diabetes, treated with insulin or glyburide (GDM), and healthy women with uncomplicated pregnancies after C-section, in Huaian Maternal and Child Health Care Center between June 2015 to June 2016. We randomly sampled villous tissues from five sites in the placenta, according to a previous study [23]. One part of the tissues was flash frozen in liquid nitrogen and then stored at -80°C. The other part was used for trophoblast cells culture. Placentas were collected from Huaian Maternal and Child Health Care Center under protocol approved by the Institutional Review Board of Huaian Maternal and Child Health Care Center. Informed consent was obtained from each patient.

Primary trophoblasts extraction

Primary villous cytotrophoblasts (CTs) were extracted from placental tissues of women with GDM and healthy women, as previously described [24]. CTs cells were grown in DMEM supplement with glucose (17 mM), 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin mix solution. Cells were incubated in a 5% CO_2 incubator at 37°C.

Cell transfection

The day before cell transfection, CTs cells from GDM women were plated into a six-well plate (5×10^4 cells per well). miR-9-5p mimic, its negative control (GenePharma Co., Ltd, Shanghai, China), and miR-9-5p mimic + Control plasmid or miR-9-5p mimic + HK-2-plasmid (GenScript, Piscataway, NJ, USA) were transfected into CTs cells using Lipofectamine2000 (Thermo Fisher Scientifc, Waltham, MA, USA, USA), according manufacturer instructions. 48 hours after cell transfection, transfected CTs cells were harvested and used for analysis.

QRT-PCR

Total RNA from placental villous tissues and CTs cells was isolated using TRIzol Reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA), following protocol provided by the manufacturer. RevertAid First Strand cDNA synthesis Kit (Fermentas, Pittsburgh, PA, USA) was carried out to synthesize cDNAs. Real-time PCR was performed with SYBR Green qRCR Mix (TOYOBO, Osaka, Japan) in a Thermal Cycler Dice Real-time system. U6 expression was used as internal control for miR-9-5p expression and GAPDH for gene mRNA ex-pression. Primer sequences were synthesized by GenScript (Piscataway, NJ, USA), as required, and listed in **Table 1**. For relative expression values calculation, $2^{-\Delta\Delta Cq}$ method was performed [25]. All experiments were repeated at least 3 times.

Western blotting

Forty-eight hours after cell transfection, CTs cells were harvested by 0.25% trypsin and total cell proteins were isolated using RIPA lysis buffer (Auragene, Changsha, China). Protein concentration was measured using BCA protein assay kit (Beyotime, China). The same amount of protein samples (30 μg) were se-

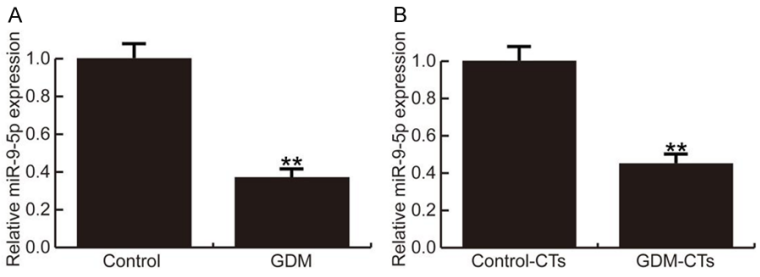

Figure 1. MiR-9-5p expression is downregulated in GDM. qRT-PCR assay was used to determine the level of miR-9-5p. A: Relative expression of miR-9-5p in placental villous tissues from GDM women and healthy control women; B: Relative expression of miR-9-5p in CTs cells from GDM women and healthy control women. Experiments were repeated three times. Data is presented as mean ± SD. **p<0.01 vs control.

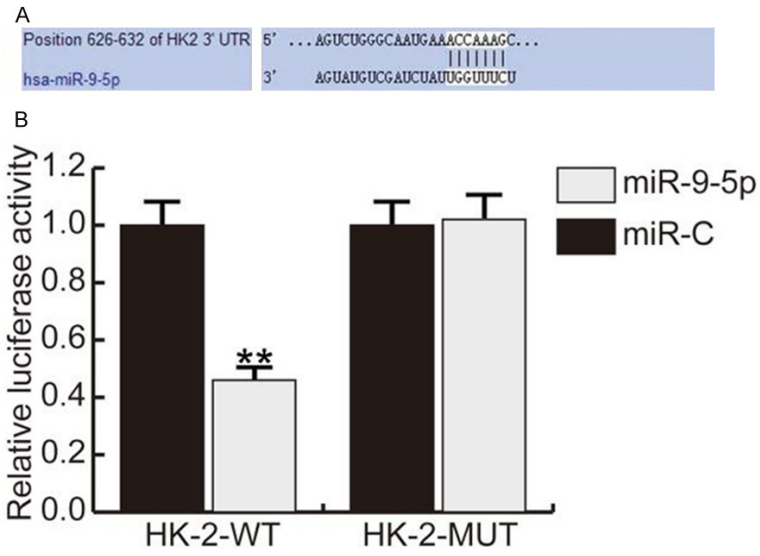

Figure 2. HK-2 is a direct target of miR-9-5p. A: Interaction between miR-9-5p and 3'UTR of HK-2 was predicted using microRNA target site prediction software; B: Luciferase activity of a reporter containing a wild-type HK-2 3'UTR or a mutant HK-2 3'UTR are presented. "HK-2-3'UTR-MUT" indicates the HK-2 3'UTR with a mutation in the miR-9-5p binding site. UTR, untranslated region. All data are presented as mean ± SD of three independent experiments. **p<0.01 *vs* control.

parated by 12% SDS-PAGE and then transferred onto the nitrocellulose membrane (Millipore, Bedford, MA, USA). After blocking with 5% skim milk, membranes were washed 3 times with TBST and then incubated with a primary antibody HK-2 (Cat no. #2867, CST, Beverly, MA, USA; dilution ratio: 1:1000), GLUT1 (Cat no. #12939, CST, Beverly, MA, USA; dilution ratio: 1:1000), PFK (Cat no. #12764, CST, Beverly, MA, USA; dilution ratio: 1:1000), LDH (Cat no. #3582, CST, Beverly, MA, USA; dilution ratio: 1:1000), complex I (Cat no. #17700, CST, Beverly, MA, USA; dilution ratio: 1:1000), complex II (Cat no. #28070, CST, Beverly, MA, USA; dilution ratio: 1:1000), complex III (Cat no. ab1098-62, Abcam, UK, dilution ratio: 1:1000), and β-actin (Cat no. #4970, CST, Beverly, MA, USA; dilution ratio: 1:1000) at 4°C overnight. Subsequently, membranes were washed again with TBST at least 3 times and then treated with a second antibody (Cat no. #7074, CST, USA; dilution ratio, 1:5000) at room temperature for about 2 hours. An ECL kit (Applygen, Beijing, China) was used to visualize protein bands, according to manufacturer protocol.

Dual luciferase reporter assay

In order to predict potential targets for miR-9-5p, TargetScan was used. To investigate whether miR-9-5p directly targets HK-2 3'-UTRs, vectors named HK-2-3'UTR-WT and HK-2-3'UTR-MUT with wild-type and mutated 3'UTR of HK-2 mRNA were established. A fragment from the 3'-untranslated region (3'-UTR) of HK-2, containing the putative binding site of miR-9-5p, was amplified by PCR from genomic DNA of 293T cells and inserted into SacI and Xba I or Xho I and Acc I restriction sites of pmirGLO dual-luciferase miRNA target expression vector (Promega). To generate 3'-UTR mutations, miR-9-5p target site ACCAAAG within the Hk-2 3'-UTR was changed to AGGTTAG using a site-directed mutagenesis kit (NEB). HK-2-3'UTR-WT or HK-2-3'UTR-MUT and miR-9-5p or its control (hsamiR-C) vector were co-transfected into 293T cells (Shanghai Institute of Cell Biology, Chinese Academy of Sciences) using Lipofectamine 2000 transfection reagent, according to the manufacturer's instructions. Dual-Luciferase Reporter Assay Kit (Promega,

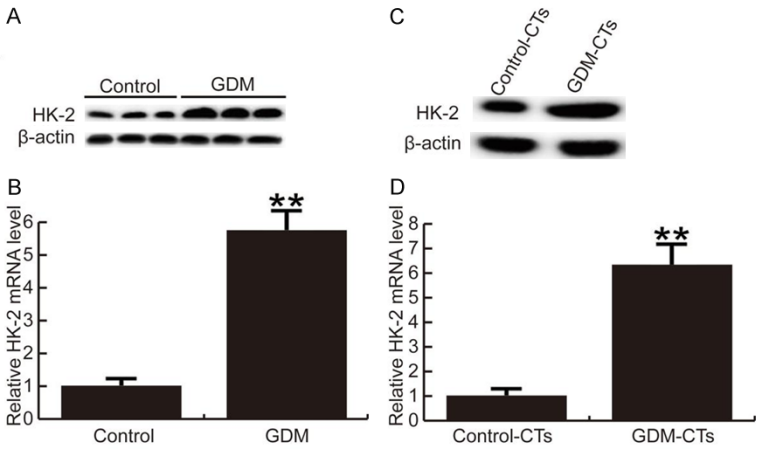

Figure 3. Expression of HK-2 is upregulated in GDM. A, B: Protein/mRNA expression of HK-2 in placental villous tissues from GDM women and healthy control women; C, D: Protein/mRNA expression of HK-2 in CTs cells from GDM women and healthy control women. All data are presented as mean ± SD of three independent experiments. **p<0.01 *vs* control.

Wisconsin, USA) was applied to assess luciferase activity, according to instructions supplied by the manufacturer.

Statistical analysis

Data are displayed as mean ± standard deviation (SD). Student's t-test or one-way ANOVA was performed to analyze differences between groups. A value of $p<0.05$ was recognized as statistically significant.

Results

miR-9-5p is downregulated in placental villous tissues and CTs cells from GDM women

To investigate expression levels of miR-9-5p in placental villous tissues and CTs cells from GDM women, qRT-PCR was performed. Our findings demonstrated that, compared with the control group, expression levels of miR-9-5p were significantly decreased in placental villous tissues and CTs cells of GDM patients (**Figure 1**). This data suggests that miR-9-5p may be involved in development of gestational diabetes mellitus.

HK-2 is a target of miR-9-5p

To investigate the potential role of miR-9-5p in GDM, we first predicted potential targets of miR-9-5p using TargetScan (http://www.targetscan.org/vert_71/) (**Figure 2A**). We then used Dual Luciferase Reporter Assay to confirm our prediction. We found that HK-2 is a potential target of miR-9-5p. Dual Luciferase Reporter Assay showed that miR-9-5p significantly reduced luciferase activity in 293T cells transfected with HK-2-3'UTR-WT, compared with the control group, and MUT HK-2-3'UTR-MUT eliminated repression by miR-9-5p (**Figure 2B**). These results indicate that miR-9-5p directly targets HK-2.

HK-2 is upregulated in placental villous tissues and CTs cells from GDM women

Next, we determined HK-2 expression in both placental villous tissues and CTs cells from GDM women. We found that HK-2 was upregulated in both placental villous tissues and CTs cells from GDM patients, compared to healthy women (**Figure 3**).

To further confirm miR-9-5p regulating HK-2 expression in CTs cells, we transfected CTs cells from GDM women with miR-9-5p mimic, negative control of miR-9-5p mimic, miR-9-5p inhibitor, or negative control of miR-9-5p inhibitor, respectively. We then applied qRT-PCR assay to determine transfection efficiency (**Figure 4A**). Results revealed that miR-9-5p mimic significantly prevented mRNA and protein levels of HK-2 in CTs cells, while miR-9-5p inhibitor enhanced HK-2 mRNA and protein expression (**Figure 4B** and **4C**). These data indicate that miR-9-5p may function in GMD via regulating HK-2 expression.

Overexpression of miR-9-5p reduces aerobic glycolysis in trophoblast cells

We next determined the effect of miR-9-5p mimic on aerobic glycolysis in CTs cells from GDM women. Our results showed that, compared with controls, miR-9-5p mimic significantly decreased expression of GLUT1, HK-2, PFK, and LDH, at both mRNA and protein levels, and the effects were eliminated by HK-2-plasmid (**Figure 5**).

Overexpression of miR-9-5p rescues mitochondrial complexes in trophoblast cells

Mitochondrial complexes in CTs cells were determined in our present study. We found that miR-9-5p mimic markedly enhanced ex-

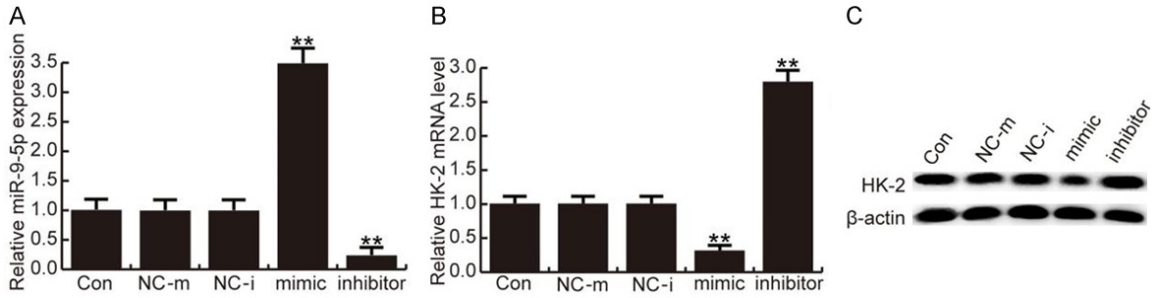

Figure 4. MiR-9-5p regulates HK-2 expression in CTs cells. CTs cells from GDM women were transfected with miR-9-5p mimic (mimic), miR-9-5p inhibitor (inhibitor), or its negative control (NC), respectively. Cells without any treatment were used as the control group (Con). 48 hours after cell transfection, expression of miR-9-5p and HK-2 in different groups were determined. A: Relative expression of miR-9-5p in CTs cells; B: Relative mRNA expression of HK-2 in CTs cells; C: Protein expression of HK-2 in CTs cells. Con: control group, cells without any treatment; NC-m: cells transfected with the negative control of miR-9-5p mimic; NC-i: cells transfected with the negative control of miR-9-5p inhibitor; mimic: cells transfected with miR-9-5p mimic; inhibitor: cells transfected with miR-9-5p inhibitor. All data are presented as mean ± SD of three independent experiments. **p<0.01 *vs* control.

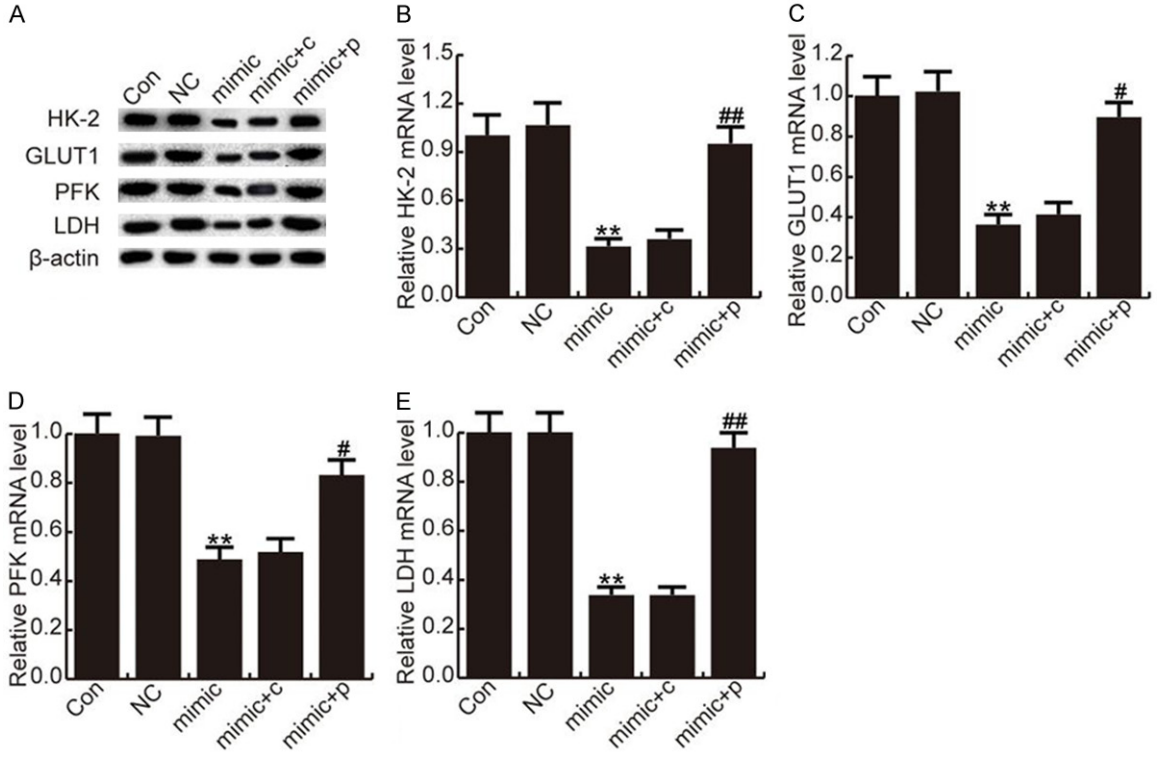

Figure 5. Overexpression of miR-9-5p reduces aerobic glycolysis in CTs cells. CTs cells from GDM women were transfected with miR-9-5p mimic (mimic), its negative control (NC), or miR-9-5p mimic + HK-2-plasmid (mimic + p), respectively. Cells without any treatment were used as control group (Con). 48 hours after cell transfection, expression of GLUT1, HK-2, PFK, and LDH in different groups were determined. A: Protein expression of GLUT1, HK-2, PFK, and LDH in CTs cells; B-E: Relative mRNA expression of HK-2, GLUT1, PFK, and LDH in CTs cells. Con: control group, cells without any treatment; NC: cells transfected with the negative control of miR-9-5p mimic; mimic: cells transfected with miR-9-5p mimic; mimic + c: cells co-transfected with miR-9-5p mimic and the control vector of HK-2-plasmid; mimic + p: cells co-transfected with miR-9-5p mimic and HK-2-plasmid. All data are presented as mean ± SD of three independent experiments. **p<0.01 vs control. #, ##p<0.05, 0.01 vs mimic.

pression of mitochondrial complex I, II, and III, at both mRNA and protein levels, and the effects were eliminated by HK-2-plasmid (**Figure 6**).

Discussion

GDM, one of the most common pregnancy complications, is considered a well known risk

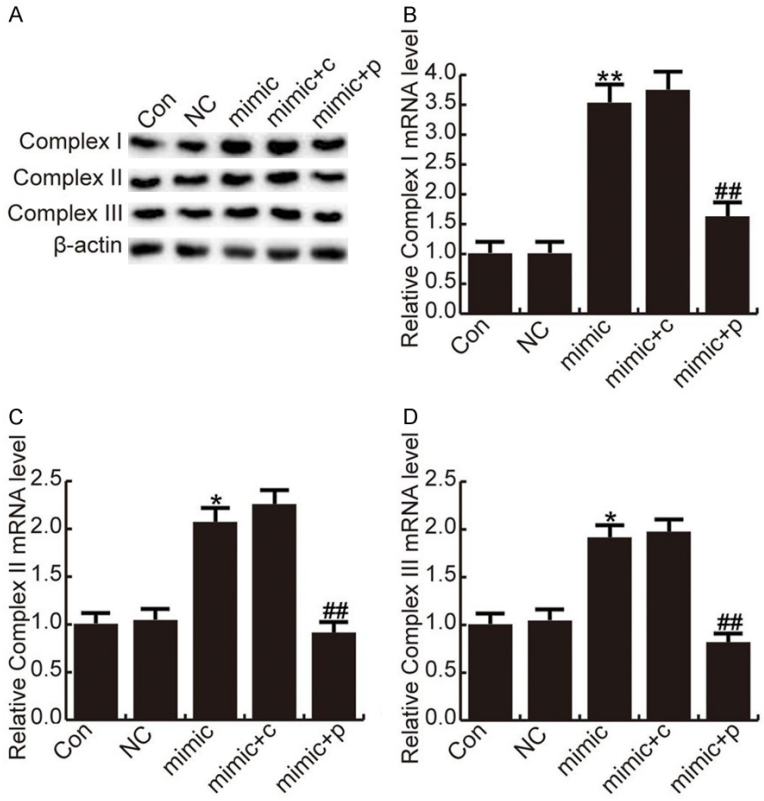

Figure 6. Overexpression of miR-9-5p enhances mitochondrial complexes in CTs cells. CTs cells from GDM women were transfected with miR-9-5p mimic (mimic), its negative control (NC), or miR-9-5p mimic + HK-2-plasmid (mimic + p), respectively. Cells without any treatment were used as control group (Con). Forty-eight hours after cell transfection, expression of mitochondrial complexes I, II, and III in different groups were determined. A: Protein expression of mitochondrial complexes I, II, and III in CTs cells; B-D: Relative mRNA expression of mitochondrial complexes I, II, and III in CTs cells. Con: control group, cells without any treatment; NC: cells transfected with the negative control of miR-9-5p mimic; mimic: cells transfected with miR-9-5p mimic; mimic + c: cells co-transfected with miR-9-5p mimic and the control vector of HK-2-plasmid; mimic + p: cells co-transfected with miR-9-5p mimic and HK-2-plasmid. All data are presented as mean ± SD of three independent experiments. *, **p<0.05, 0.01 vs control. ##p< 0.01 vs mimic.

factor for macrosomia [26]. Numerous studies have suggested that abnormal expression of miRNAs is potentially disease-specific and plays a critical role in the pathologic processes [9]. Differential microRNA expression in placental tissues of women with GDM has been identified and miR-9 has been found to be downregulated in GDM [14]. However, no previous reports have investigated the role of miR-9-5p in GDM. Thus, in our present study, we determined expression and the role of miR-9-5p in GDM.

The placenta is an organ that connects a developing fetus to the uterine wall to allow nutrient uptake, provide thermo-regulation to the fetus, eliminate waste and gas exchange via the mother's blood supply, fight against internal infection, and produce hormones to support pregnancy. It provides oxygen and nutrients to growing babies and removes waste products from the baby's blood [27]. The placenta is considered to have important roles in the pathogenesis of GDM [28]. We detected expression levels of miR-9-5p in placental villous tissues and CTs cells from GDM women. Our findings suggested that miR-9-5p significantly decreased in placental villous tissues and CTs cells from GDM women, indicating miR-9-5p may be involved in GDM.

To investigate the potential role of miR-9-5p in GDM progression, we predicted target genes of miR-9-5p using TargetScan and Dual Luciferase Reporter Assay. We found that hexokinase-2 (HK-2) was a direct target of miR-9-5p and HK-2 was significantly increased in placental villous tissues and CTs cells from GDM patients. Glycolytic pathways were found to be activated and mitochondrial function was compromised in GDM placentas [15]. In our present study, we determined the effect of miR-9-5p on expression of aerobic glycolysis related genes and mitochondrial complexes. Our results show that miR-9-5p significantly reduced expression of glucose transporter GLUT1 and glycolytic enzymes (HK-2, PFK and LDH). In addition, expression levels of mitochondrial complexes I, II, and III were notably increased by miR-9-5p mimic.

Taken together, for the first time, we found that miR-9-5p is downregulated in GDM and participates in progression of GDM through regulating glycolytic pathways and mitochondr-

ial complex expression by targeting HK-2. Our results may present a new idea for clinical treatment of GDM.

Disclosure of conflict of interest

None.

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