MiRNA-203a-3p inhibits inflammatory response in preeclampsia through regulating IL24

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Abstract. – OBJECTIVE: This study aims to investigate the protective role of miRNA-203a-3p in preeclampsia (PE) patients via inhibiting the inflammatory key protein IL24.

PATIENTS AND METHODS: Serum samples of 36 PE pregnant women and 30 normal pregnant volunteers hospitalized between 2015 and 2019 were collected to extract placental mononuclear cells and exosomes. Relative levels of microRNA-203a-3p and IL24 were examined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). In addition, the interaction between microRNA-203a-3p and IL24 was analyzed through bioinformatics analysis and Luciferase reporting assay. Finally, the underlying molecular mechanisms were further explored via immunofluorescence and Western blotting.

RESULTS: Compared with normal pregnant volunteers, microRNA-203a-3p expression in serum exosomes and placental mononuclear cells of PE patients were dramatically reduced, while IL24 was conversely up-regulated, indicating a negative correlation between microRNA-203a-3p and IL24 levels. In addition, IL24, which was down-regulated in mononuclear macrophages overexpressing microRNA-203a-3p, was indicated as a target of microRNA-203a-3p. At the same time, microRNA-203a-3p was able to suppress the proliferation capacity of LPS-stimulated mononuclear macrophages, and it exerted anti-inflammatory effects via down-regulating IL24 in THP-1 cells.

CONCLUSIONS: MicroRNA-203a-3p plays an anti-inflammatory role in PE pregnant women by down-regulating IL24 level.

Key Words: MicroRNA-203a-3p, IL24, PE, Inflammatory response.

Introduction

Preeclampsia (PE) is a common complication in pregnancy with a prevalence of 4%-7%, and severe cases may even endanger the health of pregnant women and the life of perinatal infants. The clinical manifestations of PE include persistent hypertension, proteinuria, and edema after 20 weeks of gestation. Without active treatment, PE may suddenly develop into eclampsia after varying periods of time, which endangers the lives of both mothers and infants. Eclampsia is one of the main causes of maternal death, and the maternal mortality rate in China is as high as 2%-5%. PE is considered as a complex disease caused by multiple factors, among which, changes in vascular development, placental growth, immune regulatory mechanism, and oxidative stress regulation are well-known risk factors of PE. In addition, placental factors are particularly crucial because the symptoms of pre-eclampsia can be rapidly deteriorated after removal of the placenta. However, there is still a lack of effective approach to predict the onset of PE. Therefore, it is necessary to seek abnormally expressed miRNAs in placenta and placental growth factors, which are beneficial to early detection, diagnosis, and treatment of PE.

MiRNA is a kind of non-coding, small molecule RNA, which plays a pivotal role in the field of gene expression regulation. A variety of miRNAs can be specifically expressed in human placental tissues, and their unique gene regulation functions may be significantly correlated with the incidence of PE. Currently, PE-related miRNAs are rarely reported. Therefore, it is necessary to explore the involvement of PE-related miRNAs in the occurrence and development of PE.

Interleukin-24 (IL24) is a new member of the cytokine interleukin-10 family. As it was initial-
ly found in human melanoma, it is also known as the melanoma-differentiation related factor-7 (mad-7) gene. IL24 can be secreted by a variety of non-immune cells and are involved in not only melanoma differentiation but also inflammatory diseases such as rheumatoid arthritis and psoriasis. In this paper, expression changes of miR-203a-3p and IL24 in placental tissue and serum exosomes of PE patients were examined. Moreover, the possible mechanisms of miR-203a-3p affecting the pathogenesis and progression of PE were analyzed, and thus provided a theoretical basis for further understanding the molecular mechanism of gestational hypertension and the potential diagnostic value of miRNA in PE.

**Patients and Methods**

**Patients and Controls**

From 2015 to 2019, 36 PE pregnant women were randomly selected from the hospital, and control group was composed of 30 normal pregnant volunteers enrolled in the hospital during the same period. Placental monocytes and serum exosomes were obtained from these subjects. Exosomes isolation kit (Invitrogen, Carlsbad, CA, USA) was used to extract serum exosomes. The study was approved by the Ethics Committee of the hospital and all patients signed informed consent. This research was conducted in accordance with the Declaration of Helsinki.

**Cell Lines and Reagents**

Mononuclear macrophages (THP-1) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA); Roswell Park Memorial Institute-1640 (RPMI-1640) medium and fetal bovine serum (FBS) were purchased from Life Technologies (Gaithersburg, MD, USA). Cells were cultured in a 37°C, 5% CO₂ incubator with RPMI-1640 medium containing 10% FBS.

**Transfection**

Negative control (NC) and microRNA-203a-3p mimic were purchased from GenePharma (Shanghai, China). Cells were plated in 6-well plates and grown to a cell density of 30%-40%, and then transfection was performed according to the manufacturer’s instructions. After 48 h, cells were collected for qRT-PCR and cell function experiments.

**Extraction of Serum Exosomes**

Exosomes were obtained by a series of centrifugation steps and combined with polyethylene glycol (PEG) precipitation. First, 200 µl of serum were centrifuged at 3,000 × g for 20 min at 4°C, after which the supernatant was passed through a 0.22-µm filter and incubated with 8% PeG 6000 overnight. Following further centrifugation at 10,000 × g for 1 h at 4°C, the 8% PeG 6000-serum mixture precipitate was resuspended in 0.25 m sucrose. Subsequently, the suspension was layered onto a linear sucrose gradient and ultracentrifuged at 100,000 × g for 5 h at 4°C. These fractions were incubated with 8% PeG 6000 overnight and then centrifuged at 10,000 × g for 1 h at 4°C. Ultimately, these exosomes were suspended in 200 µl of PBS. An equal volume of sample from each fraction was used for Western blotting.

**Cell Counting Kit-8 (CCK-8) Assay**

After 48 h of transfection, cells were collected and plated into 96-well plates at 2000 cells per well. After culturing for 24 h, 48 h, 72 h, and 96 h respectively, 10 µL of CCK-8 solution (Dojin-do Molecular Technologies, Kumamoto, Japan) was added per well for 2-h incubation. Optical density (OD) value of each well was measured in the microplate reader at 490 nm absorption wavelength.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

After homogenization of THP-1 cells in each treatment group, the levels of TNF-α, IL-1β, IL-6, and IL-10 were determined using an ELISA kit according to the manufacturer’s instructions (LifeSpan BioSciences, Inc., Seattle, WA, USA). Cytokine concentrations in each treatment group were quantitatively determined with reference to a standard curve.

**QRT-PCR**

Total RNA was extracted from placental monocytes using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using Primescript RT Reagent. QRT-PCR reactions were performed using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan), and StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The following primers were used for qRT-PCR reaction: microRNA-203a-3p: forward: 5′-CCG-
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GTGAAATGTTTAGGACCCTAG-3', reverse: 5'-GCCCCGTAATGTTTAGG-3'; U6: forward: 5'-CTCGCTTCGGGCAGCACA-3'; reverse: 5'-AACGCTTCACGAATTTGCGT-3'; IL24: forward: 5'-TAGGTATTGTCTACTACTCTG-3', reverse: 5'-TATATACCTCTTGTCTTCA-3'; β-actin: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-TGCCGTAGGTGTCCCTTTG-3'. Data analysis was performed using ABI Step One software and mRNA levels were calculated using the $2^{-ΔΔCt}$ method.

Western Blot

The transfected cells were lysed using cell lysis buffer, shaken on ice for 30 min, and centrifuged at 14,000 × g for 15 min at 4°C. Total protein concentration was calculated by bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA). The extracted proteins were separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. Western blot analysis was performed according to standard procedures. The primary antibodies against IL24 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the secondary antibody were all purchased from Cell Signaling Technology (Danvers, MA, USA).

Immunofluorescence

After lipopolysaccharide (LPS; 1 μg/mL) treatment for 2 h, the fluorescence intensity of IL24 THP-1 mononuclear macrophages was evaluated by confocal laser scanning microscopy.

Dual-Luciferase Reporter Assay

HEK293T cells were seeded in 24-well plates and co-transfected with microRNA-203a-3p mimic/NC and pMIR Luciferase reporter plasmids. The plasmid was then introduced into the cells using Lipofectamine 2000. After 48 h of transfection, the reporter Luciferase activity was normalized to control firefly Luciferase activity using a Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

Statistical analysis was processed using the Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) and the data were expressed as mean ± standard deviation. $p<0.05$ was considered to be statistically significant. The continuity variable was analyzed by $t$-test, and the categorical variable was analyzed by $\chi^2$-test or Fisher’s exact probability method.

Results

Expression Levels of MicroRNA-203a-3p and IL24 in PE Patients

Compared to normal pregnant volunteers, lower microRNA-203a-3p expression and higher IL24 level were observed in both serum exosomes and placental monocytes of PE pregnant women (Figure 1A-1E). In addition, we found in placental monocytes extracted from PE pregnant women, microRNA-203a-3p level was negatively correlated with that of IL24 (Figure 1F). The above results suggested that microRNA-203a-3p may be engaged in the occurrence of PE by modulating IL24.

MicroRNA-203a-3p Promotes the Proliferation of Mononuclear Macrophages and Inhibits the Inflammatory Response

MicroRNA-203a-3p mimic was constructed and its transfection efficiency was verified by qRT-PCR (Figure 2A). Subsequently, CCK-8 detected an enhanced proliferation of mononuclear macrophages induced by overexpression of microRNA-203a-3p (Figure 2B). At the same time, ELISA assay indicated that overexpression of microRNA-203a-3p could down-regulate relative levels of TNF-α, IL-1β, IL-6, and IL-10 in culture medium of mononuclear macrophages (Figure 2C-2F).

IL24 Is the Target Gene of MicroRNA-203a-3p

We considered IL24 as a potential candidate gene for microRNA-203a-3p through bioinformatics (databases of Targetscan, Microrna.org, and miRDB). IL24 is one of the most important genes in PE, and microRNA-203a-3p shares complementary seed sequence in the 3'-UTR of IL24 (Figure 3A). Currently, in our study, Dual-Luciferase reporter assay revealed that IL24 was a direct target of microRNA-203a-3p (Figure 3B). In addition, IL24 expression was dramatically down-regulated in THP-1 mononuclear macrophages after overexpression of microRNA-203a-3p, as measured by Western blotting and qPCR (Figure 3C-3D).
MicroRNA-203a-3p Promotes Proliferation of Mononuclear Macrophages and Inhibits Inflammatory Cell Infiltration by IL24

First, LPS-induced monocyte macrophage model was constructed, followed by detection of transfection efficiency in THP-1 mononuclear macrophages overexpressing microRNA-203a-3p (Figure 4A). Western blotting and qRT-PCR were performed to examine IL24 level in each treatment group (Figure 4B). In addition, CCK-8 assay revealed that LPS-induced cell proliferation was dramatically reduced, whereas overexpression of microRNA-203a-3p achieved the opposite result (Figure 4C). Subsequently, immunofluorescence assay indicated that overexpression of microR-
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NA-203a-3p enhanced the anti-inflammatory capacity of mononuclear macrophages via inhibiting IL24 expression (Figure 4D). Finally, ELISA result revealed that relative levels of TNF-α, IL-1β, IL-6, and IL-10 in LPS-induced mononuclear macrophages were elevated dramatically, while overexpression of microRNA-203a-3p reduced their levels (Figure 4E-4H).

Discussion

It is currently believed that placental superficial implantation or defective placenta contributes to PE6-9. Relevant factors such as coagulation, angiogenesis, placental development, immune regulation, regulation of lipid metabolism, and regulation of oxidative stress are markedly
associated with the pathogenesis of PE, especially the factors of placenta\textsuperscript{11-13}. Therefore, among the many causes of PE, the abnormalities of placental dysfunction and insufficient revascularization induced by multifactorial trophoblastic dysfunction may be the main reasons\textsuperscript{13,14}.

MiRNAs are specifically expressed in the body fluids of pregnant women with PE, and effective methods for extracting miRNAs and assessing their levels have been found\textsuperscript{14,15}. We also demonstrated that miRNA level in body fluids reflects the physiological conditions of PE pregnant women. It is even possible to detect pregnancy-induced hypertension by assessing the expressions of specific miRNAs. In addition, although the functions of miRNAs specifically expressed in the placenta are not clear, these miRNAs play a pivotal role in promoting the development of placental morphology and functional characteristics in PE pregnant women\textsuperscript{13-15}. MiRNAs can serve as biomarkers that are easy to detect for the diagnosis of pregnancy complications and other emergency disease, especially for PE\textsuperscript{14}, which is a hypertensive disorder complicating pregnancy and a serious gestational comorbidity worldwide. MiRNAs and their biosynthesis-related genes play a role in the occurrence and progression of various diseases\textsuperscript{16-19}. However, expression and function of microRNA-203a-3p in PE are rarely reported. In this study, microRNA-203a-3p was markedly reduced in serum exosomes and placental mononuclear cells in PE pregnant women compared with normal pregnant volunteers, which therefore can be used as a specific indicator for the diagnosis of pre-eclampsia.

Most of the discovered miRNAs are expressed in a time-specific and tissue-specific manner. Genes that produce miRNAs are not randomly distributed, and co-expressed miRNAs from the same precursor RNA are clustered\textsuperscript{16,17}. Mature miRNA recognizes and completely or incompletely complements the target mRNA sequences through 5-8 nucleotides at its 5' end. Later, miRNA induces degradation or translational inhibition of target mRNA, and thus regulates the expression of the target gene\textsuperscript{24}. Through the above-mentioned mechanism, miRNA plays a pivotal role in gene regulatory networks and biological processes such as cell proliferation, apoptosis, differentiation, and tumorigenesis\textsuperscript{18,19}.

Figure 3. IL24 is the target gene for miR-203a-3p. A, Schematic representation of miR-203a-3p sharing the same complementary seed sequence in the 3'-UTR of IL24. B, Luciferase reporter assay detected that IL24 is the target gene of miR-203a-3p. C, Western blotting detected the expression of IL24 in mononuclear macrophages after overexpression of miR-203a-3p. D, QRT-PCR was used to detect the expression level of IL24 in mononuclear macrophages after overexpression of miR-203a-3p. The data were expressed as mean ± SD, and *indicates a significant difference compared with the NC group ($p<0.05$).
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In this research, bioinformatics analysis predicted that microRNA-203a-3p can specifically bind to IL24, which was then validated by Dual-Luciferase reporter gene assay. Activation of IL24 can be initiated by a variety of extracellular factors, including growth factors, cytokines, and stress stimuli (ultraviolet light, high osmolality, ischemia-reperfusion injury). This inflammatory factor is widely involved in many biological reactions such as cell apoptosis, proliferation, metabolism, and DNA damage repair. Moreover, dysfunctional IL24 can cause neurodegenerative diseases, ischemia-reperfusion injury, diabetes, and tumors. Here, overexpression of microRNA-203a-3p down-regulated the expression of IL24. In addition, by constructing LPS-induced inflammation cell model, we found that overexpression of miR-203a-3p promoted proliferation and activation of placental macrophages via the IL24 signaling pathway, thereby attenuating the inflammatory response.
the inflammatory response. Therefore, microRNA-203a-3p may be an anti-inflammatory factor and a promising target for the diagnosis and treatment of PE.

Conclusions

This study reveals for the first time that in PE pregnancies, microRNA-203a-3p may play an anti-inflammatory role by down-regulating the expression of IL24. Therefore, microRNA-203a-3p may act as a promising marker for the diagnosis and treatment of PE.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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