Original Article
Upregulation of YWHAZ in placental tissues, blood, and amniotic fluid from patients with gestational diabetes mellitus related to downregulation of microRNA-214

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Abstract: Aims: The present study aimed to examine the expression of YWHAZ mRNA and protein in placenta, blood, and amniotic fluid from gestational diabetes mellitus (GDM) patients and how it is related to expression of microRNA (miRNA or miR)-214. Methods: Thirty pregnant women with GDM were included into the experimental group and 26 normal pregnant women with matched age, gestational weeks, and pre-pregnancy indexes were included in the control group. Human primary embryo trophoblast cells were transfected with agomiR-214 to overexpress miR-214. Quantitative real-time polymerase chain reaction was used to measure expression of YWHAZ mRNA and miR-214. Western blotting was used to determine protein expression of YWHAZ in placental tissues and transfected cells. ELISA was performed to measure levels of YWHAZ protein in blood and amniotic fluid. Dual luciferase reporter assay was used to identify direct interaction between YWHAZ and miR-214. Results: Occurrence of GDM was related to expression of YWHAZ mRNA in patients. Elevated expression of YWHAZ protein played a regulatory role in the occurrence of GDM. Levels of YWHAZ protein in blood and amniotic fluid from GDM patients were enhanced, compared with the control group. miR-214 played a regulatory role in the pathology of GDM by affecting expression of YWHAZ at a transcriptional level. miR-214 directly bound with 3'-UTR of YWHAZ to regulate its expression. Conclusion: The present study demonstrates that upregulation of YWHAZ in placental tissues, blood, and amniotic fluid from patients with GDM is related to downregulation of miR-214. miR-214 regulates occurrence of GDM via YWHAZ.

Keywords: Gestational diabetes mellitus, miRNA-214, YWHAZ

Introduction
According to the latest international diagnostic criteria in 2012, incidence of gestational diabetes mellitus (GDM) reached 17.8% of all pregnant women [1]. In addition, incidence of the disease has been increasing year after year, worldwide, especially in developing countries [2]. Insulin resistance and islet β cell dysfunction are the main pathogenesis for GDM [3]. The target organ of insulin is vascular endothelial cells, which can cause insulin resistance when the vascular endothelium is abnormal [3]. However, the exact pathogenesis of GDM remains unclear [4]. Risks of fetal macrosomia and large for gestational age (LGA) will increase with the elevation of blood glucose levels in pregnant women [5, 6]. The process of GDM is a series of changes in human miRNA and mRNA. These genes have become the focus of research on the diagnosis and treatment of this disease [5, 6]. YWHAZ is a member of 14-3-3 protein family that plays important roles in the process of cardiovascular and cerebrovascular diseases [7]. It has been reported that YWHAZ is closely related to platelet adhesion and atherosclerosis [8] and levels of YWHAZ mRNA and protein are significantly upregulated in placental tissues of GDM patients [9]. However, the roles of YWHAZ in GDM and its upstream regulatory factors have been rarely reported. The present bioinformatics prediction shows that microRNA (miR)-214 is probably an upstream miRNA that regulates YWHAZ. miR-214 also plays an important role in diabetes mellitus [10]. However, the effects
of miR-214 on YWHAZ in GDM have not been reported. The present study examined expression of YWHAZ mRNA and proteins in placenta, blood, and amniotic fluid from GDM patients, aiming to understand its relation to expression of miR-214.

Materials and methods

Patients

A total of 30 pregnant women with GDM (age range, 22-36 years; median age, 27.6 years) receiving regular birth examinations and caesarean sections, between December 2016 and March 2018, were included in the present study. In addition, 26 normal pregnant women with matched age, gestational weeks, and pre-pregnancy indexes (age range, 21-38 years; median age, 26.9 years) were included in the control group. None of the included pregnant women had infections, liver and kidney dysfunction, gestational hypertension, coronary heart disease, or polycystic ovary syndrome. Pregnant women with a history of parturition of fetal macrosomia or dead fetus were excluded. Women with abnormal blood glucose levels before pregnancy were also excluded. Diagnosis of GDM was in accordance with diagnostic criteria proposed by the International Association of Diabetic Pregnancy Study Group (IADPSG).

Peripheral blood (10-15 mL) was collected from all subjects and centrifuged at 400 × g for 10 minutes before aliquoting serum into 100 μl. During caesarean section, 5 mL amniotic fluid was collected by perforating fetal membranes and stored at 4°C. In addition, placental tissues were collected from the center of placenta 2.5 cm from the umbilical cord. All procedures performed in the current study were approved by the Ethics Committee of Gansu Provincial Maternity and Childcare Hospital. Written informed consent was obtained from all patients or their families.

Cells

To transfect human primary embryo trophoblast cells (HUM-CELL-0051; PriCells, Wuhan, China) with agomiR-214, cells (3 × 10^5) in the logarithmic growth were seeded onto 24-well plates one day before transfection and cultured in antibiotics-free F12/DMEM medium (SH30023.01B; HyClone; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) supplemented with 10% fetal bovine serum (SH30084.03; HyClone; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) until reaching 70% confluence. In the first vial, 1.5 μL agomiR-214 (20 pmol/μL; miR-214 mimics group) (Hanbio Biotechnology Co., Ltd., Shanghai, China) was mixed with 50 μL Opti Mem medium (Thermo Fisher Scientific, Waltham, MA, USA). In the second vial, 1 μL Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) was mixed with 50 μL Opti Mem medium. After standing still for 5 minutes, the two vials were combined for additional warming at room temperature for 20 minutes. Next, the mixtures were added onto cells in respective groups. Six hours later, the medium was replaced with F12/DMEM medium containing 10% fetal bovine serum. After cultivation for 48 hours, the cells were collected for further analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Tissue samples (100 mg) were ground into powder in liquid nitrogen and lysed with 1 mL TRIzol Reagent, according to manufacturer instructions (Thermo Fisher Scientific, Waltham, MA, USA). Plasma (100 μl) or cells (3 × 10^6) were directly lysed with 1 mL TRIzol Reagent. Total RNA was extracted using the phenol chloroform method. The concentration and quality of RNA was measured using ultraviolet spectrophotometry (Nanodrop ND2000, Thermo Scientific, Waltham, MA, USA). cDNA was then obtained by reverse transcription from 1 μg RNA and stored at -20°C. Reverse transcription of mRNA was performed using TIANScript II cDNA First Strand Synthesis Kit (Tiangen, Beijing, China) and reverse transcription of miRNA was carried out using miRcute miRNA cDNA First Strand Synthesis Kit (Tiangen, Beijing, China).

SuperReal PreMix (SYBR Green) qRT-PCR kit (Tiangen, Beijing, USA) was used to detect mRNA expression of YWHAZ, using β-actin as an internal reference. Sequences of YW-HAZ were 5’-CGATCAGTCACAAGCAT-3’ (forward) and 5’-AGCATGGATGACAAATGGTC-3’ (reverse). Sequences of β-actin were 5’-ATCTGTTTGACCTTCAACA-3’ (forward) and 5’-CATCTCTTGCAGTGCGTCTC-3’ (reverse). The reaction system (25 μl) was composed of 12.5 μl SYBR Premix
EXTaq, 0.5 μl upstream primer, 0.5 μl downstream primer, 1 μl cDNA, and 10.5 μl ddH₂O. PCR conditions were: initial denaturation at 95°C for 30 seconds, denaturation at 95°C for 5 seconds, and annealing at 57°C for 30 seconds (45 cycles) (iQ5; Bio-Rad, Hercules, CA, USA). Analysis of dissolution curve of PCR products was performed after the cycles (95°C for 15 seconds, 60°C for 23 seconds, and 95°C for 15 seconds). The 2^ΔΔCq method [11] was used to calculate relative expression of YWHAZ mRNA against β-actin. Each sample was tested in triplicate.

Expression of miR-214 was determined by miRcute miRNA RT-PCR Kit (Tiangen, Beijing, China), using U6 as an internal reference. Sequences of miR-214 primers were 5'-AGCATACAGCAAGCAACAGC-3' (forward), and 5'-AAAGTGTCTCTCCTCCTCAG-3' (reverse). Sequences of U6 primers were 5'-ATTGGAAACGATACAGAGAAGATT-3' (forward) and 5'-GGAACGCTTCACGAATTTG-3' (reverse). The reaction system (20 μl) contained 10 μl qRT-PCR-Mix, 0.5 μl upstream primer, 0.5 μl downstream primer, 2 μl cDNA, and 7 μl ddH₂O. Reaction protocol was: initial denaturation at 95°C for 3 minutes, denaturation at 95°C for 12 seconds, annealing at 62°C for 40 seconds, and elongation at 72°C for 40 seconds (40 cycles) (iQ5; Bio-Rad, Hercules, CA, USA). The 2^ΔΔCq method [11] was used to calculate relative expression of miR-214 against U6. Each sample was tested in triplicate.

Western blotting

Before lysis, tissues (100 mg) were ground into powder. Cells (1 × 10⁶) were trypsinized and collected. Tissue samples or cells were lysed with precooled Radio-Immunoprecipitation Assay (RIPA) lysis buffer (600 μl; 50 mM Tris-base, 1 mM EDTA, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% TritonX-100, 1% sodium deoxycholate; Beyotime Institute of Biotechnology, Shanghai, China) for 30 minutes on ice. The mixture was centrifuged at 12,000 rpm and 4°C for 10 minutes. The supernatant was used to determine protein concentrations using the bicinchoninic acid (BCA) protein concentration determination kit (RTP7102, Real-Times Biotechnology Co., Ltd., Beijing, China). The 2^ΔΔCq method [11] was used to calculate relative expression of miR-214 against U6. Each sample was tested in triplicate.

Enzyme-linked immunosorbent assay (ELISA)

YWHAZ ELISA kit (ml028324; TW Reagent, Shanghai, China; www.tw-reagent.com) was used to determine concentrations of YWHAZ. In microplates, standards (50 μl) and samples (10 μl serum and 40 μl diluent) were added into predefined wells, while blank wells were left empty. In the wells for standards and samples, horseradish peroxidase-labelled conjugates (100 μl) were added before sealing the plates for incubation at 37°C for 1 hour. After washing the plates 5 times, substrates A (50 μl) and B (50 μl) were added into each well. After incubation at 37°C for 15 minutes, stop solution (50 μl) was added into each well and absorbance of each well was measured at 450 nm within 15 minutes.

Bioinformatics

Bioinformatics prediction is a powerful tool for the study of the functions of miRNAs. To understand the regulatory mechanisms of YWHAZ, this study used miRanda (http://www.microrna.org/microrna/home.do), TargetScan (http://www.targetscan.org), PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html),
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Figure 1. Direct interaction between miR-214 and YWHAZ. Bioinformatics prediction is a powerful tool for the study of the functions of miRNAs. This study used miRanda (http://www.microrna.org/microrna/home.do), TargetScan (http://www.targetscan.org), PiTa (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html), RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/), and PICTA (http://pictar.mdc-berlin.de/) to predict target genes that might be regulated by miR-214, finding that YWHAZ was a potential target gene of miR-214.

Figure 2. Relative expression of YWHAZ mRNA in (A) placental tissues, (B) blood, and (C) amniotic fluid from healthy subjects and GDM patients. qRT-PCR was used to determine the expression of mRNA. *P < 0.05 and **P < 0.01 compared with control.

Figure 3. Relative expression of YWHAZ protein in placental tissues from healthy subjects and GDM patients. Western blotting was used to determine the expression of proteins. *P < 0.05 compared with control.

According to bioinformatics results, wild-type (WT) and mutant seed regions of miR-214 in the 3'-UTR of YWHAZ gene were chemically synthesized in vitro. Their two ends were attached with Spe-I and HindIII restriction sites and then cloned into pMIR-REPORT luciferase reporter plasmids. Plasmids (0.8 μg) with WT or mutant 3'-UTR sequences were co-transfected with agomiR-214 (100 nM; Sangon Biotech, Shanghai, China) into 293T cells. For control, 293T cells were transfected with agomiR-negative control (NC). After cultivation for 24 hours, the cells were lysed using dual luciferase reporter assay kit (Promega, Fitchburg, WI, USA), according to manufacturer instructions, and luminescence intensity was measured using GloMax 20/20 luminometer (Promega, Fitchburg, WI, USA). Using renilla luminescence activity as an internal reference, the luminescence values of each group of cells were measured.

Statistical analysis

Results were analyzed using SPSS 20.0 statistical software (IBM, Armonk, NY, USA). Data are expressed as mean ± standard deviations. Data were tested for normality. Multigroup measurement data were analyzed using one-way
ANOVA. In case of homogeneity of variance, Least Significant Difference and Student-Newman-Keuls methods were used. In case of heterogeneity of variance, Tamhane’s T2 or Dunnett’s T3 method was used. Comparison between the two groups was carried out using Student’s t-test. P < 0.05 indicates statistically significant differences.

Results

Occurrence of GDM is related to expression of YWHAZ mRNA

To determine the expression of YWHAZ mRNA, qRT-PCR was performed. Data showed that expression of YWHAZ mRNA in placental tissues, blood, and amniotic fluid from GDM patients was significantly higher than that from the control group (P < 0.05) (Figure 2A-C). Results suggest that occurrence of GDM was related to expression of YWHAZ mRNA.

Elevated expression of YWHAZ protein may play a regulatory role in the occurrence of GDM

To measure YWHAZ protein expression in placental tissues, Western blotting was carried out. Data showed that YWHAZ protein expression in placental tissues from GDM patients was significantly higher than that from the control group (P < 0.05) (Figure 3). Results indicate that elevated expression of YWHAZ protein may play a regulatory role in occurrence of GDM.

Levels of YWHAZ protein in the blood and amniotic fluid from GDM patients were elevated

To examine the levels of YWHAZ protein in blood and amniotic fluid, ELISA was employed. Data showed that levels of YWHAZ protein in the blood from GDM patients was significantly higher than the control group (P < 0.05) (Figure 4A). However, levels of YWHAZ protein in amniotic fluid from GDM patients were not significantly different from the control group (P > 0.05) (Figure 4B). Results suggest that levels of YWHAZ protein in blood and amniotic fluid from GDM patients were enhanced, compared with the control group.

miR-214 may play a regulatory role in the pathology of GDM by affecting expression of YWHAZ at a transcriptional level

To determine levels of miR-214 in placental tissues, blood, and amniotic fluid, qRT-PCR was performed. Data showed that
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used. Data showed that expression of miR-214 in placental tissues, blood, and amniotic fluid from GDM patients was significantly lower than that from the control group (P < 0.05 for all) (Figure 5A-C). Results indicate that miR-214 may play a regulatory role in the pathology of GDM by affecting expression of YWHAZ at a transcriptional level.

YWHAZ is a direct target gene of miR-214

To test whether YWHAZ is a direct target of miR-214, expression of YWHAZ mRNA and proteins was examined after overexpressing miR-214. Dual luciferase reporter assay was also performed. Data showed that YWHAZ mRNA and protein expression in cells with overexpression of miR-214 was significantly reduced, compared to the control group (P < 0.05) (Figure 6A, 6B). Dual luciferase reporter assay showed that luminescence levels of the WT group were significantly lower than that in the NC group (P < 0.05), but that of the mutant group was not different from the NC group (P > 0.05) (Figure 6C). Results suggest that miR-214 directly binds with the 3’-UTR of YWHAZ to regulate expression.

Discussion

GDM refers to abnormal glucose tolerance occurring or first discovered during pregnancy [12]. GDM increases maternal and fetal complications and perinatal mortality and some of the affected patients eventually develop type 2 diabetes [13]. Understanding the expression of differential genes in the disease process and finding new therapeutic gene targets are of great significance to patients and families troubled by this disease. At the same time, improving the early diagnosis rate of GDM and individualized treatments are keys to improving curative effects and prognosis.
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compared with that in normal lung tissues [19]. These studies suggest that the important biological functions of YWHAZ in human diseases are likely achieved through regulation of blood vessels. Present results showed that YWHAZ is upregulated in placenta, blood, and amniotic fluid of GDM patients, consistent with previous reports [9]. Levels of YWHAZ in blood and amniotic fluid can provide clear indication of early diagnosis of GDM.

For the investigation of YWHAZ regulation factors, the present study focused on the upstream miRNA molecules of YWHAZ. MiRNA is a class of intracellular endogenous, small, and non-encoding ribonucleic acids. They cut mRNA of target genes and inhibit their expression [20]. Of note, miRNA has become a biomarker for various diseases [21]. Using bioinformatics, this study found that miR-214 might be one of the upstream genes that regulate YWHAZ. Zhang et al. reported that miR-214 can be used as a prognostic factor for gastric cancer and it can also affect the proliferation and invasion of tumor cells [22]. Wang et al. discovered that miR-214 can affect the proliferation and invasion of breast cancer through P53 [23]. In addition, miR-214 can play a certain inhibitory role in bladder cancer through its target gene PDRG1 [24]. miR-214 protected cardiac myocytes in the treatment of myocardial ischemia [25]. Izawa et al. showed that miR-214 effectively alleviates thioacetamide-induced hepatic cirrhosis and has an anti-fibrosis effect [26]. In the present study, it was found that miR-214 was downregulated in placental tissues, blood, and amniotic fluid. Moreover, upregulation of miR-214 in human primary trophoblastic cells negatively regulates expression of YWHAZ mRNA and protein. Dual luciferase reporter assay revealed that miR-214 binds with the 3′-UTR of YWHAZ mRNA and regulates its protein expression.

In conclusion, the present study demonstrates that miR-214 regulates protein expression of YWHAZ by targeting its mRNA and plays important roles in occurrence and development of GDM. YWHAZ and miR-214 can become potential biomarkers of GDM for prevention and treatment of the disease.

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Written informed consents for publication of any associated data and accompanying images were obtained from all patients or their parents, guardians or next of kin.

Disclosure of conflict of interest

None.

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