

Original Article

Expression and functional study of miR-23a in placental trophoblast cells during pregnancy-induced hypertension

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Abstract: Pregnancy-induced hypertension severely threatens maternal and fetal health. MicroRNA (miR)-23a participates in the regulation of vascular endothelial cell proliferation and migration. However, the mechanisms of miR-23a in PIH have not yet been reported. Real-time PCR was used to measure expressional change of miR-23a in placental tissues from both PIH patients and normal pregnant women. Placental trophoblast cell line HTR8 was cultured *in vitro* and assigned to the NC control group, miR-23 mimics group, and miR-23a inhibitors group. Expression of miR-23a was measured by real-time PCR. MTT assays were used to quantify cell proliferation. Transwell assays measured cell invasion and flow cytometry was used to analyze cell apoptosis. Caspase 3 activity was measured by test kits. Expression of inflammatory factors tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) was measured by enzyme linked immunosorbent assay (ELISA). Western blot was used to measure expression of Bax/Bcl-2. miR-23a showed downregulation in PIH patient placental tissues ($P < 0.05$ compared to the NC group). Transfection of miR-23a mimics upregulated miR-23a expression, inhibited proliferation and invasion of HTR8 cells, and facilitated apoptotic rates, caspase 3 activity, and enhanced Bax expression, while suppressing Bcl-2 ($P < 0.05$ compared to the NC group). Transfection of miR-23a inhibitors into HTR8 cells downregulated miR-23a and exerted opposite functions ($P < 0.05$ compared to the NC group). miR-23a showed downregulation in PIH. Modulation of miR-23a can improve proliferation, apoptosis, and invasion of placental trophoblast cells, further modulating PIH disease. miR-23a, thus, may work as a potential target for PIH treatment.

Keywords: Pregnancy-induced hypertension, miR-23a, cell apoptosis, cell proliferation, cell invasion

Introduction

Pregnancy-induced hypertension (PIH) is a common disease found in gynecology clinics. PIH consists of hypertension, pre-eclampsia, eclampsia, and chronic hypertension [1, 2]. PIH can occur during initial pregnancy or during multiple pregnancies, as well as in women with primary hypertension [3]. Pathological explanations of PIH are mainly attributed to ischemia of the placenta/uterus or deficiency of prostaglandin, leading to vascular malfunction during pregnancies. This includes systemic arteriole stiffness, resulting in sharply decreased placental perfusion, premature placenta, chronic hypoxia in the uterus, premature delivery, abor-

tions, and fetal deformation. PIH has become the primary reason for deaths of pregnant women and perinatal babies [4, 5]. Clinical symptoms of PIH include hypertension during pregnancy, tissue edema, and proteinuria [6, 7]. Severe PIH can cause eclampsia, cerebrovascular dysfunction, kidney failure, and neonatal death during the perinatal period [8]. PIH has high incidence, worldwide, and is a major concern in obstetrics [9]. However, the pathogenesis and disease mechanisms of PIH have not been illustrated yet. Thus, an effective treatment approach is lacking [10]. Therefore, the study of PIH pathogenesis mechanisms is a great challenge in obstetrics.

Non-coding microRNAs (miR) can modulate expression of non-coding proteins [11]. Through inhibiting the transcription and expression of downstream target genes, miRs can form complete or incomplete pairing with target genes, thus modulating mRNA degradation or protein translation [12]. MicroRNAs can regulate various processes, including cell proliferation, differentiation, and apoptosis. They can also mediate various developmental or pathological processes, including angiogenesis, cell growth, and proliferation [13, 14]. Previous studies have shown the involvement of miR-23a in vascular endothelial cell proliferation and migration [15, 16]. However, the functional roles and mechanisms in PIH have not been illustrated. Therefore, this study analyzed expressional changes of miR-23a in PIH, aiming to reveal related mechanisms.

Materials and methods

General information

A total of 82 PIH patients, confirmed in the O&G department of Maternal and Child Health Hospital of Guiyang City (Guiyang, Guizhou, China), from June 2016 to December 2017, were recruited. All individuals belonged to a single pregnancy, aged between 23 and 45 years (average age = 30 ± 3.3 years), with gestation weeks of 32-43 (average = 35 ± 3.7 weeks). All patients presented with headaches, edema, proteinuria, and hypertension, to different degrees. Inclusion and exclusion criteria [6]: All included patients fitted PIH diagnostic guidelines. Individuals complicated with severe liver/kidney dysfunction or nephritis, malignant tumors, primary hypertension, and primary heart disease were excluded. The control group included 35 normal pregnant women. All were single pregnancies, aged between 21 and 45 years (average age = 31 ± 3.6 years), with gestation weeks between 32 and 43 (average = 36 ± 2.5 weeks). No significant differences existed in age, gestation weeks, or body weights between two groups. Thus, the two groups were comparable ($P > 0.05$). This study was approved by the Ethics Committee of Guiyang, Guizhou, China. All participants provided informed consent.

Major reagents and equipment

Placental trophoblast cell line HTR8 was purchased from ATCC (CRL-3271, US). TRIzol Reagent, RNA extraction kit, RT-PCR primer, re-

verse transcription (RT) test kit, and real-time PCR reagent were purchased from Invitrogen (US). DMEM medium, fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Hyclone (US). DMSO and MTT powder were purchased from Gibco (US). Rabbit anti-mouse Bcl-2 monoclonal antibody, rabbit anti-mouse Bax monoclonal antibody, and goat anti-rabbit horse radish peroxidase (HRP) conjugated IgG secondary antibody were purchased from Cell signaling (US). Caspase 3 activity was measured using a test kit (Beyotime Biotech, China). Transwell chambers were purchased from Corning (US). Other common reagents were purchased from Sangon Biotech (China). Annexin V-PI apoptotic assay kit was purchased from BD (US). LabSystem Version 1.3.1 microplate reader was purchased from Bio-rad (US). Real-time PCR cyclers were purchased from ABI (US). Melody flow cytometry apparatus was purchased from BD (US). CO₂ cell incubator was purchased from Thermo (US).

HTR8 cell culturing and grouping

HTR8 cell lines, preserved in liquid nitrogen, were resuscitated for passage. Cells at 3rd to 8th generation were used for assays. Cells were kept in 90% high-glucose (25 mmol/L) DMEM medium. HTR8 cells with attached growth in 10% FBS were randomly assigned into three groups. These included the NC group, cultured at normal conditions, the miR-23a mimics group, receiving miR-23a mimic transfection, and the miR-23a inhibitors group, transfected with miR-23a inhibitors.

Liposome transfection of miR-23a mimics or miR-23a inhibitors into HTR8 cells

HTR8 cells were transfected with miR-23a mimics or miR-23a inhibitors. miR-23a mimics sequence was: 5'-GGU GGA GAU CCA GUU UCA-3'; miR-23a inhibitors sequence was 5'-GGA GUG UUU ACA CUA-3'. Cells were kept in 6-well plates until reaching 70-80% confluence. Liposomes, containing miR-23a mimics or miR-23a inhibitors, were added into 200 μ l serum-free DMEM medium for complete mixture with 15 minutes of room temperature incubation. Lipo2000 mixture was then diluted for 30 minutes at room temperature. Serum was removed from the cell culture and PBS was used for gentle rinsing. Next, 1.6 mL serum-free DMEM medium was added into the system, incubated in the 5% CO₂ chamber for 6 hours at 37°C. Serum-containing DMEM medium was then applied for 48 hours of continuous incubation.

Table 1. Primer sequences

Target gene	Forward primer 5'-3'	Reverse primer 5'-3'
GAPDH	AGTAGTCACCTGTTGCTGG	TAATACGGAGACCTGTCTGGT
miR-23a	A CTTGTCTGACATCC TTGG	TCTAAGGTAGCACAGCCA

Real-time PCR for measurement of miR-23a expression in PIH placental tissues and HTR8 cells

TRIzol Reagent was used to extract total cellular mRNA from placental tissues in PIH patients, normal pregnant women, or from cultured HTR8 cells. DNA reverse transcription was performed according to manufacturer instructions. Primers were designed by PrimerPremier 6.0 based on gene sequence and were synthesized by Invitrogen (China), as shown in **Table 1**. Real-time PCR was performed on target genes. PCR conditions were: 55°C 1 minute, followed by 35 cycles each consisting of 92°C 30 seconds, 58-60°C 45 seconds, and 72°C 35 seconds. Data were collected and CT values of all samples and standards were calculated based on fluorescent quantification and GAPDH reference gene, using the built-in software of PCR cyclers. Using CT values of standards as references, the standard curve was plotted. Semi-quantitative analysis was performed using the $2^{-\Delta Ct}$ approach.

MTT assay for cell proliferation of all groups

HTR8 cells at log-growth phase were inoculated into 96-well plates using DMEM medium with 10% FBS, at a density of 5×10^3 . After 24 hours of incubation, the supernatant was discarded and 20 μ l sterile MTT, with three replicated wells at each time point. After 4 hours of continuous incubation, the supernatant was completely removed and 150 μ l DMSO was added into the well for 10 minutes vortex. After complete dissolution of violet crystals, absorbance (A) values at 570 nm wavelength were measured using a microplate reader, calculating proliferation rates for all groups.

Transwell chambers for cell invasion of all groups of cells

Based on instructions of the test kit, serum-free medium was switched. Twenty-four hours later, the chamber bottoms and upper phases of the membranes were coated with 1:5 50 mg/L Matrigel dilution buffer, followed by 4°C air-drying. Next, 500 μ l DMEM medium with

10% FBS and 100 μ l tumor cell suspensions in serum-free DMEM medium were added into the interior and exterior of the chambers, respectively. Triplicated wells were performed for each group. All chambers

were placed into 24-well plates. The control group utilized Matrigel-free Transwell chambers. After 48 hours of incubation, Transwell chambers were rinsed in PBS to remove cells on the membranes. After fixation in cold ethanol and staining in crystal violet, cells at the lower phase of the membranes were enumerated. The experiment was repeated three times.

Caspase 3 activity assay

Activity of caspase 2 was measured in all groups. Briefly, cells were digested in trypsin and centrifuged at 600 g for 5 minutes at 4°C. The supernatant was discarded. Cell lysis buffer was added for 15 minutes of lysis on ice. The cell lysate was centrifuged at 20000 g for 5 minutes at 4°C. Next, 2 mM Ac-DEVD-pNA was added. Optical density (OD) values at 405 nm wavelength were measured, calculating caspase 3 activity changes.

Flow cytometry for analysis of cell apoptosis

Flow cytometry and apoptosis assay kits were used to measure cell apoptosis in all groups. Briefly, cells from all groups were digested, enumerated, and inoculated into 50 mL culture tubes at a 5×10^5 /mL concentration. Cells were then randomly assigned into three groups, as described previously. After complete rinsing, cells were fixed in 75% pre-cold ethanol, followed by 4°C overnight incubation. Cells were rinsed and re-suspended into the mixture containing 800 μ l 1XPBS and 1% BSA. Moreover, 100 μ g/mL PI staining buffer (3.8% sodium citrate, pH 7.0) was mixed with 100 RNAase (RnaseA, 10 mg/ml) for 37°C incubation for 30 minutes. Flow cytometry was employed to measure all data, analyzed using FCSEXPRESS 3.0 software.

Western blot for signal pathway changes of Bax and Bcl-2

HTR8 cellular proteins were extracted from all groups. Briefly, cell lysis buffer was added and proteins were quantified by Bradford approach. They were kept at -20°C for Western blotting.

miR-23a in PIH

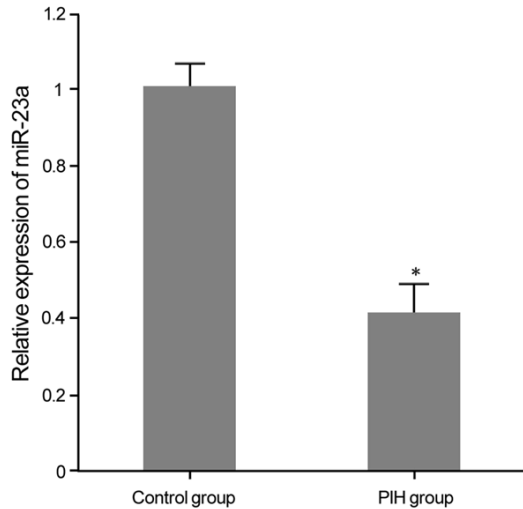


Figure 1. Expression of miR-23a in PIH. * $P < 0.05$, compared to the control group.

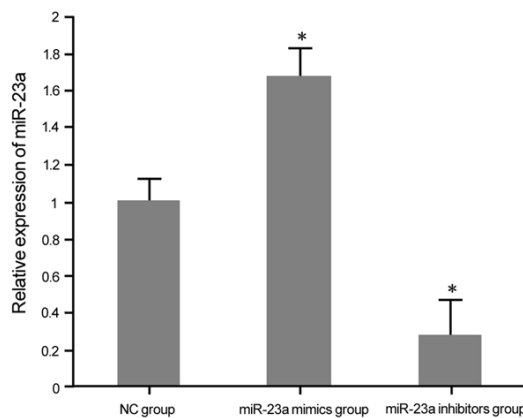


Figure 2. Regulation of miR-23a on its expression in trophoblast cell line HTR8. * $P < 0.05$, compared to the NC group.

Proteins were separated by 10% SDS-PAGE and transferred to NC membranes at 100 mA for 1.5 hours. Primary monoclonal antibody against Bax (1:2000 dilution) or Bcl-2 (1:2000 dilution) was added for 4°C overnight incubation. The membranes were rinsed in PBST at room temperature. Next, 1:2000 diluted goat anti-rabbit secondary antibody was added for 30 minutes, at room temperature, in the dark. After PBST rinsing, ECL substrate was added for 1 minute of development. X-ray exposure was performed for observation of results. Protein imaging processing system and Quantity One software was used to scan X-ray films and for measurement of band density. All experi-

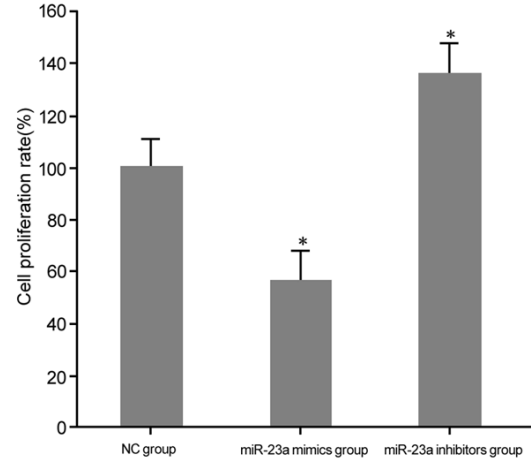


Figure 3. Effects of miR-23a regulation on proliferation in trophoblast cells. * $P < 0.05$, compared to the NC group.

ments were repeated four times ($n = 4$) for statistical analysis.

Statistical processing

SPSS 16.0 software was used for data analysis. Measurement data are presented as mean \pm standard deviation (SD). Comparisons of means across multiple groups were performed by one-way analysis of variance (ANOVA). Statistical significance is defined when $P < 0.05$.

Results

Expression of miR-23a in PIH

Real-time PCR was employed to measure expression of miR-23a in PIH. Results indicate that, compared to normal pregnant women, miR-23a showed significantly decreased expression in PIH patient placental tissues ($P < 0.05$, **Figure 1**).

Effects of miR-23a regulation on expression in trophoblast cell line HTR8

This study separately transfected miR-23a mimics or miR-23a inhibitors into HTR8 cells, using real-time PCR to measure expression of miR-23a. Results showed that, compared to the control group, transfection of miR-23a mimics upregulated miR-23a expression in HTR8 cells. There was statistical significance, compared to the NC group. Transfection of miR-23a

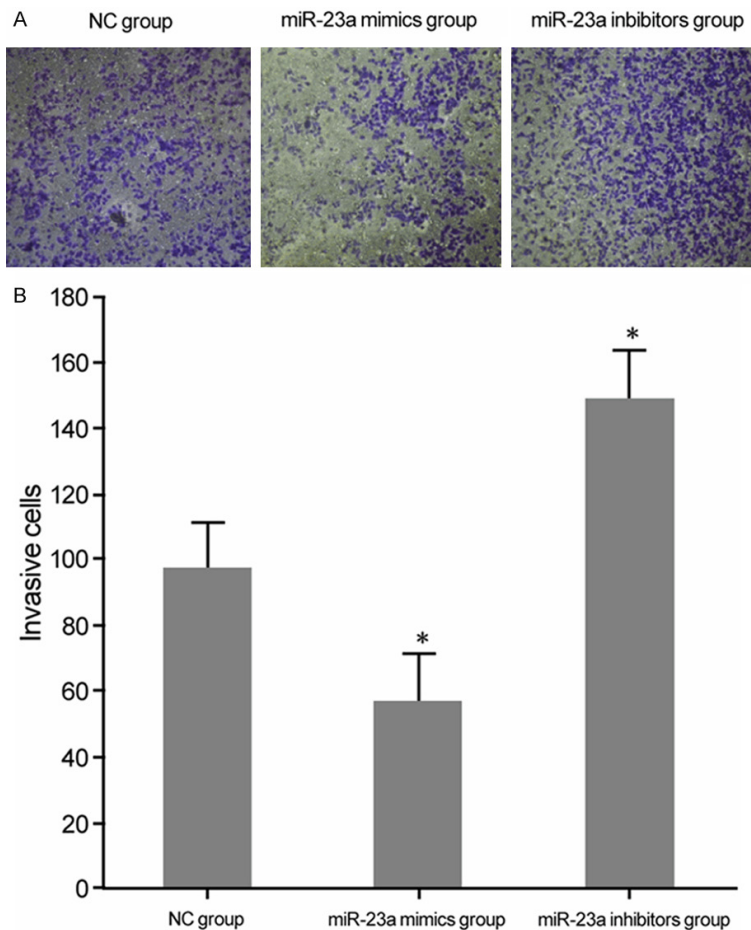


Figure 4. Effects of miR-23a regulation on invasion of trophoblast cell line HTR8. A. Transwell chamber analysis for the effects of miR-23a on HTR8 cell invasion. B. Statistical analysis for the effects on HTR8 cell invasion. * $P < 0.05$.

inhibitors downregulated miR-23a expression in HTR8 cells ($P < 0.05$, **Figure 2**).

Regulation of miR-23a on trophoblast cell HTR8 proliferation

After transfecting miR-23a mimics or inhibitors into HTR8 cells, MTT assay was used to analyze effects on proliferation of trophoblast cell line HTR8. Results showed that transfection of miR-23a mimics upregulated miR-23a expression and significantly suppressed HTR8 cell proliferation ($P < 0.05$ compared to the NC group). Transfection of miR-23a inhibitors downregulated miR-23a expression and remarkably facilitated HTR8 cell proliferation ($P < 0.05$, compared to the NC group, **Figure 3**).

Regulation of miR-23a on HTR8 cell invasion

After transfecting miR-23a mimics or miR-23a inhibitors into HTR8 cells, the effects on inva-

sion of trophoblast cell HTR8 were analyzed. Results showed that transfection of miR-23a mimics upregulated miR-23a expression and significantly inhibited HTR8 cells invasion ($P < 0.050$, compared to the control group). Transfection of miR-23a inhibitors suppressed miR-23a expression and remarkably facilitated HTR8 cell invasion ($P < 0.05$, compared to the NC group, **Figure 4**).

Effects of miR-23a regulation on HTR8 cell apoptosis

After separately transfecting miR-23a mimics or miR-23a inhibitors into HTR8 cells, flow cytometry was used to analyze effects on trophoblast cell HTR8 apoptosis. Results showed that transfection of miR-23a mimics increased miR-23a expression and facilitated HTR8 cell apoptosis ($P < 0.05$, compared to the NC group). Transfection of miR-23a inhibitors suppressed miR-23a expression and remarkably suppressed HTR8 cell apoptosis ($P < 0.05$, compared to the NC group, **Figure 5**).

Effects of miR-23a regulation on caspase 3 activity of trophoblast cell HTR8

After transfecting miR-23a mimics or miR-23a inhibitors into HTR8 cells, a test kit was used to analyze the effects on caspase 3 activity in trophoblast cell HTR8. Results showed that transfection of miR-23a mimics upregulated miR-23a expression and remarkably facilitated caspase 3 activity ($P < 0.05$, compared to the NC group). Transfection of miR-23a inhibitors downregulated miR-23a expression and significantly suppressed caspase 3 activity of HTR8 cell ($P < 0.05$, compared to the NC group, **Figure 6**).

Regulation of miR-23a on expression of Bax and Bcl-2 in trophoblast cell HTR8

After transfecting miR-23a mimics or miR-23a inhibitors into HTR8 cells, Western blotting was

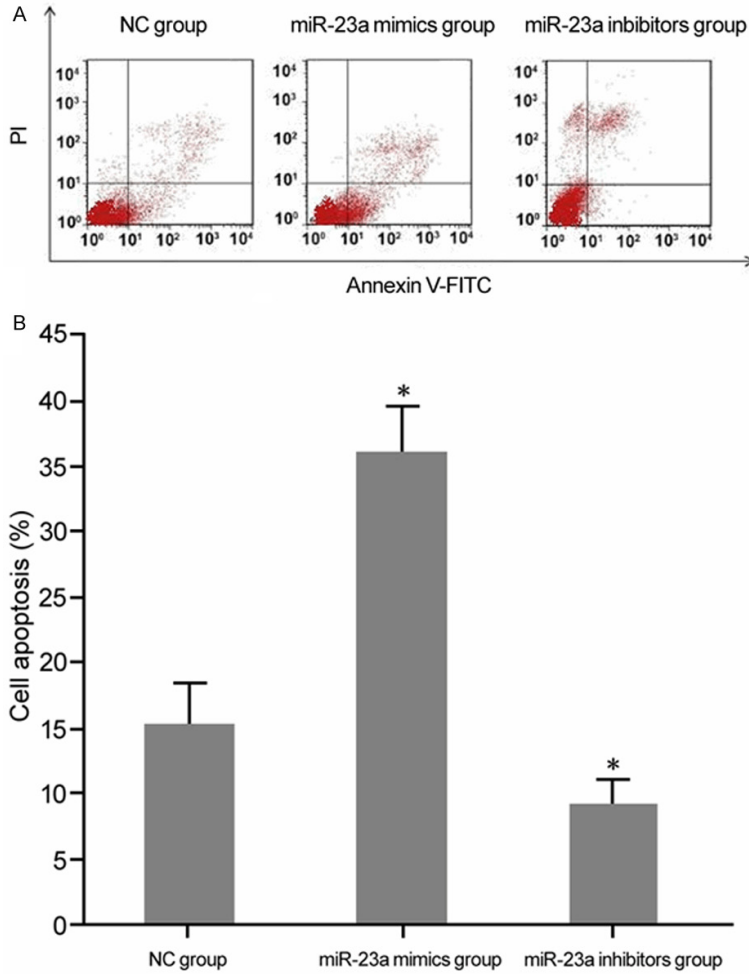


Figure 5. Regulation of miR-23a on apoptosis of trophoblast cell HTR8. A. Flow cytometry for the effects of miR-23a on apoptosis of trophoblast cell HTR8. B. Statistical analysis for HTR8 cell apoptosis. * $P < 0.05$, compared to the NC group.

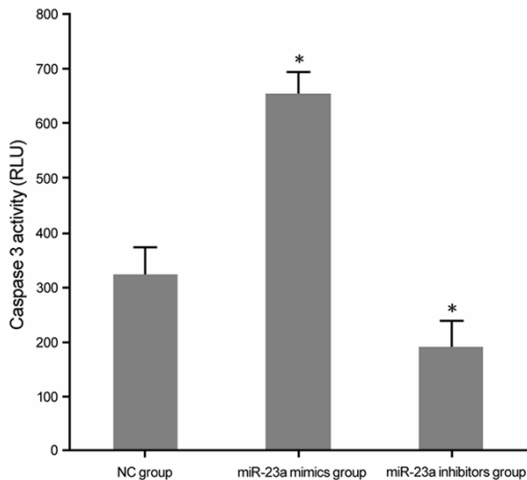


Figure 6. Regulation of miR-23a on caspase 3 activity of trophoblast cell line HTR8. * $P < 0.05$, compared to the NC group.

used to analyze the effects of miR-23a on expression of Bax and Bcl-2 in trophoblast cell HTR8. Results showed that transfection of miR-23a mimics into HTR8 cells upregulated miR-23a expression, facilitated Bax expression, and decreased Bcl-2 ($P < 0.05$, compared to the NC group). Transfection of miR-23a inhibitors into HTR8 cells exerted opposite effects. It reduced Bax expression and elevated Bcl-2 ($P < 0.05$, compared to the NC group, **Figure 7**).

Discussion

PIH is a common and severe obstetrics complication. Having multiple inducing factors, the precise pathogenic mechanisms of PIH have not been fully illustrated. As PIH can occur in pregnant women at any age and can cause severe trouble for both pregnant women and perinatal babies, early prevention and treatment of PIH is a critical challenge [17, 18]. MiRNAs can regulate both normal and pathological conditions of cell behaviors, including growth, proliferation, cell cycle, and cell apoptosis, making it an important regula-

tory factor for pathogenesis and progression of human diseases [19]. However, few studies have been performed regarding miRNA regulation in PIH or related mechanisms. miR-23a is known to regulate vascular endothelial cells. Thus, it is probably related with occurrence and progression of hypertension [16]. This study first analyzed expression of miR-23a in PIH placental tissues. It demonstrated downregulation of miR-23a in PIH, indicating its possible role in PIH pathogenesis and its effects on maternal-fetal health.

This study further analyzed the function of miR-23a and related mechanisms in PIH. This study utilized placental trophoblast cells as research subjects. The placenta is the sole interface between the maternal and fetal body. It works as the exchange station for substances bet-

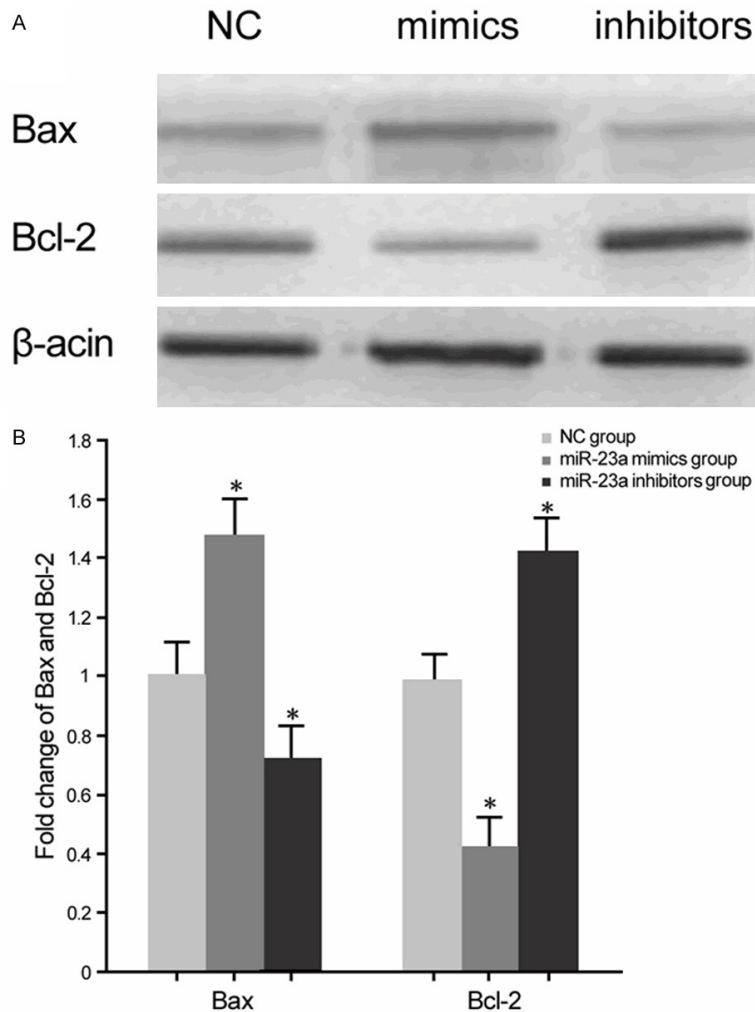


Figure 7. Effects of miR-23a regulation on Bax and Bcl-2 expression in trophoblast cell line HTR8. A. Western blot for analysis of the effects of miR-23a on Bax/Bcl-2 expression in trophoblast cell line HTR8. B. Statistical analysis for Bax/Bcl-2 expression. * $P < 0.05$, compared to the NC group.

between the maternal and fetal body, providing routes for circulation of nutrients, gas, and blood. It is of critical importance for maintaining and protecting normal development of the fetus [20, 21]. By transfecting miR-23a mimics or miR-23a inhibitors into placental trophoblast cell line HTR8 to modulate miR-23a expression, this study analyzed its effects on cell proliferation, apoptosis, and invasion. Transfection of miR-23a mimics into HTR8 cells remarkably elevated miR-23a expression, further inhibiting HTR8 cell proliferation and invasion and facilitating apoptosis. On the other hand, transfection of miR-23a inhibitors into HTR8 cells exerted opposite effects. Present results suggested that the modulation of miR-23a expression can regulate proliferation, apoptosis, and invasion

of trophoblast cell line HTR8, further affecting the placental function of PIH patients. Bax is a water-soluble related protein with Bcl-2 homology. It is the pro-apoptotic gene in the Bcl-2 gene family. Overexpression of Bax can antagonize the protective effects of Bcl-2, thus making cells susceptible to programmed death. Therefore, it is a critical factor for the inhibition of cell apoptosis. Caspase 3, on the other hand, is a critical enzyme modulating cell apoptotic pathways [22, 23]. This study further analyzed the related mechanisms of miR-23a in regulating PIH, demonstrating that upregulation of miR-23a expression could facilitate Bax expression, decrease Bcl-2, and increase caspase 3 activity in HTR8 cells. On the other hand, downregulation of miR-23a expression can inhibit Bax, facilitate Bcl-2 expression, and suppress caspase 3 activity in HTR8 cells, suggesting the involvement of miR-23a in onset and progression of PIH via modulating apoptotic pathways. Further studies should be performed to investigate the related mechanisms of miR-23a in PIH and related target genes, providing references for determination of PIH targets.

Conclusion

miR-23a shows downregulation in PIH patients. Modulation of miR-23a can mediate PIH via improving proliferation, apoptosis, and invasion of placental trophoblast cells. miR-23a, therefore, may be a potential target of PIH treatment.

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Disclosure of conflict of interest

None.

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