Original Article
Mechanism underlying the regulation of trophoblast-like cell invasion by miR-183 screened from the peripheral blood of pregnant women with preeclampsia

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Abstract: In recent years, microRNAs (miRNAs) have been shown to have critical regulatory roles in preeclamptic placentas. However, the effect and mechanisms of miRNAs on preeclamptic (PE) have not been well understood. This study aimed to test whether miR-183 could inhibit trophoblast-like cell invasion and reveal its underlying molecular mechanism. Differentially expressed miRNAs in the peripheral blood of preeclamptic compared with healthy pregnant women were validated using quantitative real-time polymerase chain reaction. The peripheral blood samples from patients with preeclampsia were analyzed for levels of a specific differentially expressed miRNA (miR-183). The effects of miR-183 were assessed by silencing and over-expressing the miRNA in vitro. Our study showed that miR-183 was specifically up-regulated in preeclampsia placentas. We found that overexpression of miR-183 inhibited the invasive capabilities of BeWo and JAR cells, whereas enhanced by knockdown of miR-183. We also confirmed that MMP9 as a direct target of miR-183 in BeWo cells by using the dual-luciferase assay. Furthermore, restoration of MMP9 expression in BeWo cells could partially reverse the cell invasion by miR-183 overexpression, indicating that miR-183 inhibits cytotrophoblast invasion via targeting MMP9. Our study suggests miR-183 could be a promising therapeutic target for preeclampsia.

Keywords: Preeclampsia, miR-183, MMP9, invasion, trophoblast-like cells

Introduction
Preeclampsia (PE) is a systemic maternal disease, characterized by hypertension and proteinuria, it can involve many organs and is a leading cause of considerable maternal morbidity and perinatal morbidity and mortality worldwide [1, 2]. Many studies have shown that the disease is mainly related to the invasiveness descent and invasion failure of the trophoblastic cells [3-6]. However, the molecular mechanism related to insufficient trophoblastic invasion remains largely elusive.

MicroRNAs (miRNAs) are a class of endogenous small noncoding RNAs with 18-22 nt in length. Mature miRNAs bind to the 3’-UTR of target mRNAs and repress translation of target mRNAs or induce degradation of target mRNAs leading to repressed translation or induced mRNA cleavage of the target genes [7-9]. A handful of studies demonstrated that microRNAs play critical roles in many biological processes involving in the pathogenesis of various human diseases [10]. Recent studies confirmed the involvement of microRNAs in the pathogenesis of PE [9, 11, 12]. In 2007, it was the first report that preeclampsia was associated with altered expression of microRNAs in placenta [13]. Thereafter, more investigations were performed with regards to placental miRNAs in PE and numerous differential placental miRNAs were identified, including miR-210, miR-152 and miR-584 [14-16]. These studies suggesting that aberrant expression of miRNAs may contribute to the pathogenesis of PE [17].

In a previous study, Shi et al [18] revealed a promotional role of miR-183 in endometrial stromal cells and inhibition of miR-183 on cell invasive ability. Recent studies have evaluated miR-183 expression in preeclampsia [19]. These
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studies found increased miR-183 expression in preeclamptic placentas. However, the detailed role of miR-183 in the pathogenesis of preeclampsia remains unclear. In this study, we will employ BeWo and JAR cells to explore the effects of miR-183 on the invasion of trophoblast cells and the underlying mechanism. Our results reveal the potential roles of miR-183 as a therapeutic target and a novel biomarker for PE.

Materials and methods

Cell culture and transfection

Human trophoblast-like cells BeWo and JAR obtained from Cell Bank of Wuhan University (Wuhan, China). BeWo was maintained in F12 medium (Invitrogen, Barcelona, Spain). JAR was maintained in RPMI-1640 (Hyclone, USA). All of them containing 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂. Lipofectamin 2000™ (Life Technologies) was used for miRNA and plasmid transfection according to the manufacturer’s protocol. Forty eight hours after transfection, the total protein was extracted for Western Blot and the total RNA was isolated for qRT-PCR.

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 who underwent perinatal care in Henan Provincial People’s Hospital from August 2010 to October 2012. Totally 20 severe preeclamptic patients who delivered at 35th to 39th weeks and 33 normal pregnant women who delivered at 37th to 39th weeks were enrolled in this study. Their placentas at deliveries and plasma samples at gestational weeks 15 to 18 and weeks 35 to 38 were used.

Transwell invasion assay

Cell invasion assays were performed using Transwell chambers (8.0 μm pore size; Millipore, MA), which were coated with Matrigel (4 × dilution; 60 μl/well; BD Bioscience, NJ), in 24-well plates. Transfected cells were starved overnight and then seeded in the upper chamber at a density of 2×10⁴ cells/ml in 400 μl of medium containing 0.5% FBS. Medium with 10% FBS (600 μl) was added to the lower chamber. Following a 24 h-incubation at 37°C with 5% CO₂, non-invading cells in the upper chamber were removed with a cotton swab, and invading cells were fixed in 100% methanol and stained with 0.5% crystal violet in 2% ethanol. Photographs were taken randomly for at least four fields of each membrane. Thereafter, the stained cells were counted in five random areas in multiple wells.

RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was extracted from peripheral blood samples and cell lines using TRIzol reagent (Life Technologies) following the manufacturer’s instructions. Reverse transcription of mRNA and miRNAs were performed using PrimeScript RT Master Mix and SYBR PrimeScript miRNA RT-PCR Kits, respectively (Takara Biotechnology, Dalian, China). For miR-183 expression level detection, total RNA was reverse-transcribed with a miR-183-specific RT primer (RiboBio, China) and amplified with PCR primers (RiboBio, China) on the ABI 7300 Real-Time PCR System (Life Technologies, USA). The relative expression of miR-183 was normalized against U6. qRT-PCR was carried out in Applied Biosystems 7500 (Life Technologies) using SYBR ExScript qRT-PCR Kit (Takara, Dalian, China).

Protein extraction and Western blot analysis

Cells were lysed on ice for 30 min in RIPA buffer (50 mm TrisHCl, pH 7.4, 1% Igepal CA-630, 0.5% sodium deoxycholate, 150 mm NaCl, 1 mm EDTA, 0.1% SDS) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany). Lysates were cleared by centrifugation at 4°C for 10 min at 10,000 × g. Equal amount of protein was separated on 10% SDS-PAGE gels and transferred electrophoretically to nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% (w/v) nonfat milk in Tris-buffered saline with 0.1% Tween-20. After incubation with primary antibodies against rabbit polyclonal antibodies MMP9 (1:400 diluted; Boster, Wuhan, China) overnight at 4°C, the membranes were washed
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and incubated with corresponding secondary antibodies. Finally, the protein blots were visualized with an enhanced chemiluminescence (ECL) Kit (Sevensea Pharmtech Co., Ltd, Shanghai, China). β-actin was used as the loading control. Quantification of immunoreactive bands was performed using Quantity One software (Bio-Rad, Hercules, CA, USA).

Luciferase activity assay

The 3′-UTR regions of human MMP9 containing the miR-183 binding site predicted by the TargetScan (http://www.targetscan.org) were amplified by PCR from BeWo cell cDNA and then cloned into the pcDNA3.1 vector (Ambion, Austin, TX, USA). This set of plasmids was named wild type (WT). Then mutant (MT) plasmids containing nine single nucleotide mutations in the predicted miR-183 binding sites of the 3′-UTR regions were also constructed. For the luciferase assay, BeWo cells were co-transfected with 400 ng of either miR-NC (miRNA negative control) or miR-183 along with target gene 3′-UTR WT or MT plasmid, and Renilla plasmid using Lipofectamine 2000. The Renilla luciferase plasmid was used as an internal control to correct differences in both transfection and harvest efficiencies. Whether miR-183 interacted with the 3′UTR of MMP9 was determined with a Dual-luciferase Reporter Kit (Promega) according to standard supplier’s protocols.

MMP9 rescue experiments

The coding sequence of MMP9 excluding its 3′-UTR was inserted into the pcDNA3.1+ (pcDNA3.1-MMP9). The BeW0 cells were co-transfected with negative control oligos or miR-183 mimics and with empty pcDNA3.1 vector or pcDNA3.1-MMP9. Forty-eight hours after transfection, the cells were harvested and analyzed using transwell invasion assay, which was described above. Western blotting was also used to signify the expression of MMP9.

Statistical analysis

All data are. All the results were presented as the mean ± SD. Data from different groups were compared with each other by using one-way analysis of variance followed by the Bonferroni post hoc test. Only a P-value < 0.05 was considered statistically significant.

Results

Screening of differentially expressed microRNA in peripheral blood of patients with PE

To identify the specific preeclampsia (PE)-related biomarkers, 6 microRNAs (miRNAs) were selected based on their reported aberrant

Figure 1. Differential expression of microRNA in peripheral blood from patients with PE. A: Quantitative real time PCR to reveal miR-183, miR-21, miR-320, miR-200, miR-145, miR-183 and miR-126 in peripheral blood from patients with PE and Control. U6 gene served as an internal control. The relative gene expression was calculated using 2-ΔΔCt method. Data represent the mean ± SD of three independent experiments, **P < 0.01, compared to the control group. B: miR-183 expression was validated by real-time PCR in 20 peripheral blood samples. The relative expression of miR-183 was normalized to the endogenous control U6. Each sample was analyzed in triplicate. (**P < 0.01).
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expression associated with tumor invasion. qPCR was used to analyze the expression of six miRs (miR-21, miR-320, miR-200, miR-145, miR-183 and miR-126) in peripheral blood from patients with PE. As shown in Figure 1A, the relative quantification of miR183 was elevated in peripheral blood of patients with PE, whereas the expression of miR-21, miR-320, miR-200, miR-145 and miR-126 showed no significant differences between PE and control. Further validation of aberrant miR-183 was determined using qPCR analysis in peripheral blood from 20 patients with PE and 20 healthy women with normal pregnancies. As shown in Figure 1B, the miR-183 level in pregnancies with PE was higher than that in the healthy.

Figure 2. Effect of miR-183 on the invasiveness of trophoblast-like cells. A, B: The expression levels of miR-183 in BeWo and JAR trophoblast-like cells transfected with miR-183 mimics, miR-NC or control, and in cells transfected with miR-183 inhibitor, miR-NC or control were determined with quantitative real-time PCR. The relative expression levels of miR-183 in cells were normalized to U6. C: Overexpression of miR-183 inhibited invasiveness of BeWo and JAR cells. Representative of cell invasion results detected with the matrigel-coated transwell inserts in indicated groups (magnification 200×). Data were expressed in mean ± SD. **P < 0.01 vs. the corresponding NC group. D: Suppressed miR-183 expression promoted invasiveness of BeWo and JAR cells. Representative of cell invasion results detected with the matrigel-coated transwell inserts in indicated groups (magnification 200×). Data were expressed in mean ± SD. **P < 0.01 vs. the corresponding NC group.
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pregnancies. These data strongly suggest that miR-183 may be involved in the pathogenesis of PE.

MiR-183 inhibits the trophoblastic invasion in vitro

To further study the role of miR-183 in the regulation of trophoblast invasion, we examined cell invasion in two trophoblast-like cell lines, BeWo and JAR cells that were transfected with miR-183 mimics or its inhibitor. As shown in Figure 2A and 2B, the relative expression level of miR-183 was significantly increased in miR-183 mimics-transfected cells, whereas decreased in those transfected with the miR-183 inhibitor. Transwell assay demonstrated that overexpression of miR-183 significantly reduced the inva-
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Figure 4. MMP9 overexpression counteracts the inhibitory effect of miR-183 on BeWo cell invasion. A: Representatives of BeWo cell invasion determined with transwell insert assays (magnification 200×). Cells were transfected with miR-183 mimics/mimics-NC and pcDNA3.1-vector/pcDNA3.1-MMP9 as indicated. B: The invasive cells were counted in five random areas in multiple wells. Data were expressed in mean ± SD. **P < 0.01.

In order to identify the potential targets of miR-183, we carried out an unbiased computational screening using TargetScan, and identified MMP9 as a potential target of miR-183 (Figure 3A). To test whether MMP9 is a direct target of miR-183, we cloned wild type (WT) and mutant (MT) 3′-UTR regions of MMP9 into luciferase reporter vectors and were cotransfected into BeWo cells along with miR-183 mimic, miR-183 inhibitor or negative control. Data showed that miR-183 mimic significantly decreased the activity when cotransfected with WT reporter plasmid, compared with control, whereas increased in those transfected with the miR-183 inhibitor. Meanwhile, when transfected with negative control miRNA, there was no significant difference between WT or mutant vector (Figure 3B). The data strongly validated MMP9 as target gene of miR-183 in BeWo cells.

To further confirm the inhibitory regulation of miR-183 on MMP9 expression, we performed Western blot analysis. We found that overexpression of miR-183 significantly reduced the protein levels of MMP9 (Figure 3C), whereas knockdown of miR-183 promoted the levels of MMP9 (Figure 3D). Taken together, these data clearly indicated that MMP9 was direct downstream targets of miR-183 in BeWo cells. These results indicated that miR-183 modulated invasion in trophoblast-like cells.
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MMP9 expression by directly targeting MMP9 mRNA.

Overexpression of MMP9 attenuates the invasion inhibiting effect of miR-183 in BeWo cells

Next, we tried to figure out whether MMP9 directly participated in the invasion-promoting effect of miR-183, we transfected BeWo cells with miR-183 together with a MMP9 overexpressing vector to perform the rescue experiment. It was shown that overexpression of MMP9 could partly block the invasion-promotion of BeWo cell (Figure 4A, 4B, P < 0.01). These results suggested that MMP9 was directly involved in the trophoblast invasion induced by miR-183.

Discussion

In the present study, we report that miR-183 may contribute to the development of preeclampsia by altering trophoblast invasion. First, we found that an increased placental expression of miR-183 was associated with preeclampsia. Then, we found that overexpression of miR-183 suppressed trophoblast-like cell invasion and knock down of miR-183 enhanced trophoblast-like cell invasion. More importantly, MMP9 was identified as a novel direct target of miR-183 in trophoblast cell. Overexpression of MMP9 could reverse the inhibitory effect of miR-183 on the invasion of trophoblast cells. These findings provide a clear starting point for future investigations into the role of miR-183 in the pathogenesis of preeclampsia.

Due to the difficulty involved in obtaining large amounts of freshly isolated trophoblast cells, use of appropriate cell lines enables the investigators to study the trophoblastic invasion in vitro. In this study, two trophoblast-like cell lines, BeWo and JAR cells were used to explore the role of miR-183 in trophoblastic invasion. These two cell lines have been previously used as models to study different aspects of trophoblast physiology and pharmacology [20].

Several reports demonstrated differentially expressed microRNAs in preeclamptic placentas, compared with normal pregnant ones [13, 14, 16]. In a study by Muralimanoharan et al, increased expression of miR-210 was found in preeclampsia, and data revealed that they are related to angiogenesis and trophoblast cell invasion/migration [21]. By far, it remains to be clarified that how the deregulated miRNAs involve in the development of preeclampsia. Here, we found that miR-183 was upregulated in peripheral blood from patients with PE and preeclamptic placentas, suggesting that abnormal expression of miR-183 might contribute to the pathogenesis of preeclampsia.

It is generally accepted that invasiveness descent and invasion failure of the trophoblastic cells maybe result in the recast disturbance of the uterus helicine arteries, which is the most important pathophysiology mechanism of the preeclampsia occurrence [22]. Therefore, we examined the effect of miR-183 on the invasion of trophoblast cells. Our results showed that transfection of miR-183 mimics inhibit the invasion of BeWo and JAR cells, whereas transfection of miR-183 inhibitor enhanced the invasion. These finding indicate that miR-183 could suppress the invasiveness of trophoblast cells.

The molecular mechanism by which miR-183 regulates trophoblast invasion is likely complex because miRNAs can target hundreds of genes. Several evidences in this study prove that MMP9 is a direct functional target of miR-183 in trophoblast cells. First, MMP9 expression could be reduced by transfection of miR-183 mimics in BeWo cells. Second, luciferase assay vector pcDNA3.1 MMP9 was responsive to miR-183. Additionally, overexpression of MMP9 could reverse the invasion inhibiting effect of miR-183 in trophoblast cells. These findings indubitably indicated that MMP9 is one of the critical targets, at least, in mediating the invasion-promoting role of miR-183 in human trophoblast cells.

Matrix metalloproteinases (MMPs) are a group of zinc-dependent endopeptidases and they are involved in the decomposition of extracellular matrix (ECM). Among these MMPs, MMP9 is a proteolytic enzyme that degrades the ECM [23]. Previous studies showed that MMP9 expression was decreased in patients with pre-eclampsia and the decrease was negatively correlated with the severity of the condition [24, 25], suggesting that MMP9 plays an important role in normal placental development. Moreover, MMP9 has been reported to participate in trophoblast cell invasion that is mediated by microRNAs, such as miR-144 and miR-204 [26,
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27]. Here, we reported for the first time that invasion-inhibiting effect of miR-183 is mediated, as least in part, by suppressing MMP9.

In summary, our study provide new evidence that upregulated miR-183 contribute to preeclampsia by inhibiting the invasion of trophoblast cells via directly targeting MMP9. Therefore, miR-183 and MMP9 may be developed to be potential clinical predictive and therapeutic targets for preeclampsia. Given that a single microRNA can target many genes, we believe that miR-183 also has multiple targets. It will greatly help us to better understand the mechanism of miR-183 in human preeclampsia after more functional targets are identified.

Disclosure of conflict of interest

None.

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