Original Article

MicroRNA-195 is downregulated in the peripheral blood of pregnant women with pregnancy-induced hypertension and inhibits the trophoblast apoptosis through targeting iNOS

Li Wang¹, Huanping Wang¹, Yu Wang¹, Juan Wang²

¹Department of Obstetrics and Gynecology, Henan Provincial People’s Hospital, Zhengzhou, China; ²Department of Neurology, Henan Staff and Workers Hospital, Zhengzhou, China

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Abstract: Reports indicate that pregnancy induced hypertension (PIH) is known to have long term adverse effects both in the mother and offspring. At present, the pathogenesis of PIH is incompletely understood. Recent studies on microRNA (miRNA) offer the possibility for developing a new class of molecular markers for diagnosis of PIH. However, the function of miRNAs in the development of PIH remains unknown. In the present study, differentially expressed miRNAs in the peripheral blood of healthy pregnant women and women with PIH were screened by PCR array and validated using quantitative real-time polymerase chain reaction. Then, the effects of miRNA-195 on the apoptosis of trophoblast cells were assessed by silencing and over-expressing the miRNA. Our study showed that miR-195 was specifically down-regulated in the peripheral blood of women with PIH. We found that overexpression of miR-195 inhibited the apoptosis of trophoblast cells, whereas apoptosis was enhanced by knockdown of miR-195. And the activity of caspase 3/8/9 were inhibited or enhanced in trophoblast cells transfected with miR-195 inhibitor or miR-195 mimic. We also confirmed that inducible nitric oxide synthase (iNOS), which playing important roles in cells apoptosis, as a direct target of miR-195 by using the dual-luciferase assay. Furthermore, we found that expression of iNOS was up-regulated in peripheral blood samples from patients with PIH and inversely correlated with miR-195 expression. Our study demonstrated that the miR-195-inhibited apoptosis of trophoblast cells may contribute to PIH and miR-195 can be considered to be a promising therapeutic target for PIH.

Keywords: Pregnancy-induced hypertension, miR-195, apoptosis, trophoblast cell, iNOS

Introduction

Pregnancy induced hypertension (PIH) includes a group of hypertensive disorders which develop during pregnancy and is one of the most common obstetric complication [1]. PIH includes gestational hypertension, pre-eclampsia and eclampsia [2, 3]. It has been estimated that worldwide about 76,000 pregnant women die each year from pre-eclampsia and related hypertensive disorders, resulting in a very large disease burden [4]. Inadequate trophoblast invasion into the uterine spiral arteries and impaired placentation affect fetal growth and lead to serious pregnancy complications, such as intrapartum growth restriction (IUGR), pre-eclampsia (PE), preterm birth and spontaneous abortion [5]. Additionally, increased apoptosis of trophoblast cells can also be observed in the placentas of patients who have been affected by pregnancy complications [6]. However, the molecular mechanism for the regulation of trophoblast behavior and the pathogenesis of PIH remains largely elusive.

MicroRNAs (miRNAs) are a class of endogenous small noncoding RNAs with 18-22 nt in length that have been discovered recently as fundamental and posttranscriptional regulators of gene expression [7, 8]. There is an accumulating body of evidence that miRNAs are implicated in a variety of biological processes, such as cell proliferation, differentiation, apoptosis, tumorigenesis, etc [9]. Increasing evidence supports that altered miRNA expression profiles are closely related to the pathophysiology of
placenta [10, 11]. And, kinds of miRNAs have been confirmed to participate in the initiation and progression of pregnancy induced hypertension [12-15]. Zhu X et al. found that miR-18a expression suppression led to a decrease in JEG-3 cell invasion and an increase in JEG-3 cell apoptosis, by inducing ESRα expression [16]. And a study from Zou Y et al. demonstrated that miR-101 regulates apoptosis of trophoblast HTR-8/SVneo cells by targeting endoplasmic reticulum (ER) protein 44 during preeclampsia [17]. These findings suggest that miRNAs play important roles in the development of PIH. However, the potential effects and mechanisms by which miRNAs regulate trophoblastic cell function are poorly characterized and need to be investigated further.

Given the potential role of miRNAs, their expression was profiled in the peripheral blood from patients with normal pregnancies and those with PIH by PCR array. The differentially expressed miRNAs were then selected and validated. We also revealed the regulatory mechanisms associated with miR-195 during this process and found that iNOS was a direct target of miR-195. These results showed that miR-195 inhibited the apoptosis of trophoblast cells by regulating the expression of iNOS. These findings highlight the importance of miR-195 in the pathogenesis of PIH and provide new insight into the limited understanding biological process of PIH.

**Materials and methods**

**Cell culture**

HTR-8/SVneo cells (purchased from the Cell Bank of the Chinese Academy of Sciences, China) were maintained in RPMI-1640 medium (Gibco BRL, New York, USA) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin and 100 U/mL penicillin under standard culture conditions (37°C and 5% CO₂ incubator). Shortly before transfection, 1.5 × 10⁵ cells were seeded per well in a six-well plate in 2 ml DMEM culture medium containing serum and antibiotics. Before transfection, the cells were incubated under normal growth conditions (typically 37°C and 5% CO₂). Then, miR-195 mimics, miR-195 inhibitor or miR negative control (GenePharma, Shanghai, China) were pre-incubated with HiPERFect transfection reagent (Qiagen, Suzhou, China) with the final concentration of miRNA analogs at 100 nmol/L.

**Clinical specimen collection**

The collection of human peripheral blood specimens was performed with the permission of the local ethical committee in the Institute of Zoology, Chinese Academy of Sciences, and informed consent was obtained from all patients enrolled in this study. Peripheral blood samples from normal pregnant and preeclamptic women were obtained from pregnant women who underwent perinatal care in Peking University Third Hospital from August 2014 to October 2015. Totally 20 severe preeclamptic patients who delivered at 35th to 39th weeks and 20 normal pregnant women who delivered at 37th to 39th weeks were enrolled in this study.

**RNA extraction**

Total RNA was extracted from the peripheral blood from patients with PIH using an RNeasy® mini kit (Qiagen GmbH, Hilden, Germany). Potential genomic DNA contamination was removed from the samples by treatment with RNase-free DNase (Qiagen) for 15 min at room temperature. Concentration and purity were determined using a NanoDrop 1000™ spectrophotometer (Thermo Fisher, Dubuque, IA, USA), while the integrity of miRNA was further assessed using an Agilent 2100 Bioanalyzer (Agilent Tech, Palo Alto, CA).

**PCR array**

The human RT² profiler PCR autophagy array (Qiagen) was used to study the expression of 21 genes related to PIH. Briefly, using an RT² first Strand kit (Qiagen), 1 μg total RNA obtained from the peripheral blood from patients with PIH was incubated with the kit’s genomic DNA elimination mixture at 42°C for 5 min and then transferred to ice for no less than 1 min to remove any residual DNA contamination. The kit’s reverse transcription mixture was added to the purified RNA sample. The mixture was incubated at 42°C for 15 min and then 95°C for 5 min to convert total RNA back into cDNA. After cDNA synthesis, real-time RT-PCR was performed using RT2 SYBR® Green Master mix (Qiagen), according to the manufacturer’s instructions. The amplification data (fold changes in the threshold cycle [Ct] values of all the genes) were analyzed by the ΔΔCt method.
Real-time PCR
For miRNAs analysis, total RNA was isolated from lungs using the MicroRNA Extraction and Purification Kit (Novland, Shanghai, China). Real-time PCR was performed using two-step Stemaim-it miR qRT-PCR Quantitation Kit (SYBR Green) (Novland, Shanghai, China) on BIO-RAD IQ5 real-time PCR instrument. Specific primers and probes for mature microRNAs and snRNA RNU6B were obtained from GenePharm, Shanghai, China. All reactions were conducted in triplicate. Quantitative normalization was performed on U6 and β-actin for miRNA and mRNA detection, respectively.

Apoptosis assay
To detect the effects of miR-195 on HTR-8/SVneo cell apoptosis, the cells (50-60% confluent) were transfected with miR-195 mimics, inhibitor or negative control. After treatment, the cells were washed with 1 x PBS for three times. Then, an Annexin-V FITC–PI Apoptosis Kit (Invitrogen) was applied to determine the apoptotic rate by flow cytometry. This assay employs fluorescein-labeled Annexin-V in concert with propidium iodide to detect the cells undergoing apoptosis.

Luciferase reporter assay
Dual luciferase assays were conducted in a 24 well plate format. pGL3-iNOS 3’UTR report/pGL3-iNOS 3’UTR Mutant report + TK100 Renilla report were transfected into 70% confluent HTR8/Svneo cells, along with miR-195 mimic, miR-195 inhibitor or each control. After 48-h transfection, firefly and renilla luciferase were quantified sequentially using the Dual Luciferase Assay kit (Promega, USA) following the manufacturer’s recommendations.

Western blots
Protein extracts from HTR-8/SVneo cells were subjected to 10% SDS-PAGE and subsequently transferred to a PVDF membrane. The membrane was blocked with 5% (w/v) nonfat milk and incubated sequentially with the primary antibodies against caspase 3, caspase 8, caspase 9 (rabbit, 1:5000, Abcam, Cambridge, UK) in TBST containing 5% bovine serum albumin overnight at 4°C. Anti-β-actin antibody was used as an internal control. After washing three times with TBST, the membrane was incubated at room temperature for 2 hours with horse-radish peroxidase-conjugated secondary antibody (anti-rabbit, 1:2000, Cell Signaling Technology) diluted with TBST. The detected protein signals were visualized using an enhanced chemiluminescence (ECL) system western blot kit (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis
Statistical analyses were performed with SPSS 13.0 software. The results were evaluated by χ² test and the other data were evaluated by Student’s t-test and expressed as the mean ± SD from three independent experiments. A P-value of less than 0.05 was considered statistically significant.

Results
MiRNA expression profiles and qPCR validation in peripheral blood of healthy pregnant women and women with PIH
Hierarchical clustering showed systematic variations in the expression of miRNAs in peripheral blood of healthy pregnant women and women with PIH (Figure 1A). To validate the miRNA PCR array analysis findings, we selected 4 upregulated and 4 downregulated miRNAs and analyzed their expression using quantitative real-time polymerase chain reaction (qRT-PCR) in the peripheral blood. These data confirmed that miR-130a, miR-125b-5p, miR-378, and miR-212 were over-expressed in peripheral blood from pregnant women with PIH, whereas the expression of miR-195 miR-20a, miR-19a and miR-27b was decreased, which corroborated the expression patterns seen using the PCR array (Figure 1B). Thus, our data indicate that a set of miRNAs is frequently aberrantly expressed in peripheral blood from pregnant women with PIH. Among them, miR-195 has been reported to be down-regulated in pre-eclamptic placentas compared with normal pregnant ones, indicating possible association of this small molecule with placental pathology of preeclampsia [18], therefore, we focused on miR-195 for further study. According to the qPCR results, we also found that miR-195 expression was significantly decreased in peripheral blood from pregnant women with PIH when compared to healthy pregnant women.
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Based on this, we hypothesized that miR-195 may be involved in the progress of PIH.

MiR-195 inhibited apoptosis of HTR-8/SVneo cells

Because previous studies have established that increased apoptosis in HTR-8/SVneo trophoblast cells contributed to pre-eclampsia [19]. Thus, we examined the effect of miR-195 on apoptosis in HTR-8/SVneo cells. As shown in Figure 2A and 2B, knockdown of miR-195 significantly promoted HTR-8/SVneo cells apoptosis when compared with inhibitor NC group, whereas promotion of miR-195 would inhibited cell apoptosis. To confirm the involvement of caspases in miR-195-induced apoptosis, we measured the activities changes of caspases 3, 8 and 9. Our results showed that inhibiting miR-195 levels in HTR-8/SVneo cells with the miR-195 inhibitor increased the activity of caspases 3, 8 and 9, whereas increasing miR-195 with the miR-195 mimic decreased the activity of caspases 3, 8 and 9 (Figure 2C, 2D). Taken together, these results indicate that miR-195 may inhibit apoptosis in HTR-8/SVneo cells partially by caspase dependent process.

iNOS is a direct target of MiR-195

To investigate the mechanism by which miR-195 regulates the apoptosis of trophoblast cells, bioinformatics algorithms TargetScan and miRanda were applied to identify the direct target genes of miR-195. The bioinformatics analysis revealed a putative miR-195 binding site in 3'UTR of iNOS (Figure 3A). It is known that iNOS is a well-known regulator of apoptosis and has been shown to inhibit the activity of many caspases under both in vitro and in vivo conditions. To determine whether miR-195 directly targets iNOS, we checked the direct binding of miR-195 to iNOS mRNA 3'UTR by

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Using luciferase report assay. The results showed that miR-195 inhibitor significantly increased the luciferase activity in pGL3-iNOS 3′-UTR transfected cells and miR-195 mimics decreased the luciferase activity in pGL3-iNOS 3′-UTR transfected cells (Figure 3B). No significant difference in luciferase activities was observed in cells co-transfected with pGL3-iNOS and miR-195 mimic, miR-195 inhibitor or each control. To further evaluate whether miR-195 regulated iNOS expression, we detected the protein expression level of iNOS in miR-195 mimic or miR-195 inhibitor transfected cells.

Western blot analysis showed that miR-195 overexpression markedly decreased the protein level of iNOS, whereas miR-195 inhibition increased the protein expression of iNOS (Figure 3C). Taken together, we showed that the iNOS mRNA is directly regulated by miR-195 via conserved seed-matching sequences.

Relationship between miR-195 expression and the activity of iNOS

Previous study demonstrated that the exaggerated upregulated iNOS is probably associated

Figure 2. miR-195 inhibited the apoptosis of HTR-8/SVneo cells via caspases. A, B. A flow cytometry assay, using annexin V-FITC and PI staining, was performed to detect the apoptotic cells transfected with miR-195 inhibitor and miR-195 mimic or negative control. C, D. The activity of caspases 3, 8, and 9 is assessed at 24 h after transfection with miR-195 inhibitor and miR-195 mimic or negative control. Data represent the means ± SEM. **P < 0.01 vs inhibitor NC or mimic NC.
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As shown in Figure 4A and 4B, we found that expression of iNOS was up-regulated in peripheral blood samples from patients with PIH and inversely correlated with miR-195 expression. We therefore postulate that miR-195 affects the apoptosis of HTR8/Svneo cells via directly regulation iNOS.

Discussion

In the present study, we demonstrated that miR-195 was significantly down-regulated in the peripheral blood of pregnant women with pregnancy-induced hypertension. Furthermore, downregulation of miR-195 may contribute to the occurrence of PIH by promoting trophoblast cell apoptosis via directly up-regulating iNOS. This results suggesting miR-195 may have potential as a predictive or therapeutic target for PIH.

Recently, several studies have used miRNA microarray approaches to identify differentially expressed miRNAs in placentas from patients with normal pregnancies and those with PIH [21]. MiR-29b participates in the progress of PE by repressing trophoblast cell invasion and angiogenesis and enhancing cell apoptosis through targeting MMP-2, Integrin beta 1 (ITGB1) and vascular endothelial growth factor A (VEGF-A) [12]. And a study from Jie Ding et al. confirmed that upregulation of miR-519d-3p may contribute to the development of preeclampsia by inhibiting trophoblast cell migration and invasion via targeting MMP-2, and indicated that miR-519d-3p may participate in the pathological processes underlying PE [22]. These findings suggest
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that miRNAs play important roles in the development of PIH. In this study, we observed that miR-195 was significantly down-regulated in the peripheral blood of pregnant women with PIH. MiR-195 is clustered with miR-497 [23] and belongs to miR-15 family [24], which is one of the differential miRNAs in preeclamptic placentas reported by Zhu et al. [15]. To date, functional studies on miR-195 are mainly in cancer, whereas little is known about its role in PIH.

It is generally accepted that increased trophoblast cell apoptosis in the placenta contributed to the pathogenesis of PIH [25]. In order to study the function of miR-195, we examined the effects of miR-195 on the apoptosis of trophoblast cells in vitro by transiently transfecting a trophoblast cell line, HTR8/SVneo, with a miR-195 mimic or miR-195 inhibitor. Knockdown of miR-195 potently promoted trophoblast cell apoptosis. In addition, inhibition of endogenous miR-195 significantly increased the activity of caspase-3/8/9. Combined with our findings that the expression of miR-195 is downregulated in the peripheral blood of pregnant women with PIH compared to those of healthy pregnant women, these observations indicate that miR-195 may play an important role in abnormal trophoblast apoptosis during the pathological processes leading to PIH.

Given that a single microRNA can target many genes, we believe that miR-195 also has multiple targets. It will greatly help us to better understand the mechanism of miR-195 in PIH after more functional targets are identified. In our study, we demonstrated that miR-195 interacted with its partially complementary sequence in the 3’UTR of iNOS using luciferase reporter assays, and confirmed that miR-195 negatively regulated iNOS protein expression using Western blot analyses. Furthermore, to confirm the regulation of iNOS by miR-195, we analyzed the expression of miR-195p and iNOS in the peripheral blood of pregnant women with PIH and those of patients with healthy pregnancies, and observed an inverse correlation between the expression of iNOS and miR-195. These findings demonstrated that miR-195 regulates trophoblast cell apoptosis by targeting iNOS.

In conclusion, our data demonstrated that miR-195 inhibited trophoblast cell apoptosis via targeting iNOS in vitro. This study provided novel insights into the mechanism of PIH progression and suggested a new therapeutic strategy against PIH by targeting miR-195.

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Disclosure of conflict of interest

None.

Address correspondence to: Li Wang, Department of Obstetrics and Gynecology, Henan Provincial People’s Hospital, 7 Weiwu Road, Zhengzhou 450003, China. Tel: +86 371-65580014; E-mail: wanglihigh@163.com

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