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Correlation of MiR-152 expression with VEGF expression in placental tissue of preeclampsia rat and its influence on apoptosis of trophoblast cells

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Abstract. – OBJECTIVE: To detect the expression of micro ribonucleic acid (miR)-152 in the placental tissues of preeclampsia (PE) rats and its correlation with the expression of vascular endothelial growth factor (VEGF), and to investigate the influence of miR-152 on the apoptosis of trophoblast cells.

MATERIALS AND METHODS: A Sprague Dawley (SD) rat model of PE was established, the total RNA and total protein in the placental tissues were extracted, and reverse transcription-polymerase chain reaction (RT-PCR) was applied to determine the expression level of miR-152 in the placental tissues. The rats were divided into miR-152 high expression group (High group) and miR-152 low expression group (Low group) according to the expression level of miR-152. RT-PCR, immunohistochemistry and Western blotting assay were performed to detect the expressions of VEGF messenger RNA (mRNA) and protein, respectively, in the placental tissues of the two groups of rats. Meanwhile, the BeWo trophoblast cell lines were used in *in-vitro* experiment, which were divided into Control group and miR-152 mimic group. 10 μL miR-152 mimic were added into each well plate in miR-152 mimic group, and 24 h later, TUNEL staining and flow cytometry were utilized to assess the cell apoptosis in both groups. At the same time, the expressions of B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax) in the two groups of cells were measured using Western blotting assay.

RESULTS: The expression level of miR-152 in the placentas of PE rats was increased markedly compared with that of normal rats. The expression levels of VEGF mRNA and protein in the rat placenta in High group were notably higher than those in Low group. *In vitro* experiment results indicated that miR-152 mimic could promote the apoptosis of BeWo trophoblast cells, up-regulate the pro-apoptotic gene Bax and inhibit the anti-apoptotic gene Bcl-2 simultaneously.

CONCLUSIONS: The expression of miR-152 is increased in the placental tissues of PE rats, and it is positively correlated with VEGF. In addition, the increased miR-152 expression can promote the apoptosis of trophoblast cells.

Key Words
Preeclampsia, miR-152, Trophoblast cells, Apoptosis.

Introduction

Preeclampsia (PE), a disease specific to pregnant women, is the leading cause of rising incidence and mortality rates among pregnant women and perinatal infants[1]. PE generally occurs at or after 20 weeks of pregnancy, which can lead to the impairments of multiple organs such as heart, kidney, brain and liver[2]. However, the specific molecular mechanism of the occurrence and development of PE has not been completely clarified yet, nor can an individual pathophysiological factor thoroughly elucidate the pathogenesis of PE[3]. There are many hypotheses about the pathogenesis of PE at present, including abnormal placental function, impaired infiltration of trophoblast cells, abnormal spiral arterial remodeling, endothelial dysfunction and abnormal increase in the apoptosis of trophoblast cells[4,5]. Micro ribonucleic acids (miRNAs), as a new category of small non-coding single-stranded RNA molecules that are highly conserved, can repress gene expressions by degrading specific messenger RNA (mRNA) of gene or inhibiting translation process[6]. Previous investigations have manifested that miRNAs play vital roles in the occurrence and development of a variety of diseases, including cardio-

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vascular disease, tumor, endocrine disorder and pregnancy-associated disease[7]. Recent works[8,9] have revealed that some miRNAs are capable of affecting the occurrence and development of PE through regulating the apoptosis, proliferation, angiogenesis, invasion and mitochondrial respiratory chain of trophoblast cells. For example, miR-128a can accelerate the apoptosis of HTR-8/SVneo trophoblast cells by virtue of the mitochondrial apoptotic pathway, ultimately causing the occurrence of PE[10]. In this research, the rat model of PE was established, the expressions of miR-152 in the placental tissues of normal and PE rats were detected, and the correlation between the miR-152 expression level and vascular endothelial growth factor (VEGF) in the placenta was further analyzed. Meanwhile, an *in-vitro* experiment was conducted to explore the influence and mechanism of overexpressed miR-152 on the apoptosis of BeWo trophoblast cells, so as to provide certain references for the clinical treatment and prevention of PE in the future.

Materials and Methods

Establishment of PE Model

A total of 30 rats with a gestation of 2 weeks were divided into 2 groups using a random number table, including normal pregnancy group (Control group, n=15) and PE group (n=15). In PE group, 50 mg/kg L-NAME were injected into the rats at multiple subcutaneous points at 14-19 d of pregnancy, so as to induce PE. In Control group, an equal dose of normal saline was injected into the rats. This study was approved by the Animal Ethics Committee of The First People's Hospital of Wujiang District Suzhou Animal Center.

Collection of Placental Specimens

The rats with a gestation of 20 d were intraperitoneally injected with chloral hydrate (10%, 3 mL/kg) for anesthesia. Then, the placentas and fetal rats were taken out from the uterus, and the placentas in each group were washed with the normal saline to remove bloodiness, cut into pieces, put into Eppendorf (EP) tubes, and stored in a refrigerator at -80°C.

Detection Via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

(1) The total RNA in the placental tissues was extracted using TRIzol reagent method (Invitrogen, Carlsbad, CA, USA), whose concentration and purity were measured by virtue of an ultraviolet spectrophotometer, and the RNA with A_{260}/A_{280} =1.8-2.0 was eligible for use. (2) The mRNA was synthesized into complementary deoxyribonucleic acid (cDNA) through reverse transcription, and the cDNA was stored in the refrigerator at -80°C. (3) RT-PCR system: 2.5 μL 10× Buffer, 2 μL cDNA, 0.25 μL forward primer (20 μmol/L), 0.25 μL reverse primer (20 μmol/L), 0.5 μL dNTPs (10 mmol/L), 0.5 μL Taq polymerase (2×10^6 U/L) and 19 μL ddH_2O. The RT-PCR amplification system was the same. The primers are shown in Table I.

Immunohistochemistry

The sliced placental tissue sections were baked in an oven at 60°C for 30 min, followed by deparaffinization in xylene (5 min × 3 times) and dehydration with 100%, 95% and 70% ethanol for 3 times, respectively. The activity of endogenous peroxidase was inhibited by 3% hydrogen peroxide-methanol, and then the tissues were sealed in goat serum for 1 h. The anti-VEGF antibody was diluted at 1:200 with phosphate-buffered saline (PBS) and incubated at 4°C overnight, followed by washing with PBS on a shaking table for 4 times. After the addition of secondary antibodies, the diaminobenzidine was adopted for color development. After that, 6 samples were randomly selected from each group, and 5 fields of vision were randomly selected from each sample for photography under a light microscope (400×).

Table I. Primer sequences of indexes for RT-PCR.

Target gene	Primer sequence
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Forward 5'-GACATGCCGCCTGGAGAAAC-3' Reverse 5'-AGCCCAGGATGCCCTTTAGT-3'
VEGF	Forward 5'-TGCACTACGTAGCGTTCCTT-3' Reverse 5'-AAGGCTGATCGATGCGGGAAGT-3'
MiR-152	Forward 5'-GTCCACGAACCCGTAAGGT-3' Reverse 5'-CATCTTACACGATCGCGTCCA-3'

Cell Culture

BeWo trophoblast cell lines were purchased from the Institute of Microbiology, Chinese Academy of Sciences. PBS, trypsin, fetal bovine serum and Ham's F-12 medium containing dextran were bought from Gibco Company (Rockville, MD, USA). MiR-152 mimic was purchased from Guge Bio-Technology Co., Ltd., (Wuhan, China). The BeWo trophoblast cells were cultured in a cell incubator with 5% CO_2 at 37°C, and they were subjected to digestion and subculture with 0.25% trypsin-ethylene diamine tetraacetic acid (EDTA) after the culture dish was covered with the cells.

Western Blotting Assay

(1) The liquid in the medium was discarded first, and the medium was washed with PBS for 3 times. (2) 1,000 μL lysis buffer was added into each dish and shaken sufficiently for 20 min. (3) Cells on the bottom of the dish were scrapped completely using a brush and placed into the prepared Eppendorf (EP) tubes. (4) The harvested cells were lysed with an ultrasonication instrument for about 15 s. (5) After standing for 15 min, the cells were centrifuged at 12,000 r/min for 0.5 h. (6) The supernatant was taken and subpackaged into the EP tubes, the protein concentration was measured by virtue of bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA) and ultraviolet spectrophotometric assay, and the volume of all the sample proteins was maintained at equal concentration. (7) The proteins were subpackaged and preserved in the refrigerator at -80°C. Note: As for the extraction of total protein in the placental tissues, the placental tissues in each group needed to be sufficiently ground in the lysis buffer, and the subsequent steps were the same as the steps 4-7 for the extraction of total protein in the cells. After the total protein in the BeWo trophoblast cells or placental tissues was extracted, it could be subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After that, the protein in the gel was transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), followed by incubation with primary antibody at 4°C overnight, incubation with goat-anti-rabbit secondary antibody in the dark for 1 h, and scanning and quantification of protein bands using Odyssey membrane scanner. The level of the protein to be detected was corrected *via* GAPDH.

Detection of Cell Apoptosis Via Flow Cytometry

The BeWo trophoblast cells in the logarithmic phase were fetched, digested and prepared into suspension with 0.25% trypsin-EDTA, and seeded into the medium with a 6-well plate. Loading was performed according to the operation steps in the cell apoptosis detection kit Annexin V-FITC PI (Beyotime, Shanghai, China), and the apoptosis rate was calculated.

Terminal Dexynucleotidyl Transferase (TdT)-Mediated dUTP Nick End Labeling (TUNEL) Staining of Cells

The cells on the coverslips were fixed in fixative for 1 h and then washed with PBS for 3 times. After permeabilization, the TUNEL reagent (Beyotime, Shanghai, China) was prepared, and two negative controls were set. Then the cells were observed, photographed and counted under a fluorescence microscope after the staining.

Statistical Analysis

All the data were analyzed using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA), the measurement data were presented as mean ± standard deviation, and *t*-test was performed for comparison of data between two groups. $p<0.05$ suggested that the difference was statistically significant.

Results

Expression of miR-152 in Placental Tissues of Normal Pregnant Rats and PE Rats

The results of RT-PCR (Figure 1) indicated that the expression level of miR-152 in the placental tissues of rats in PE group was remarkably higher than and about 6.85 times that in Control group ($p<0.05$), suggesting that miR-152 may have certain impacts on the incidence of PE.

Levels of VEGE mRNA in miR-152 High and Low Expression Groups

Considering that angiogenesis plays an important role in PE, the 15 rats in PE group were divided into miR-152 high expression group (High group) and miR-152 low expression group (Low group) according to the average value of miR-152 expression level at (14.23±2.17). Later, the expression levels of VEGF mRNA in both groups were detected *via* RT-PCR. It was shown that the

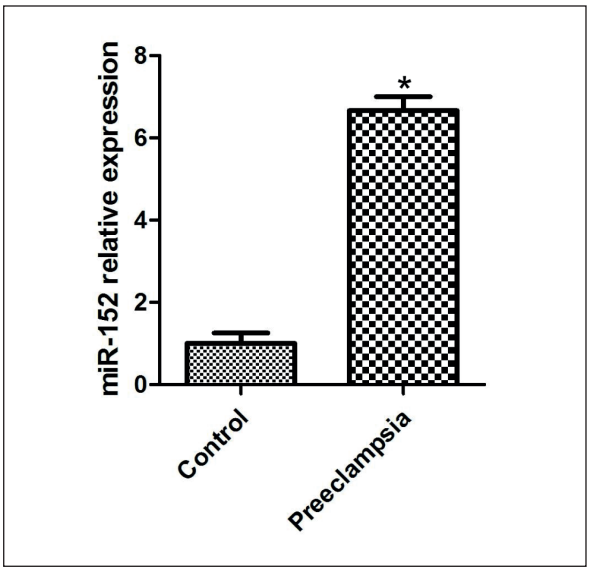

Figure 1. Expression of miR-152 in placental tissues of normal pregnant rats and PE rats. Control group: healthy controls, Preeclampsia: PE group, *p<0.05 vs. Control group, with a statistical difference.

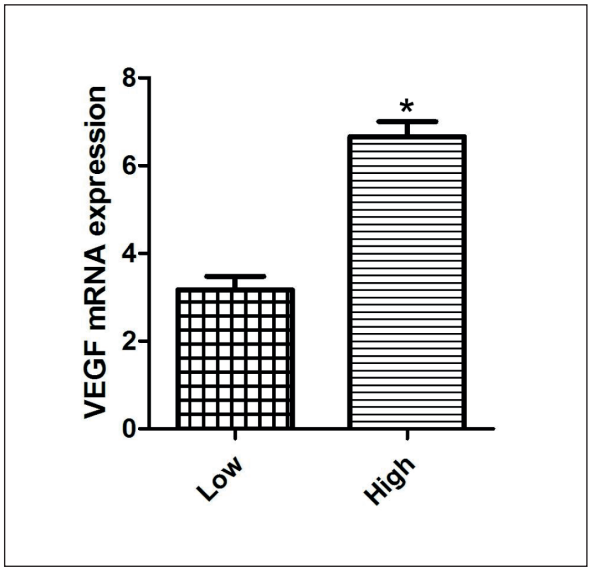

Figure 2. Expression of VEGF mRNA in the placental tissues of rats in miR-152 high and low expression groups. Low group: miR-152 low expression group, High group: miR-152 high expression group, *p<0.05 vs. Low group, with a statistical difference.

expression level of VEGE mRNA in High group was approximately 2.61 times as high as that in Low group (p<0.05) (Figure 2).

Levels of VEGE Protein in miR-152 High and Low Expression Groups

In order to explore whether the VEGF was changed at the protein level in Low group and High group, the expression of VEGE protein in both groups was detected by means of Western blotting assay, and then semi-quantified. As shown in Figure 3, the expression level of VEGF protein in High group was evidently higher than that in Low group (about 3.22 times) (p<0.05).

Results of Immunohistochemical Staining for VEGE in miR-152 High and Low Expression Groups

For the purpose of observing the expression of VEGF in miR-152 high and low expression groups and tissue distribution more intuitively, the immunohistochemical staining was performed for different groups of placental tissues. The results manifested that the VEGF expression in High group (brown) was increased significantly compared with that in Low group (Figure 4).

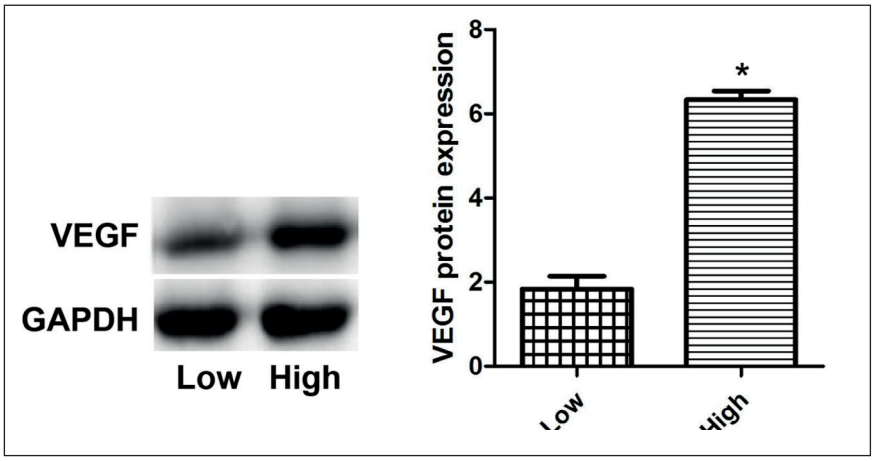

Figure 3. Expression of VEGF protein in the placental tissues of rats in miR-152 high and low expression groups. Low group: miR-152 low expression group, High group: miR-152 high expression group, *p<0.05 vs. Low group, with a statistical difference.

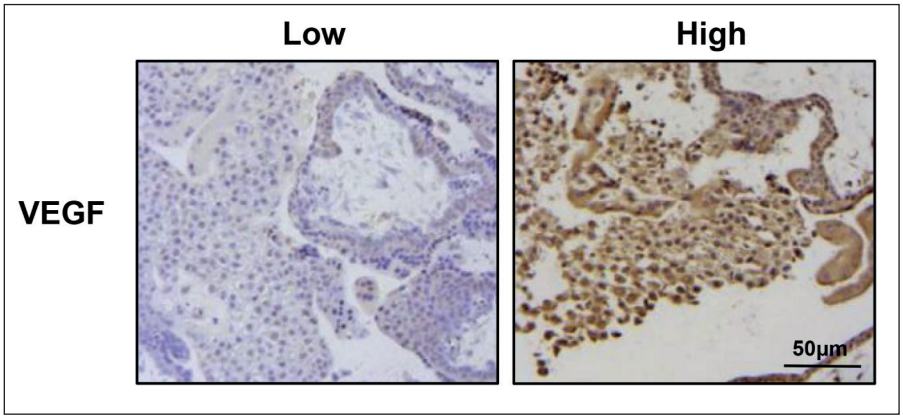

Figure 4. Immunohistochemical results for VEGF in the placental tissues of rats in miR-152 high and low expression groups. (Magnification: 200×). Low group: miR-152 low expression group, High group: miR-152 high expression group. Positive results are in brown.

Influence of miR-152 Over-expression on BeWo Trophoblast Cells Detected Via TUNEL

The apoptosis of trophoblast cells in the placenta is a crucial reason for the incidence of PE. Therefore, the BeWo trophoblast cells were cultured *in vitro*, and a certain amount of miR-152 mimic was added to simulate the occurrence of miR-152 overexpression, thus further investigating whether miR-152 overexpression exerted effects on the apoptosis of trophoblast cells. According to the results of TUNEL staining (Figure 5), the number of TUNEL positive cells after miR-152 overexpression was notably larger than that in Control group [(8.34±1.11) *vs.* (2.39±1.62)] ($p<0.05$).

Apoptosis of BeWo Trophoblast Cells Quantified Via Flow Cytometry

Since the TUNEL is a semi-quantitative method for the quantification of cell apoptosis, the apoptosis of the two groups of BeWo trophoblast cells was further detected *via* flow cytometry, and the results (Figure 6) were consistent with those in 3.4. The cell apoptosis in miR-152 mimic group was evidently higher than that in Control group (about 10.04 times) ($p<0.05$).

Influence of miR-152 Overexpression on Expressions of Apoptosis-Related Genes of BeWo Trophoblast Cells

The cell apoptosis can be regulated by multiple pathways, of which the most classical is the

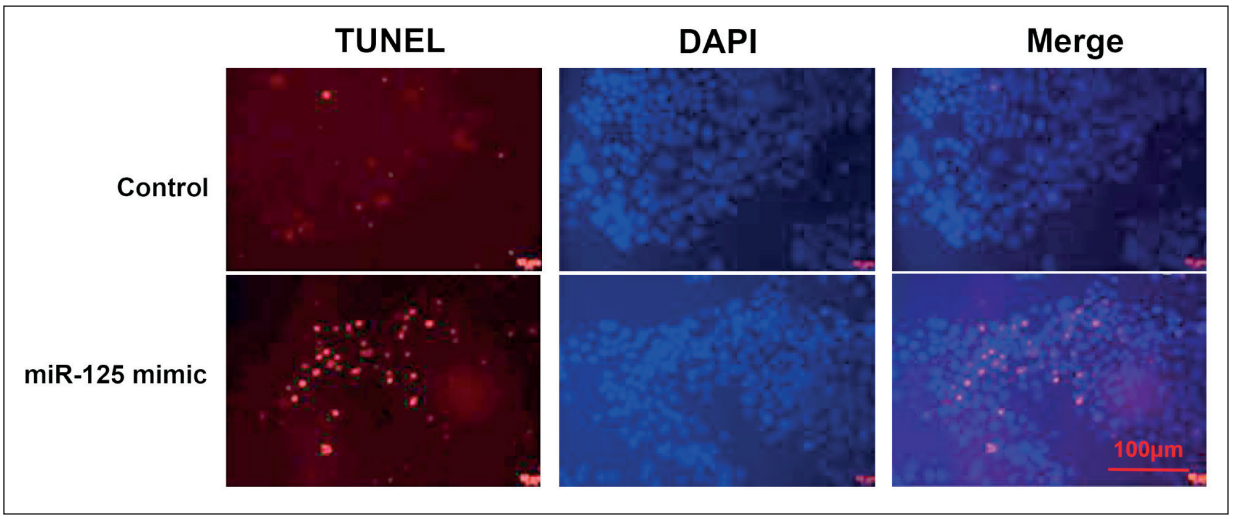

Figure 5. Results of TUNEL staining for the influence of miR-152 mimic on apoptosis of trophoblast cells. Control group: blank control group, miR-152 mimic group: miR-152 overexpression group, red: TUNEL positive, blue: nucleus.

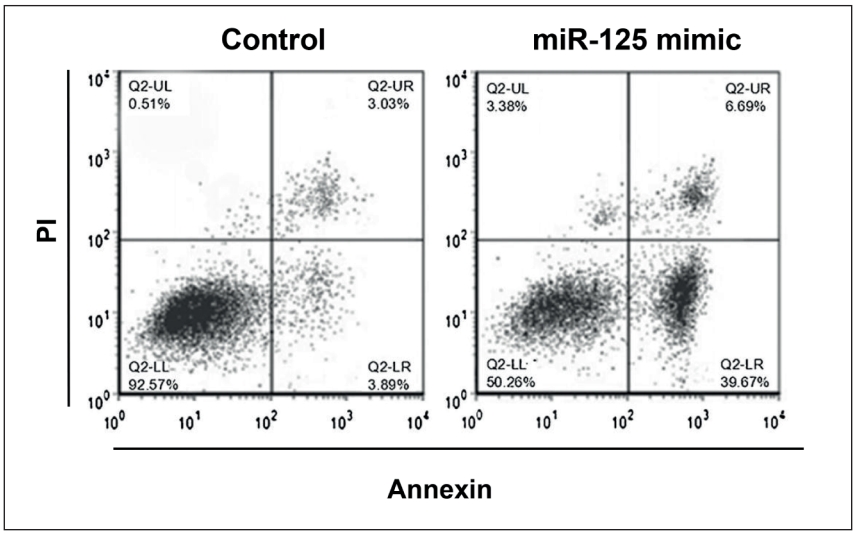

Figure 6. Results of flow cytometry for the influence of miR-152 mimic on apoptosis of trophoblast cells. Control group: blank control group, miR-152 mimic group: miR-152 overexpression group.

mitochondria-dependent apoptotic pathway. So does miR-152 overexpression also promote the apoptosis of BeWo trophoblast cells by depending on the mitochondria? Hence, the expressions of the classical proteins B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax) in the mitochondria-dependent apoptotic pathway were measured. The results indicated that miR-152 overexpression could markedly up-regulate the expression of pro-apoptotic gene Bax and down-regulate the expression of anti-apoptotic gene Bcl-2 (Figure 7), manifesting that miR-152 accelerates the apoptosis of trophoblast cells by depending on the Bcl-2 signaling pathway.

Discussion

PE is a complex pregnancy-specific disease induced by various factors[11]. Enquobahrie et al[12] have demonstrated that the occurrence of PE is closely related to the abnormal development of placenta, in which the abnormal expressions of multiple miRNAs exist. According to early investigations, many miRNAs play vital roles in the occurrence and development of PE[13]. For instance, miR-29b can regulate the invasion, apoptosis and angiogenesis of trophoblast cells by targeting and inhibiting myeloid cell leukemia sequence 1, vascular endothelial growth factor

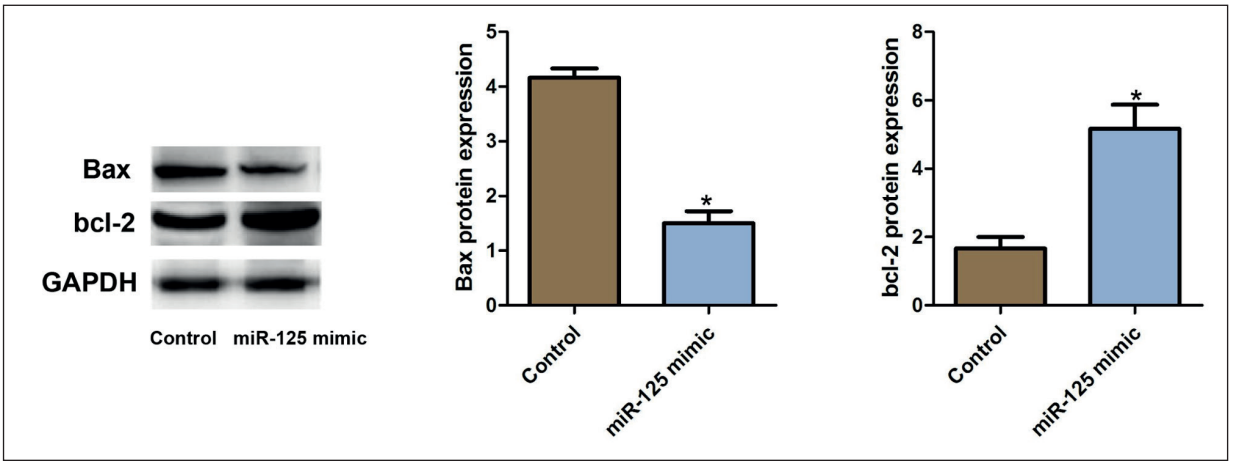

Figure 7. Influence of miR-152 mimic on Bax and Bcl-2 expressions in trophoblast cells. Control group: blank control group, miR-152 mimic group: miR-152 overexpression group, *p<0.05 vs. Control group, with a statistical difference.

A and integrin β1[14]. MiR-155 is able to repress the invasion of trophoblast cells through regulating the expression of endothelial nitric oxide synthase, ultimately leading to the occurrence of PE[15]. MiR-101 is capable of suppressing the apoptosis of trophoblast cells caused by endoplasmic reticulum stress by means of targeting and inhibiting endoplasmic reticulum protein 44[16]. In this research, it was discovered that the expression level of miR-152 in the placental tissues of PE rats was elevated significantly compared with that in healthy rats. Gunel et al[17] findings are consistent with those in this research, which revealed that the expression level of miR-152 is increased notably in the serum of PE patients. Yang et al[18] also indicated that miR-148a/152 can mediate the inhibition of DNMT1 expression, thereby up-regulating FABP4 expression and finally aggravating PE. The cell apoptosis is regulated by multiple signaling pathways, so in order to explore the molecular mechanism of miR-152 in promoting the apoptosis of trophoblast cells, the mitochondria-dependent apoptotic signaling pathways were detected. As an important energy supply station in cells, mitochondria are capable of providing necessary adenosine triphosphate (ATP) for the vital activities of cells. Mitochondria can participate in the cell apoptosis through several signaling pathways, such as release of cytochrome enzyme from mitochondrion to cytoplasm, disorder of mitochondrial membrane potential, ATP depletion and increased production of intra-cellular reactive oxygen species[19, 20]. Those mitochondria-dependent apoptotic-signaling pathways can be directly or indirectly regulated by Bax and Bcl-2 by virtue of many pathways. For example, Bax can form Bax dimers through the nuclear translocation from cytoplasm to mitochondrion, which can stimulate the mitochondrion to release large quantities of cytochrome enzymes and thus trigger the cell apoptosis[21]. On the other hand, Bcl-2 can repress the abnormal decrease of mitochondrial membrane potential, release of cytochrome enzymes and activation of Caspase at the same time, ultimately inhibiting the cell apoptosis[22]. In this research, both flow cytometry and TUNEL staining verified that miR-152 could accelerate the apoptosis of trophoblast cells. The detection of protein levels of Bax and Bcl-2 revealed that miR-152 overexpression prominently promoted the Bax expression and repressed Bcl-2 expression simultaneously, indicating that the miR-152-regulated apoptosis of trophoblast cells possibly relies on the mitochondrial pathway. Nevertheless, there were still some limitations in this research: 1) Only one kind of cell line was adopted, so other different cell lines or primary cells should be utilized. 2) The direct action targets for miR-152 in regulating cell apoptosis were not found.

Conclusions

We observed that miR-152 is highly expressed in the placental tissues of PE rats, and its expression level has a positive correlation with VEGF. Meanwhile, miR-152 can promote the apoptosis of trophoblast cells through the mitochondria-dependent apoptotic pathways.

Conflict of Interests

The authors declared no conflict of interest.

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