**Abstract.** – **OBJECTIVE:** The aim of this study was to explore the role of micro ribonucleic acid (miR)-133 in the apoptosis of human placental trophoblasts through the Ras homolog gene family (Rho)/Rho-associated coiled-coil forming protein kinase (ROCK) signaling pathway.

**PATIENTS AND METHODS:** The plasma samples were collected from 30 patients with pre-eclampsia (PE) undergoing treatment and 30 healthy subjects (control group) who received physical examination in our hospital. The Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was utilized to measure the expression of miR-133 in PE patients and healthy people. Meanwhile, blood pressure, urine protein content, liver function, and kidney function were detected in patients of both groups as well. Subsequently, the placental trophoblasts were extracted and transfected with inhibitors and miRNA mimics to suppress and overexpress miR-133, respectively. The transfection efficiency was determined by RT-PCR. The levels of interleukin-6 (IL-6), IL-1, and tumor necrosis factor-alpha (TNF-α) were measured in both groups. The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay was performed to determine the apoptosis of trophoblasts. Next, the RT-PCR and Western blotting were carried out to detect the expressions of the Rho/ROCK pathway. Furthermore, the influence of miR-133 on the apoptosis of trophoblasts in human placenta tissues through Rho/ROCK was comprehensively observed.

**RESULTS:** In vivo experiments demonstrated that the urinary protein content, miR-133 level, systolic blood pressure, diastolic blood pressure, and liver function and renal function indexes were significantly elevated in pre-eclampsia (PE) patients in comparison with normal subjects (p<0.05). After transfection of mimics and inhibitors, the expression of miR-133 was remarkably up- and down-regulated, respectively. The content of the inflammatory factors in miR-133 mimics group was overtly higher than the other two groups. The TUNEL staining results showed that the number of apoptotic cells significantly increased and decreased in the miR-133 mimics group and miR-133 inhibitors group, respectively. Subsequent experiments indicated that the expressions of apoptosis gene Caspase3, pathway gene, and protein ROCK1 were notably up-regulated in miR-133 mimics group. However, they were evidently down-regulated in miR-133 inhibitors group than in the control group. In addition, a consistent trend was observed in the protein expression level.

**CONCLUSIONS:** MiR-133 participates in the development and progression of PE through the Rho/ROCK signaling pathway, which may affect the apoptosis of trophoblasts in the placenta tissues.

**Key Words:** MiR-133, Rho/ROCK pathway, Human placental trophoblasts, Apoptosis, Pre-eclampsia (PE).

**Introduction**

For a successful pregnancy, it is crucial to maintain the normal function of the fetal trophoblasts. Pre-eclampsia (PE) is often correlated with poor spiral artery remodeling and placental hypoxia caused by excessive apoptosis and invasion of trophoblasts. It is reported that the morbidity rate of PE is 7-12% all over the world, with 50,000 deaths annually. PE is one of the most common pathological complications of pregnancy, which is also one of the leading causes of pregnancy-related death. In addition, PE complicates the pregnancy, giving rise to a relatively high disease burden on pregnant women. PE is characterized by new onset of hypertension and proteinuria. Meanwhile, it is able to cause a series of maternal complications, thereby leading to severe damage to multiple or-
MiR-133 in trophoblasts of human placenta tissues

Trophoblasts, which also play an important role in the proliferation and apoptosis of placental trophoblasts, are major contributors to maternal and perinatal morbidity and mortality. Moreover, the involvement of placenta in PE increases the possibility of fetal complications, including low birth weight, premature delivery, intrauterine growth restriction, oligohydramnios, bronchopulmonary dysplasia, and perinatal death. Currently, there is no gold standard for the clinical treatment of PE except for the placenta. During the past few years, the pathogenesis of PE has been largely explored. Multiple studies have revealed that various mechanisms participate in the development and progression of PE. The disorder of the placental functions, abnormally increased apoptosis of trophoblasts, and other theories have been put forward. Among these theories, abnormally increased apoptosis and impaired migration and invasion abilities of trophoblasts have been widely accepted by researchers. However, the precise molecular pathogenesis of PE has not been fully elucidated. Interestingly, micro ribonucleic acids (miRNAs) can serve as non-invasive biomarkers for pregnancy-related diseases. Therefore, a better understanding of the roles of miRNAs in regulating trophoblasts and its underlying mechanisms may be conductive to clarify the development and therapeutic goals of new biomarkers for PE diagnosis. This may help to understand the pathogenesis of PE. In addition, these findings can provide theoretical support for the application of miRNAs in the treatment of eclampsia and other pregnancy-related diseases in the future.

With the advances in genetic research in recent years, the development of miRNAs has been noticed by researchers. MiRNAs are a type of widely distributed small non-coding RNAs with approximately 22 nucleotides in length, which can regulate gene expression. Numerous researchers have focused on the exploration of the specific biological effects of miRNAs in diseases. Increasing studies have proved that miRNAs regulate many human genes, thereby participating in the specific regulation of protein-coding and non-coding genes. In addition, miRNAs are capable of regulating various cellular processes involved in normal development and disease onset, such as cell cycle, development, metabolism, and immune responses. They are deemed to contribute to the development of many diseases. In fact, miR-133 is able to participate in the regulation of placental trophoblasts, which also plays an important role in the proliferation and apoptosis of cells. However, the potential role of miR-133 in the proliferation and apoptosis of placental trophoblasts are still unclear so far. Currently, Bharadwaj et al. have demonstrated that Ras homolog gene family (Rho) and its most studied effector Rho kinases (Rho-associated coiled-coil forming protein kinase ROCKI and ROCKII) are involved in several cellular functions, including smooth muscle contraction and apoptosis. Meanwhile, the Rho/ROCK pathway exerts vital function in axon-guided cell contraction and apoptosis regulation. However, the exact role of the Rho/ROCK pathway in cell apoptosis remains unclear. Scholars have shown that miR-133 plays a key role in many processes in pregnancy, such as implantation and placental homeostasis. Furthermore, it acts as an important regulator in placental development. Recent research has found that placental-specific miR-133 may be an important pathway for the development and progression of abnormal placental apoptosis. Moreover, Rho/ROCK plays an important role in PE and participates in the formation of the placenta. However, the role and mechanism of miR-133 in the apoptosis of placental trophoblasts through the Rho/ROCK pathway have not been fully elucidated.

Therefore, the aim of this study was to explore the role of miRNA-133 in the apoptosis of placental trophoblasts and its effects on the Rho/ROCK signaling pathway. In vivo and in vitro experiments were used in this study to elucidate its influence on cell apoptosis. Our findings might provide experimental and theoretical guidance for subsequent studies of pregnancy-related diseases.

 Patients and Methods

Collection of Plasma Samples

A total of 30 PE patients aged (28.36 ± 4.78) years old admitted to our hospital from October 2017 to February 2018 were enrolled in this study. Meanwhile, 30 healthy people aged (24.34 ± 2.87) years old (control group) were enrolled as normal controls. Informed consent was obtained from each subject before the study. The venous blood (5 mL) was collected, added with sodium citrate for anticoagulation and cryopreserved in a refrigerator at -20°C for use. The blood pressure and 24 h urine protein content were detected. This study was approved by the Ethics Committee of Qilu Hospital of Shandong University.

Liver and Renal Function

Previous studies have manifested that the function of kidney and liver is abnormal in the case of...
PE. To predict the clinical development of PE and to provide important references for early diagnosis, the liver function indexes, alanine aminotransferase (ALT) and alkaline phosphatase (ALP), and the renal function indicators, creatinine (Cr) and blood urea nitrogen (BUN), were determined in this study. The plasma samples stored in the low-temperature refrigerator were taken out, thawed in gradients and dispensed into centrifuge tubes. Next, an automatic biochemical analyzer with an operation program set was used to detect changes in content. In addition, RT-PCR technology was applied to detect the content of miR-133 in the PE group and control group, respectively.

Cell Culture, Transfection, and Grouping

The placental trophoblasts were extracted from placenta tissues of pregnant women. The collected cell suspension was slowly added to the upper layer of the Percoll separation medium (Santa Cruz, CA, USA). The cells were cultured in an incubator and divided into negative control group (NC group), miR-133 inhibitor group (inhibitors group), and miR-133 mimic group (mimics group). Subsequently, the transfected substances were added to trophoblasts in each group, followed by continuous culture for 36 h and starvation treatment. The cells in the three groups were then selected. Finally, the content of miR-133 was determined via Real Time-Polymerase Chain Reaction (RT-PCR) to observe the transfection efficacy.

Detection of Cellular Inflammatory Factors

After stimulation, the well-grown cells in the three groups were first selected. The medium was discarded, and the cells were collected using a disposable cell scraper, followed by centrifugation. Next, the supernatant was collected in an Eppendorf (EP) tube. The levels of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and IL-1 were determined according to the instructions of ELISA (Novus, Littleton, CO, USA) kit. The absorbance of each group was detected using a microplate reader, and the standard curve was plotted. Finally, the optical density (OD) value in each group was calculated.

Detection of Apoptosis Via Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate-Biotin Nick End Labeling (TUNEL)

An in-situ cell death detection kit (Roche, Basel, Germany) was employed to determine the apoptosis of cells in the paraffin sections according to the following specific procedures. Briefly, the paraffin sections were deparaffinized and washed with Phosphate-Buffered Saline (PBS). After added with proteinase K working solution, the sections were immersed in blocking solution, followed by osmosis with 0.1% Triton X-100. Next, FITC end labeling of the apoptotic fragment deoxyribonucleic acids (DNAs) was conducted using a TUNEL detection kit (Beyotime Institute of Biotechnology, Beijing, China). Finally, the images of FITC-labeled TUNEL-positive cells were observed under a fluorescence microscope at an excitation wavelength of 488 nm and a wavelength of 530 nm.

Detection of MiR-133 Content Via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The total RNA was first extracted from cells treated differently. After the purity met relative criteria, the DNAs were synthesized in accordance with PrimeScript™ Kit (TaKaRa, Otsu, Shiga, Japan). Then, the single-stranded complementary deoxyribonucleic acids (cDNAs) were amplified using a conventional reaction system and placed at -20°C for use. 20 μL PCR amplification reaction system was prepared, including: 2 μL cDNA, 10 μL mix, 2 μL primer, and 6 μL ddH2O. The primer sequences of the target genes and the internal reference GAPDH were designed based on the sequences on GenBank. The expression levels of the target genes were calculated by the 2-ΔΔCT method.

Western Blotting (WB)

The well-grown cells of the three groups were partially taken out, and the supernatant was discarded. Then, the cells collected using the cell scraper were used for total protein extraction. The cells treated and collected in the previous stage were dispensed into 10 mL EP tubes to extract proteins. After that, the proteins samples were subjected to a water bath for 8 min, followed by centrifugation at 1,000 g for 5 min. 10% separation gel and 5% spacer gel were prepared. The total protein samples were separated by electrophoresis and transferred onto membranes by the semi-dry method. After blocking, the membranes
were incubated with primary antibody overnight. On the next day, the membranes were incubated with the corresponding secondary antibody. Enhanced chemiluminescence (ECL) imaging analysis system was used for color development. The protein bands were scanned and quantified using an Odyssey membrane scanner. GAPDH was utilized to correct the level of protein to be tested.

**Statistical Analysis**

The Statistical Product and Service Solutions (SPSS) 20.0 (IBM Corp., Armonk, NY, USA) software was used for all statistical analyses. The experimental results were expressed as mean ± standard deviation (±SD). p<0.05 was considered statistically significant. GraphPad Prism 5.0 (La Jolla, CA, USA) was employed for the histogram.

**Results**

**Arterial Blood Pressure and Urine Protein Content**

Compared with the control group, the systolic and diastolic blood pressures were significantly elevated in the PE group. Meanwhile, the same changes were observed in 24 h urine protein expression (p<0.05) (Table II).

<table>
<thead>
<tr>
<th>Group</th>
<th>Systolic blood pressure (mmHg)</th>
<th>Diastolic blood pressure (mmHg)</th>
<th>Urine protein (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>112.5 ± 3.8</td>
<td>75.8 ± 3.9</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>PE group</td>
<td>146.8 ± 2.4*</td>
<td>108 ± 4.3*</td>
<td>7.25 ± 0.42*</td>
</tr>
</tbody>
</table>

Note: The systolic blood pressure, diastolic blood pressure and 24 h urine protein content are notably higher in PE group than those in control group (p<0.05).
Levels of Cellular Inflammatory Factors in Each Group

The levels of TNF-α, IL-6, and IL-1 were shown in Table IV. Mimics group displayed evidently up-regulated levels of TNF-α, IL-6, and IL-1 compared with the other two groups. However, the levels of the three indexes were significantly reduced in the inhibitor group ($p<0.05$).

Apoptosis Detected Via TUNEL Assay

TUNEL results (Figure 3) showed that no evident TUNEL-positive cells were observed in both the NC group and inhibitor group. However, the number of TUNEL-positive cells in mimics group was significantly higher than the other two groups ($p<0.05$). These results suggested that the transfection of miR-133 mimics promoted the abnormal apoptosis of trophoblasts in PE.

Expressions of Apoptotic Genes and Rho/ROCK1 in Trophoblasts via RT-PCR

The expression levels of the apoptotic genes and Rho/ROCK1 in human placental trophoblasts were measured via RT-PCR. The results (Figure 4) showed that the inhibitor group exhibited evidently reduced levels of Caspase3 and ROCK1 in human trophoblasts ($p<0.05$). However, the expression of the anti-apoptotic gene Bcl-2 was significantly up-regulated in the inhibitor group ($p<0.05$).
Expressions of Apoptotic Proteins and Rho/ROCKI in Human Placental Trophoblasts via WB

WB was employed to measure the expression levels of the apoptotic proteins and ROCKI in human placental trophoblasts. It was found that the protein expressions of Caspase3 and ROCKI in human trophoblasts were significantly down-regulated in the inhibitor group ($p<0.05$), whereas were notably up-regulated in the mimics group ($p<0.05$).

Discussion

Most miRNAs, also known as endogenous short RNA molecules, are ubiquitously expressed. They have been found to play important roles in the cellular process regulation. The best characteristic function of miRNAs is to “fine-tune” gene activity post-transcriptionally. To this end, mature miRNAs are incorporated into a fine ribosome structure called RNA-induced signal complex (RISC). Once loaded with miRNAs, RISC uses its seed sequence to find matching miRNAs. Multiple miRNAs are expressed in a tissue-specific manner, including muscle-specific and cardiac-specific expression patterns. Tissue-specific expression of miRNAs is regulated at the transcriptional level. This indicates that the precise spatiotemporal regulation of miRNA expression is important for their function. MiR-133a is expressed in cardiac and skeletal muscle in the form of double cervical clusters. Meanwhile, it is transcriptionally regulated by myogenic differentiation factors. However, miR-133b is only considered expressed in skeletal muscles. Previous studies have proved that miR-133 is expressed in early embryonic development, which also shows functions like regulating proliferation and apoptotic activities. However, it exerts opposite functions in late embryonic development. Nevertheless, the development of the placenta depends on both the absolute concentration of certain miRNAs and their correct
spatial and temporal expression\textsuperscript{23}. Recent studies have discovered that miRNAs can regulate various cellular processes in normal development and disease onset. They have also been considered as contributors to the progression of various diseases and important regulators in the gene expression. Currently, many miRNAs have been found expressed in human placental trophoblasts. During the entire pregnancy, early placental progression occurring in relatively hypoxic environment promotes trophoblast invasion and angiogenesis. Moreover, PE is characterized by hypertension and proteinuria\textsuperscript{24}. In this study, the abnormal expressions of miRNA-133 and its target genes were observed in PE placenta. These results implied that miR-133 played a vital role in regulating placental development. Moreover, systolic blood pressure, diastolic blood pressure, and urinary protein expression were significantly upregulated in the PE group. Therefore, it was hypothesized that differentially expressed miRNA-133 might be involved in placental hypoxia, thus leading to eclampsia. To predict the development of PE in advance in clinical practice, plasma AST, ALP, ALT, Cr, and BUN content was determined. It was found that the content of the above indexes was notably higher in the PE group than in the control group ($p<0.05$). This suggested that liver function and renal function indicators were significantly elevated during the development and progression of PE. Our findings might provide important references for the early diagnosis of PE. However, the mechanism of action of miR-133 in regulating trophoblastic function and placental development remains unclear. It is extremely important to further investigate the underlying mechanism, which may help to understand the profound pathogenesis of PE and to provide theoretical support for clinical treatment of pregnancy-related diseases including eclampsia. Therefore, the mechanism of action was further investigated in this study through many molecular biology techniques.

The common pathological complications during pregnancy increase the disease burden on pregnant women, causing a series of maternal complications and forming a vicious cycle\textsuperscript{25}. In addition, the close connection between the placenta and the mother increases the possibility of fetal complications such as premature birth. As a result, it is essential to maintain the normal function of fetal trophoblasts for a successful pregnancy. Multiple studies have found that PE is usually associated with poor spiral artery remodeling and placental hypoxia due to excessive apoptosis and invasion of trophoblasts. Meanwhile, it is a leading cause of maternal and perinatal morbidity and mortality\textsuperscript{26}. Researches have manifested that the development and progression of PE involve many mechanisms. Rho/ROCK is important for PE and is involved in the formation of the placenta. Rho and its most studied effector (Rho kinases ROCKI and ROCKII) are involved in cell apoptosis. Meanwhile, the Rho/Rho kinase pathway plays a key role in the development and progression, which also functions in axon-guided cell contraction and apoptosis regulation\textsuperscript{17}. However, its exact role in apoptosis remains unclear. The role of miR-133 in the placenta and its mechanism through the Rho/ROCK pathway in the apoptosis of placental trophoblasts have not been reported. Therefore, in this report, we investigated the role of miR-133 in the regulation of trophoblast apoptosis through the Rho/ROCK. This might help to elucidate the development and therapeutic goals of novel biomarkers for PE diagnosis. Besides, the transfection efficiency of miR-133 showed that the expression of miR-133 significantly increased in the mimics group, whereas it was re-
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markedly reduced in the inhibitor group (p<0.05). These data suggested that the transfection effect was clear, and that the relevant subsequent validation tests could be carried out. The results of ELISA revealed that the levels of TNF-α, IL-6, and IL-1 were significantly higher in mimics group than those in the other two groups. However, they were notably declined after the addition of inhibitors, which were similar to the findings of previous studies27. The TUNEL staining findings showed that the number of TUNEL-positive cells in mimics group was evidently higher than the other two groups (p<0.05). This implied that the transfection of miR-133 mimics facilitated the abnormal apoptosis of trophoblasts in PE. Apoptosis, tightly regulated active programmed cell death (PCD), plays a crucial role in the homeostasis and pathological processes of multicellular organisms28. Such a controlled cell suicide process is triggered by extracellular and intracellular stimuli. Once triggered, the cells inevitably die. Meanwhile, the cells have established a set of complex mechanisms that can avoid the initiation of pro-apoptotic procedures by activating Caspase-activated survival factors. Apoptosis correlation and its negative regulators are important for the regulation of system homeostasis29. In this study, the levels of Caspase3 and ROCK1 in human trophoblasts in the inhibitor group were significantly down-regulated (p<0.05). The protein results were basically consistent with the data on the genetic level, which were also in line with the findings of the previous studies30,31. To sum up, this study demonstrated that miR-133 was involved in apoptosis through the Rho/ROCK signaling pathway. Our findings complemented and improved the theoretical basis for the effects of miR-133 on trophoblast apoptosis and Rho/ROCK signaling pathway. Furthermore, our results indicated that the miR-133/Rho/ROCK axis regulated the apoptosis of trophoblasts, providing new potential targets for the gene therapy for pregnancy-related diseases.

Conclusions

MiR-133 regulates the apoptosis of trophoblasts by activating the Rho/ROCK signaling pathway. In addition, the miR-133/Rho/ROCK axis plays a crucial role in the pregnancy-related diseases by regulating the apoptosis of trophoblasts. Therefore, the Rho/ROCK pathway can be used to evaluate the treatment effect and prognosis in patients, which lays an experimental and theoretical basis for the finding of new targets.

Conflicts of interest

The authors declare no conflicts of interest.

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