Low expression of microRNA-21 inhibits trophoblast cell infiltration through targeting PTEN


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Abstract. – OBJECTIVE: We investigate whether microRNA-21 could increase the infiltration ability of trophoblast cells via regulating PTEN expression, thus participating in the occurrence and development of preeclampsia.

PATIENTS AND METHODS: MicroRNA-21 expression in the placenta tissues of preeclampsia women and normal pregnant women was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The effects of microRNA-21 on cell proliferation and infiltration were examined by cell counting kit-8 (CCK-8) and transwell assay, respectively. The dual-luciferase reporter gene assay was used to determine the binding relationship between microRNA-21 and PTEN. Western blot was performed to detect PTEN and microRNA-21 in trophoblasts.

RESULTS: QRT-PCR results showed that the microRNA-21 expression was significantly lower in the placenta of the preeclampsia women than those of normal pregnant women. Overexpression of microRNA-21 in HTR-8/SVneo cells had no effect on cell proliferation, but enhanced cell infiltration ability. Inhibition of microRNA-21 in trophoblasts showed the opposite effects. The results of luciferase activity assay and Western blot showed that microRNA-21 could target PTEN and downregulate its expression. Overexpression of PTEN in HTR-8/SVneo cells partially reversed the enhanced invasive ability induced by microRNA-21 overexpression.

CONCLUSIONS: Low expression of microRNA-21 attenuated cell infiltration of trophoblasts via direct regulating PTEN expression.

Key Words: MicroRNA-21, Trophoblast cells, Preeclampsia, Cell infiltration, PTEN.

Introduction

Preeclampsia is an important cause of morbidity and mortality in pregnant women and fetal death1. Although abnormalities of placenta development are considered to be greatly involved in preeclampsia development, the specific pathogenesis still remains unclear2. Defective trophoblast cell function, such as decreased cell proliferation, excessive apoptosis, abnormal differentiation, limitation of migration and uterine infiltration, and inadequate vascular spiral artery remodeling are closely related to the onset of preeclampsia3-6. The occurrence of preeclampsia and the specific molecular mechanisms of its progression are required for the further exploration.

MicroRNAs are small non-coding RNAs with 18-24 bp in length. Specific recognition and binding to the 3’untranslated region (3’UTR) of the target mRNA lead to miRNA degradation or inhibited translation of the target mRNA at post-transcriptional level. MiRNAs play an important role in cell growth, development, aging and other life processes7-9. MicroRNA-21 is overexpressed in non-small cell lung cancer, laryngeal squamous cell carcinoma, glioblastoma, gastric cancer, ovarian cancer, osteosarcoma, and chronic lymphocytic leukemia10-14. As one of the most commonly overexpressed miRNAs in solid tumors, microRNA-21 may serve as an oncogene in tumor development. Previous studies15 have reported that the expression of microRNA-21 in the placenta of preeclampsia women is significantly lower than that of normal pregnant women. Its specific mechanism remains to be fully studied.

PTEN is an important tumor-suppressor gene with a mutation/deletion rate similar to that of p5316. PTEN-encoded protein exhibits dual-specific phosphatase activities in the cytoplasm, manifesting as protein tyrosine phosphatase activity and lipid phosphatase activity. PTEN regulates multiple intracellular and nuclear signal transduction pathways, thus participating in the proliferation, apoptosis and drug resistance of tumor

PTEN has become one of the most important tumor-suppressor genes in human tumors. Previous studies have shown that PTEN mainly inhibited cell proliferation and induced apoptosis by interfering with PKB/AKT pathway in hematological malignancies.

We aim to elucidate the effect of microRNA-21 on the infiltration of the trophoblast cell lines. We also aim to verify whether microRNA-21 could exert its biological function through target regulation of PTEN expression.

Patients and Methods

Patients
Placental samples were obtained from pregnant women who were undergoing Antenatal Care in Obstetrics and Gynecology at The Third Hospital of Ji’nan from July 2013 to September 2017. During the caesarean section, clinical specimens were obtained from the fetus near the umbilical cord. A total of 28 patients with severe pre-eclampsia and 34 age-matched normal pregnant women were selected. All specimens were stored in liquid nitrogen. All subjects voluntarily participated in the study and signed written informed consent. This study has been approved by the Hospital Ethics Committee.

Cell Culture
Human trophoblast cell line HTR-8/SVneo was purchased from ATCC (American Type Culture Collection) (Manassas, VA, USA). The cells were cultured in RPMI-1640 (Roswell Park Memorial Institute-1640) medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and 1% penicillin + streptomycin and incubated in a 37°C, 5% CO2 incubator. The medium was changed every 2 days.

Transfection
HTR-8/SVneo cells in logarithmic growth phase were selected and seeded. Transfection was performed according to Lipofectamine 2000 instructions (Invitrogen, Carlsbad, CA, USA). Cells were transfected with microRNA-21 mimics, microRNA-21 inhibitor, pcDNA-PTEN or corresponding negative controls, respectively. All transfection reagents were designed and synthesized by GenePharma (Shanghai, China). After 48 hours of transfection, cells were collected for further assays.

RNA Extraction
Appropriate amount of cells and tissues were collected and mixed with 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) and 250 μL of chloroform. After gentle shaking for 30 s and centrifuged at 4°C, the aqueous phase was aspirated and an equal volume of pre-cooled isopropanol was added. After centrifugation, the precipitate was gently washed with 75% ethanol. The precipitate was then dissolved in 20 μL of DEPC (diethyl pyrocarbonate) water (Beyotime, Shanghai, China). The RNA concentration was measured using a spectrophotometer and placed in a refrigerator at -80°C.

QRT-PCR (Quantitative Real-Time Polymerase Chain Reaction)
A reverse transcription reaction system was prepared on ice and the reaction was completed according to the instruction of the PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). The miRNA quantitative PCR procedure was performed according to the miScript SYBR Green PCR Kit (TaKaRa, Otsu, Shiga, Japan) instructions with a total reaction system of 10 μL. PCR amplification conditions were as follows: pre-denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 90 s. The primer sequences used in this study were as follows: microRNA-21 (F: 5’-GTGATCTAGTGCAGGGTCCGAGGTATTCGCACTGGGATACGACTCAACA-3’, R: 5’-CGGCGGTTTCGACGACTTATCAGACTGATGT-3’), PTEN (F: 5’- TGGATTCGACTTAGACTGATGT-3’, R: 5’-GGTGGGTTATGGTCTTCAAAAGG-3’), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (F: 5’-AGCCACATCGCTCAGACAC-3’, R: 5’-GCCCAATACGACCAAATCC-3’), Cell Counting Kit-8 (CCK-8) Assay
Transfected HTR-8/SVneo cells were seeded in 96-well plates (1×104/well) with approximately 100 μL of cell suspension per well. 5 replicate wells were set for each group. A total of 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) were added at 0 h, 24 h, 48 h, and 72 h after incubation at 37°C and 5% CO2, respectively. After incubation for another 2 h under the above conditions, the absorbance of each well was measured at 450 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). The growth curve was plotted with the culture time as the horizontal axis and the average absorption value as the vertical axis.
Transwell Assay

The final concentration of Matrigel was adjusted to 1 mg/mL with pre-cooled serum-free medium at 4°C. Matrigel was pre-coated on the upper surface of transwell chamber and maintained at 37°C for 3-5 h. Cells in logarithmic growth phase were digested and washed once with PBS (phosphate-buffered saline) and serum-free medium. The cells were suspended in serum-free medium and adjusted to a concentration of $2 \times 10^5$ / mL. 600 μL of medium containing 10% FBS and 100 μL of cell suspension were added to the lower and upper chamber, respectively. After 24 hours of culture, the medium in the upper chamber was removed. Cells were fixed with formaldehyde for 30 min and stained with crystal violet for 15-30 min. Penetrating cells were observed under an optical microscope (Nikon, Tokyo, Japan).

Dual-Luciferase Reporter Gene Assay

The 3’UTR sequence of PTEN was downloaded from the NCBI (National Center of Biotechnology Information) website to construct the wild-type PTEN (PTEN-WT) and the mutant-type PTEN (PTEN-MUT) sequence. The cells were then seeded in 96-well plates and co-transfected with 50 pmol/L microRNA-21 mimics or negative controls and 80 ng plasmid containing PTEN-WT or PTEN-MUT, respectively. After 48 hours of co-transfection, dual-luciferase activity assay was performed to detect fluorescence intensity.

Western Blot

After the cells were disrupted by ultrasound, the lysate was centrifuged and the supernatant was taken. Proteins were separated on a SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gel, transferred to a PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, MA, USA) and blocked in 5% skim milk. The specific primary and secondary antibodies were used to incubate with protein bands before imaging.

Statistical Analysis

SPSS 19.0 (Statistical Product and Service Solutions) statistical software (IBM, Armonk, NY, USA) was used for analysis. $\chi^2$-test was used for the comparison of classification data and $t$-test was used for the comparison of measurement data. Data were expressed as mean±standard deviation. $p < 0.05$ indicated the difference was statistically significant.

Results

**MicroRNA-21 was Significantly Decreased in the Placenta Tissues of Preeclampsia Patients**

MicroRNA-21 expression in the placenta tissues was determined by qRT-PCR. The results showed that microRNA-21 expression was reduced in the mother surface of preeclampsia placenta compared with that of normal pregnancies (Figure 1A). There was no significant change in microRNA-21 expression in the fetal surface of preeclampsia placenta (Figure 1B). Besides, transfection of microRNA-21 mimic significantly increased microRNA-21 expression in HTR-8/SVneo cells, while microRNA-21 inhibitor transfection obtained the opposite effect (Figure 1C, 1D).

**MicroRNA-21 Promoted Trophoblast Cell Infiltration**

Proliferative ability was detected after microRNA-21 overexpression or knockdown in HTR-8/SVneo cells by CCK-8 assay. The results showed that microRNA-21 did not affect cell proliferation (Figure 2A, 2B). Subsequently, the effect of microRNA-21 on cell infiltration was determined. The results exhibited that overexpression of microRNA-21 remarkably promoted trophoblast cell infiltration, while inhibition of microRNA-21 expression was able to attenuate the infiltration of cells (Figure 2C, 2D).

**MicroRNA-21 Directly Bound to mRNA Sequence of PTEN**

Through bioinformatics prediction and functional analysis, we found that PTEN was a potential target gene for microRNA-21 (Figure 3A). Subsequently, we detected PTEN expression in the placenta tissues by qRT-PCR. The results showed that PTEN expression in the mother surface of preeclampsia placenta was significantly elevated in comparison with that of normal pregnancies (Figure 3B). On the contrary, no remarkable change of microRNA-21 expression was observed in the fetal surface of the placenta of preeclampsia patients (Figure 3C). Dual-luciferase reporter gene assay indicated that the luciferase activity in the PTEN-WT group was decreased after transfection of microRNA-21 in HTR-8/SVneo cells. However, no significant difference in the luciferase activity was found in the PTEN-MUT group, suggesting that microRNA-21 might directly bind to PTEN (Figure 3D).
of PTEN markedly decreased in HTR-8/SVneo cells transfected with microRNA-21 mimics compared with those of controls (Figure 3E, 3F). Western blot results also showed that overexpressed microRNA-21 significantly reduced protein level of PTEN (Figure 3G, 3H). These results indicated that microRNA-21 could directly bind to PTEN and downregulate its protein level.

**Overexpression of PTEN Can Reverse the Effect of MicroRNA-21 on Cell Infiltration**

PTEN expression was detected after transfection of pcDNA-NC or pcDNA-PTEN in HTR-8/SVneo cells. We found that pcDNA-PTEN transfection significantly increased both mRNA and protein levels of PTEN in HTR-8/SVneo cells (Figure 4A, 4B). We found the infiltration ability of cells was significantly enhanced after microRNA-21 overexpression. However, overexpression of PTEN partially reversed the promoted cell infiltration induced by microRNA-21 overexpression (Figure 4C). Above data demonstrated that microRNA-21 increased cell infiltration ability by inhibiting PTEN expression.

**Discussion**

Preeclampsia, eclampsia and pregnancy-induced hypertension are severe pregnancy diseases with less than 10% of the occurrence rate.
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Among them, preeclampsia is particularly severe. The major manifestations of preeclampsia are hypertension and proteinuria after 20 weeks of pregnancy, which may result in systemic multiple organ dysfunction and functional failure. As the main cause of maternal and perinatal mortality, the pathogenesis of preeclampsia is still unknown, which has been a research hotspot. Clinical studies have found that the above symptoms gradually disappear with the end of the pregnancy in preeclampsia patients. Only a small part of preeclampsia pregnancies progresses to chronic hypertension even after giving birth. It is generally believed that the placenta exerts a vital role in the pathogenesis of preeclampsia. Further studies have suggested that the decreased invasiveness of embryonic trophoblast cells results in the failure of uterine spiral artery remodeling and placental malformation, which are also the initial pathological changes in preeclampsia. In recent years, scholars\textsuperscript{20-24} have found that there are a lot of specific and differentially expressed microRNAs in placental tissue of preeclampsia. It is suggested that some certain microRNAs may be involved in the occurrence and development of preeclampsia.

**Figure 2.** MicroRNA-21 promoted trophoblast cell infiltration. **A-B,** Overexpression or knockdown of microRNA-21 did not affect the proliferation of the HTR-8/SVneo cells. **C-D,** Transwell assay showed that overexpression of microRNA-21 significantly promoted cell infiltration, while inhibition of microRNA-21 was significantly decreased the cell infiltration.
This study focused on detecting microRNA-21 expression in the placenta of preeclampsia patients and exploring its possible mechanisms that influence the abnormal biological functions of trophoblast cells. Our data suggested that microRNA-21 expression was significantly decreased in the placenta of preeclampsia patients than those of normal pregnant women. However, there was no significant change in microRNA-21 expression in the fetal surface of placenta from preeclampsia patients. These results suggested that microRNA-21 was closely related to the development of preeclampsia. Subsequent functional studies also showed that microRNA-21 had no effect on the proliferation of HTR-8/SVneo cells, but promoted cell infiltration. PTEN is the first tumor-suppressor gene found to exert phosphatase activity with dual-substrate specificities, that is, the activities of protein phosphatase and lipid phosphatase. Therefore, we hypothesized that PTEN may affect cell proliferation and infiltration by regulating AKT/PI3K pathway. It is reported that PTEN was closely related to the occurrence and development of various tumors. Jin et al. detected protein expression of PTEN in 68 colorectal cancer tissues by immunohistochemistry. The results revealed that PTEN was absent in 67.6% of the colorectal cancer samples, indicating a marked decreased of PTEN in colorectal cancer. This study demonstrated that microR-
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NA-21 directly targeted the mRNA sequence of PTEN, which played a vital role in the development of preeclampsia. Firstly, overexpression of microRNA-21 showed no effect on the cell proliferation, while significantly enhanced the ability of cell infiltration. Furthermore, the direct binding relationship between microRNA-21 and PTEN was predicted and detected by bioinformatics analysis. In addition, PTEN expression was found to be regulated by microRNA-21. Increased PTEN exhibited partial reversion of the enhanced cell infiltration induced by microRNA-21 in HTR-8/SVneo cells, indicating the regulatory effect of microRNA-21 on trophoblast infiltration relying on PTEN expression. Our study demonstrated that microRNA-21 participated in the regulation of cellular activities by regulating PTEN. However, the mechanism un-

Figure 4. Overexpression of PTEN can reverse the effect of microRNA-21 on cell infiltration. A-B, After pcDNA-PTEN was transfected into the HTR-8/SVneo cells, PTEN expression level was significantly increased. C, Overexpression of microRNA-21 enhanced the infiltration ability of the cells, while overexpression of PTEN decreased the cell infiltration increased by microRNA-21.
underlying differential expression of microRNA-21 in the placenta of the preeclampsia patients still needs further study.

Conclusions

We found that microRNA-21 expression was significantly reduced in the placenta of preeclampsia patients than that of normal pregnant women. Downregulation of microRNA-21 inhibited cell infiltration via regulating PTEN expression. Our study partially clarified the role of microRNA-21 in the development of preeclampsia and provided new suggestions for the specific diagnosis and treatment.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References


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