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MiR-148a affects the expression of MMP-9 in trophoblast cells by targeting PTEN.

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Abstract

This study aimed to investigate the role of microRNA-148a in the development of placenta and in the pathogenesis of preeclampsia (PE), and to explore the underlying molecular mechanism. The expression level of miR-148a in the placenta of 20 preeclamptic patients was detected by qRT-PCR, and we found miR-148a was significantly decreased in the preeclamptic placentas. To investigate the role of miR-148a in trophoblast cells, miR-148a mimic/inhibitor was used. The results suggested that miR-148a over-expression enhanced JEG-3 cell proliferation, migration and invasion, while miR-148a down-regulation inhibited JEG-3 cell proliferation, migration and invasion. In addition, we revealed that miR-148a directly targets PTEN in JEG-3 cells. Moreover, miR-148a mimic could decrease PTEN expression, thereby enhancing the expression of matrix metalloproteinase-9 (MMP9). While miR-148a inhibitor increased PTEN expression, and decreased MMP-9 expression. Also we found that PTEN-siRNA could reverse the decrease of MMP9 induced by miR-148a inhibitor. Taken together, the results suggested that miR-148a affects MMP-9 expression in trophoblast cells by targeting PTEN, which influences the trophoblast cell invasion ability and participates in the pathogenesis of PE.

Keywords: Preeclampsia (PE), miR-148a, PTEN, Matrix metalloproteinase-9 (MMP-9).

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Introduction

Preeclampsia (PE) is a human pregnancy-specific multi-system involvement of the disease. The main clinical manifestation is in the pregnancy weeks after the emergence of high blood pressure and protein urine retention [1,2]. PE endothelium damage and vascular spasm caused by reduced organ perfusion can cause acute renal failure, cerebral hemorrhage, pulmonary edema, syndromes and other serious complications [3,4]. About 5-8% of all pregnancies were complicated by this pregnancy-related disease and this disease mixed both the morbidity and mortality of maternal and neonatal in the world [5]. Although studies of the etiology and prevention of PE have made some progress, the clinical treatment in addition to conventional antispasmodic, anti-hypertensive, termination of pregnancy has not yet the most effective prevention and treatment. At present, it is generally recognized that the placenta is "shallow bed" that is due to the infiltration of gestational trophoblastic cells caused by superficial uterine spiral small artery recast insufficient [6]. Studies have shown that the occurrence of severe PE was associated with the decrease of trophoblast invasion ability [7].

MicroRNAs (miRNAs), a kind of endogenous small non-coding RNAs (22 nucleotides in length), play important roles in the post-transcriptional regulation of various physiological activities *via* targeting mRNAs for cleavage or translational prevention [8]. MiRNAs can regulate one-third of all mammalian genes expression. A growing number of evidence revealed that miRNAs play an important role in regulating cell growth, proliferation, apoptosis, differentiation, migration and metabolism, etc. [9]. Evidence has indicated that compared with the normal placentas, a number of miRNAs are abnormally expressed in the preeclamptic placentas [10]. Some miRNAs have been found to play critical roles in the regulation of trophoblastic invasion *in vitro* [11]. MiR-148a has been well studied in tumor growth, metastasis, and invasion [12-14]. Studies have suggested that miR-148a inhibits the epithelial-to-mesenchymal transition of the non-small cell lung cancer cells [15,16]. To date, the role of miR-148a in trophoblast biology is still unclear.

In this study, we first detected the expression level of miR-148a in preeclamptic placentas, and the results suggested that miR-148a was decreased in the preeclamptic placentas. Then, the role of miR-148a in trophoblast proliferation,

migration and invasion were determined by up or down-regulating miR-148a in JEG-3 cells. In addition, we identified that miR-148a directly targets PTEN in JEG-3 cells, and verified that preventing of PTEN expression by miR-148a mimics significantly enhanced JEG-3 invasion ability. PTEN was identified as the upstream suppressor of MMP-9 which is facilitated with cell migration [17]. The data suggest that miR-148a may play an important role in regulating trophoblast invasion *via* inhibiting PTEN, and indicate that miR-148a may be participating in the pathological processes of PE.

Materials and Methods

Tissue collection

This study was approved by the Clinical Research Ethics Committee of the Affiliated Hospital of GuiYang Medical University. And all procedures in the present study were strictly applied following the approved protocol. Both the preeclamptic patients (participants placentas) and the healthy controls (healthy placentas) (n=20 each) wrote the inform consent. Fragments from the placental sub-chorial zone were dissected, and floating villi were washed with PBS (pH 7.2) and subjected to RNA extraction.

Real-time PCR

Total RNA was extracted from the placenta tissues and cells by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. PrimeScript RT Master Mix and SYBR PrimeScript MiRNA RT-PCR Kits (Takara Biotechnology, Dalian, China) were used for the reverse transcription of mRNA and miRNAs, respectively. Quantitative real-time PCR was carried out by using SYBR Premix Ex Taq (Tli RNaseH Plus; Takara Biotechnology, Dalian, China) following the manufacturer's instructions. β-actin (mRNA) or U6 (miRNA) was used as an internal control.

Cell culture and transfection

The human trophoblast tumor cell line JEG-3 was obtained from the Cell Center of Shanghai Institute of Life Science, Chinese Academy of Science. JEG-3 cells were grown in DMEM-F12 medium (Hyclone, Logan, USA) containing 10% foetal bovine serum (Gibco, Carlsbad, CA, USA) in 5% CO_2 at 37°C, and cells were transmitted every 2-3 d.

For cell transfection, JEG-3 cells were seeded into a 6-well plate the day before transfection, then the cells were transfected with miR-148a mimic, miR-148a inhibitor or miR-148a inhibitor+PTEN-siRNA using Lipofectamine2000 reagent (Invitrogen, Carlsbad, CA, USA). Fresh cell culture medium was replaced 4 h after the transfection and the transfected cells were collected for following analysis 24 h after transfection.

Cell proliferation

JEG-3 cell proliferation ability was assessed by using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assays kit (SigmaAldrich, St. Louis, MO, USA) according to the manufacturer's instructions. For cell growth detection, ~5,000 cells were seeded into 96 well plates. 20 μl MTT (5 mg/ml; SigmaAldrich) was added to every well at 0, 24, 48 and 72 h after cell transfection and then incubated for 4 h. Subsequently, the MTT medium was replaced by 150 μl dimethyl sulfoxide. At the end of the test, the optical density (OD) at 490 nm was analyzed using an Enzyme Immunoassay Instrument (Model 680 microplate reader; BioRad Laboratories, Inc., Hercules, CA, USA).

Cell invasion

The influence of miR-148a on JEG-3 cell invasion was determined by using a 24-well transwell plate (8 mm pore size, Corning, New York, USA). For cell invasion ability detection, chamber inserts were coated with matrigel. JEG-3 cells transfected with miR-148a mimics, miR-148a inhibitor or miR-148a inhibitor+PTEN-siRNA were collected and then re-suspended in DMEM medium containing 10% FBS. The cell suspension was plated into the upper chamber and DMEM medium supplemented with 20% FBS was added to the lower chamber. After incubation for 48 h at 37°C, the cells on the upper chambers were removed. Cells on the lower chambers, which indicating the invading cells, were then fixed with 100% methanol for 15 min and stained with hematoxylin-eosin solution. Finally, the invading cells were counted under a microscope (Olympus) and then analyzed. All experiments were performed in triplicate.

Luciferase reporter assay

To confirm miR-148a directly targets the 3′-UTR of PTEN, the vectors named PTEN-3′UTR-WT and PTEN-3′UTR-MUT with wild-type and mutated 3′UTR of PTEN mRNA were conducted. For luciferase reporter assay, JEG-3 cells were plated into a 24-well plate, and then co-transfected with PTEN-3′UTR-WT or PTEN-3′UTR-MUT and miR-148a mimic or miR-148a mimic control by using Lipofectamine2000 (Invitrogen). The luciferase activity was determined by performing dual luciferase assay.

Western blotting

JEG-3 cells were transfected with miR-148a mimics, miR-148a inhibitor or miR-148a inhibitor+PTEN-siRNA for 24 h, then total cell proteins were extracted by using RIPA lysis buffer (Thermo Scientific), and bicinchoninic acid (BCA) assay (Thermo Scientific) was carried out for protein concentration determination. Protein samples were resolved on 12% SDS-PAGE gels, and then transferred onto PVDF membranes (Millipore, Bedford, MA). Membranes were incubated overnight at 4°C with primary antibodies PTEN, MMP-9, β-actin (All buy from Cell Signaling Technology) and then incubated with a horseradish peroxidase (HRP) conjugated secondary antibody. Enhanced chemiluminescence detection system was applied to observe the immunoreactive bands. The gray values of the stripes were captured using Chemilmager

5500 software which was performed to capture the gray values of the bands.

Statistical analysis

SPSS17.0 software was applied to analyze the data. Values are expressed as mean ± SD. Data were analyzed by one-way ANOVA or Student's t-test. Statistical significance was defined as $p<0.05$.

Results

The expression of miR-148a decreased in PE placenta

To detect the level of miR-148a in PE, qRT-PCR was performed. As shown in Figure 1, the expression level of miR-148a in the PE placentas was significantly lower than that in the healthy placentas. The results indicated that miR-148a may be involved in the pathogenesis of PE.

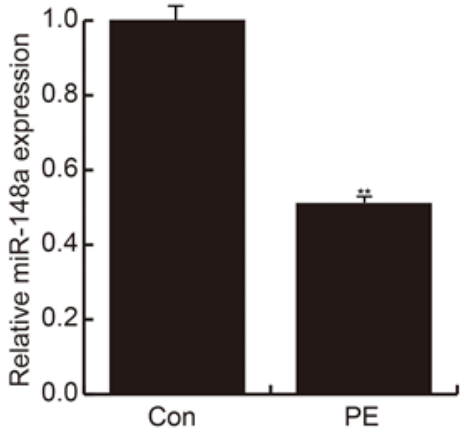

*Figure 1. Relative expression of miR-148a in PE patients. The relative expression of miR-148a in PE patients (PE) and the healthy control patients (Con) was determined by qRT-PCR. **P<0.01 vs. Con.*

Over-expression of miR-148a elevated proliferation of JEG-3 cells

To determine the role of miR-148a in PE process, human trophoblast cell line JEG-3 was transfected with miR-148a mimics, miR-148a inhibitor or their negative control vectors (NC), and miR-148a mimic significantly increased the level of miR-148a, and miR-148a expression significantly decreased in miR-148a inhibitor group (Figure 2). Over-expression of miR-148a in JEG-3 cells significantly elevated cell proliferation rate, and inhibition of miR-148a significantly reduced cell proliferation rate compared with the NC group (Figure 3). Indicating that miR-148a can regulate JEG-3 cell proliferation.

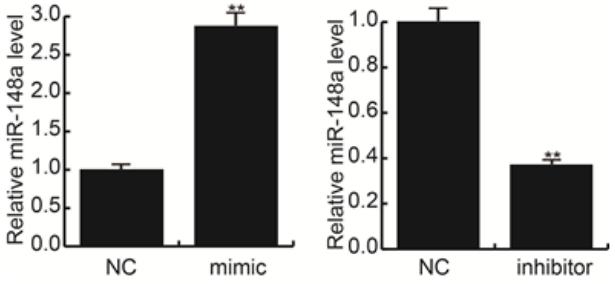

*Figure 2. Relative expression of miR-148a in JEG-3 cells. Human trophoblast cell line JEG-3 was transfected with miR-148a mimics (mimic), miR-148a inhibitor (inhibitor) or their negative control vectors (NC), and 24 h after transfection, the level of miR-148a was determined by qRT-PCR. **P<0.01 vs. NC.*

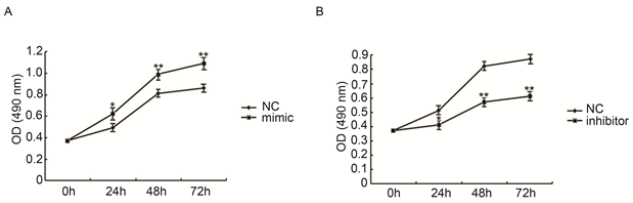

*Figure 3. Effects of miR-148a on JEG-3 cell viability. 0, 24, 48, 72 h after cell transfection, JEG-3 cell viability was determined by MTT assay. NC: JEG-3 cells were transfected with the negative control vectors; mimic: JEG-3 cells were transfected with miR-148a mimics; inhibitor: JEG-3 cells were transfected with miR-148a inhibitors. Data are presented as the mean ± SD. *, **P<0.05, 0.01 vs. NC.*

MiR-148a enhance the invasive ability of JEG-3 cells

To investigate the metastatic effect of miR-148a on JEG-3 cells, invasion assay was performed. The results showed that the invasive ability of JEG-3 cells were notably enhanced in response to miR-148a over-expression (Figure 4). Meanwhile decrease of miR-148a significantly suppressed cell invasion compared with the NC group.

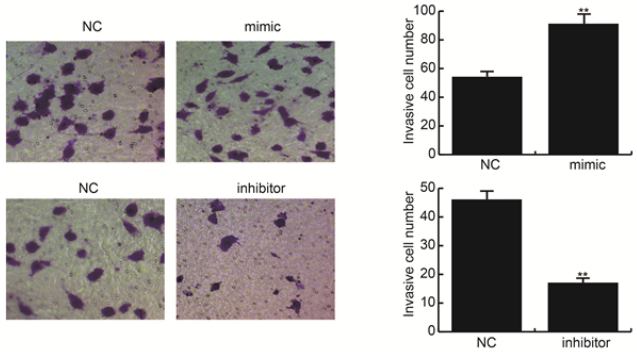

*Figure 4. Effects of miR-148a on JEG-3 cell invasion. 24 h after cell transfection, JEG-3 cell invasion was determined by cellular transwell invasion assay. NC: JEG-3 cells were transfected with the negative control vectors; mimic: JEG-3 cells were transfected with miR-148a mimics; inhibitor: JEG-3 cells were transfected with miR-148a inhibitors. Data are presented as the mean ± SD. **P<0.01 vs. NC.*

PTEN is a direct target of miR-148a in JEG-3 cells

To investigate the underlying molecular mechanism for miR-148a in the invasiveness of JEG-3 cells, TargetScan was used to predict the targets of miR-148a, and we found PTEN is a potential target of miR-148a (Figure 5A). Furthermore, dual luciferase reporter assay revealed the direct binding of miR-148a on the 3'-untranslated region (UTR) of PTEN mRNA (Figure 5B). The result confirmed miR-148a direct target PTEN. As a tumor inhibitor gene, *PTEN* has lipid phosphatase activity, dual specific phosphatase activity, and protein phosphatase activity. Protein phosphatase function can affect cell proliferation, adhesion and cell migration, whether PTEN protein was also involved in the invasion of trophoblast cells is still unclear [18]. Thus, we determined whether miR-148a mediated trophoblast-like cell invasion by regulating PTEN expression. Our results indicated that both mRNA and protein levels of PTEN were markedly decreased in JEG-3 cells transfected with miR-148a mimics and the PTEN expression was enhanced by miR-148a inhibitor (Figures 5C-5G). These results demonstrated that *PTEN* is a target gene of miR-148a in human trophoblast-like cells.

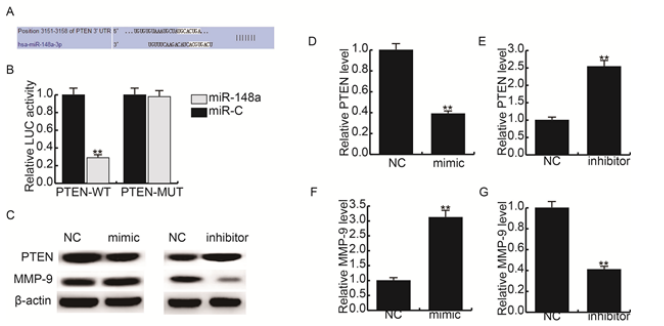

Figure 5. *PTEN is a direct target of miR-148a. (A) http://www.microrna.Org/microrna/home.do was applied to predict the interaction between miR-148a and 3'UTR of PTEN; (B) Luciferase activity was detected by Dual luciferase assay. Here, "MUT" indicates the PTEN 3' UTR with a mutation in the miR-148a binding site. UTR, untranslated region. All data are presented as the mean ± SD. $^{**}P<0.01$ vs. control; (C) Effect of miR-148a on PTEN and MMP-9 protein expression in JEG-3 cells was determined by Western blotting; (D-G) Effect of miR-148a on PTEN/MMP-9 mRNA expression in JEG-3 cells was determined qRT-PCR. NC: JEG-3 cells were transfected with the negative control vectors; mimic: JEG-3 cells were transfected with miR-148a mimics; inhibitor: JEG-3 cells were transfected with miR-148a inhibitors. Data are presented as the mean ± SD. $^{**}P<0.01$ vs. NC.*

PTEN regulate JEG-3 cell invasion through MMP9

To further explore the underlying molecular mechanism of miR-148a in the regulation of cell invasion in JEG-3 cells, MMP9, a matrix degrading enzyme that is regarded as a critical regulator in tumor invasion, was determined [19]. JEG-3 cells were transfected with miR-148a mimic or miR-148a inhibitor and then Q-PCR and Western blot analysis were used to detect the expression of MMP9. The results showed that MMP9 increased when over-expression of miR-148a, whereas down-regulation of miR-148a could decrease the expression of MMP9 (Figures 5C and 5F). We have found that PTEN is a direct target of miR-148a, so we want to know the association between PTEN and MMP9. JEG-3 cells were further co-transfected with miR-148a inhibitor and PTEN-siRNA. As shown in Figure 6, the level of MMP9 protein was markedly reduced in the cells transfected with miR-148a inhibitor, and the level have not changed when co-transfected miR-148a inhibitor with PTEN-siRNA. This indicates that PTEN inhibit cell invasion through regulating MMP9. Moreover, *PTEN* is a direct target gene for miR-148a, miR-148a inhibitor can increase the expression of PTEN and decrease the expression of MMP9, PTEN-siRNA could reverse the decrease of MMP9 induced by miR-148a inhibitor. In summary, the above results suggest that miR-148a affects the expression of MMP-9 in trophoblast cells by targeting PTEN, which affects the invasive ability of trophoblast cells and participates in the pathogenesis of PE.

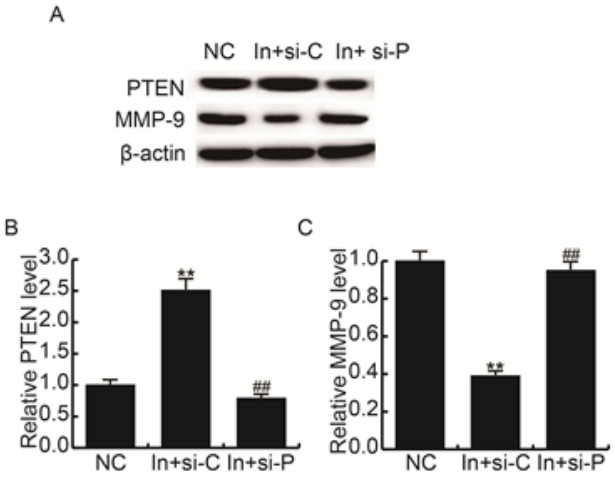

Figure 6. *Effects of PTEN-siRNA on PTEN and MMP-9 expression. (A) The protein; (B-C) mRNA expression level of PTEN and MMP-9 in different groups was determined by western blot analysis and qRT-PCR respectively. NC: Negative Control group; In+si-C: JEG-3 cells co-transfected with miR-148a inhibitor and Control-siRNA; In+si-P: JEG-3 cells co-transfected with miR-148a inhibitor and PTEN-siRNA. Data are presented as the mean ± SD. $^{**}P<0.01$ vs. NC; $^{\#\#}P<0.01$ vs. In+si-C.*

Discussion

PE is a specific complication of pregnancy, and its incidence of China is about 9%, is also the main cause of premature birth [20]. Decreased trophoblastic invasion during pregnancy can lead to uterine spiral arterial physiological recoating process obstacles, thus causing trophoblastic ischemia hypoxia and diseases [21]. MiRNAs have been reported to play critical roles in the development of many organs, including the placenta, by regulating massive genes and involving in a variety of biological processes. In this study, we detected the expression of miR-148a in 20 PE patients and 20 healthy control subjects. The results showed that the expression of miR-148a in PE placentas was significantly lower compared to the healthy placentas. So we further investigated the function of miR-148a in the pathogenesis of PE.

The trophoblastic invasion was investigated *in vitro* by using JEG-3 cells in the present study. JEG-3 cells, which were derived from human choriocarcinomas and have extravillous trophoblast phenotypes, are able to secrete human chorionic gonadotropin, estrogen, and placental lactogen [22]. In this study, we found that miR-148a mimic significantly enhanced the invasive capabilities of JEG-3 cells, while miR-148a inhibitor suppressed JEG-3 cell invasion ability. These results suggesting the critical roles of miR-148a in regulating trophoblast-like cell invasion suggested that down-regulation of miR-148a might contribute to the development of PE.

To investigate the molecular mechanisms underlying the impaired trophoblast invasion, we predicted the target genes of miR-148a. And we found PTEN is a direct target of miR-148a. Although studies have found that PTEN can inhibit the invasion of tumor cells, its function on trophoblast invasion is still unclear [23,24]. Therefore we detected the expression level of PTEN after transfected with miR-148a mimic or miR-148a inhibitor in JEG-3 cells. The results showed that miR-148a could down-regulate PTEN, thereby proving trophoblast cell invasion.

MMPs are major proteolytic enzymes required for tumor invasion and angiogenesis. Among MMPs, MMP9 secretion is observed in different types of cancer, and its production is regulated by extracellular stimuli, such as growth factors and cytokines [25]. In the current study, it was observed that MMP-9 plays an important role in the process of trophoblastic invasion and is involved in the reconstruction of the uterine spiral artery and placental formation. MMP-9 expression is regulated by the upstream regulator PDCD4 and PTEN [26]. So we detected the expression of MMP-9 by q-PCR and Western blotting, and the results found that miR-148a mimic can decrease the expression of PTEN and increase the expression of MMP9, whereas miR-148a inhibitor can promote the expression of PTEN and decrease the expression of MMP9. Further, we co-transfected PTEN-siRNA and miR-148a inhibitor into JEG-3 cells, and we found that PTEN-siRNA was able to reverse the increase in PTEN and the decrease in MMP9 caused by miR-148a inhibitor. These findings supported that PTEN is an essential target of miR-148a, and is participated in the invasion of trophoblast cells through regulating MMP9.

Taken together, our study provides new evidence that inhibition of miR-148a contribute to PE by preventing the invasion of trophoblast cells *via* directly targeting PTEN. Therefore, miR-148a and PTEN may be developed to be potential clinical predictive and therapeutic targets for PE treatment.

Disclosure of Conflict of Interest

None.

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