MicroRNA-132 stimulates the growth and invasiveness of trophoblasts by targeting DAPK-1

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Abstract. – OBJECTIVE: The purpose of this study was to elucidate the regulatory effects of microRNA-132 on the growth and invasiveness of trophoblasts, thus influencing the development of preeclampsia (PE).

PATIENTS AND METHODS: Placenta tissues from 24 PE pregnancies and 24 healthy pregnancies were collected. Expression levels of microRNA-132 and DAPK-1 in collected placenta tissues were detected. Then, the regulatory effects of microRNA-132 and DAPK-1 on expression levels of apoptosis-associated genes, viability, and invasiveness in trophoblastic were assessed. Finally, through Dual-Luciferase reporter assay, the binding relationship between microRNA-132 and DAPK-1 was determined.

RESULTS: The results showed that microRNA-132 was downregulated in placenta of PE pregnancies, while DAPK-1 was upregulated. Overexpression of microRNA-132 stimulated viability and invasiveness, and inhibited apoptosis in trophoblasts. However, it was found that DAPK-1 was the target of binding microRNA-132, and a negative correlation was identified between their expression levels. Notably, the expression of DAPK-1 inhibited viability and invasiveness, but stimulated apoptosis in trophoblasts.

CONCLUSIONS: microRNA-132 stimulates proliferative capacities and inhibits apoptosis in trophoblasts by targeting DAPK-1.

Key Words: MicroRNA-132, DAPK-1, Trophoblasts, Preeclampsia.

Introduction

Preeclampsia (PE) is a multisystemic vascular syndrome, which is one of the leading causes of maternal and fetal mortality worldwide. The incidence of PE is approximately 2-8%, and it leads to a higher risk for developing hypertension, chronic kidney disease, and premature birth. The pathogenesis of PE is multifactorial, involving genetic, environmental, and placental factors. PE is characterized by an inflammatory response, oxidative stress, and placental dysfunction.

MicroRNAs (miRNAs) are small, single-chain non-coding RNAs containing 19-25 nucleotides. By binding 3’-untranslated region (3’-UTR) of target mRNAs, miRNAs regulate post-transcriptional expressions of mRNAs by degrading them or inhibiting their translation. During the pregnancy, multiple miRNAs are dynamically expressed to regulate various processes in the placenta, including trophoblastic differentiation and invasion.

The present study aimed to elucidate the regulatory effects of microRNA-132 on the growth and invasiveness of trophoblasts, thus influencing the development of preeclampsia (PE). The results showed that microRNA-132 was downregulated in placenta of PE pregnancies, while DAPK-1 was upregulated. Overexpression of microRNA-132 stimulated viability and invasiveness, and inhibited apoptosis in trophoblasts. However, DAPK-1 was found to be the target of binding microRNA-132, and a negative correlation was identified between their expression levels. Notably, the expression of DAPK-1 inhibited viability and invasiveness, but stimulated apoptosis in trophoblasts.

The results of this study provide new insights into the role of microRNA-132 in the development of preeclampsia. Understanding the regulatory mechanisms of microRNA-132 in trophoblastic proliferation and invasion may lead to the development of novel therapeutic strategies for preventing PE.
in placenta tissues. Vital functions of miRNAs in placenta development and functions have been identified. Previous papers have reported the involvement of miRNA-132 in many types of human diseases. In bladder cancer, microRNA-132 inhibits metastasis and epithelial-mesenchymal transition (EMT) via the TGF-β1/SMAD2 pathway. By targeting E2F5, microRNA-132 inhibits proliferative and migratory abilities in vascular smooth muscle cells with high-glucose induction.

DAPK-1 is a kinase associated with cell death, which is involved in tumor suppression and cell death. In this paper, it was found that microRNA-132 was able to affect viability, invasiveness, and apoptosis in trophoblasts. Moreover, DAPK-1 was proven to be the target gene binding microRNA-132 and involved in trophoblast behaviors. The results of this study provide novel ideas for prevention and treatment of PE.

**Patients and Methods**

**Sample Collection**

A total of 24 PE pregnancies and 24 healthy pregnancies undergoing regular prenatal examination in Zibo Maternal and Child Health Hospital from January 2016 to December 2018 were enrolled, and their placenta tissues were collected. This study was approved by the Ethics Committee of Zibo Maternal and Child Health Hospital. Signed written informed consents were obtained from all participants before the study. This study was conducted in accordance with the Declaration of Helsinki.

**Cell Culture**

HTR-8/SVneo cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) containing 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, HyClone Laboratories, South Logan, UT, USA) in a 5% CO₂ incubator at 37°C.

**Cell Transfection**

Transfection plasmids, including microRNA-132 mimic, microRNA-132 inhibitor, overexpression plasmid of DAPK-1 and NC, were provided by GenePharma (Shanghai, China). Transfection was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

**Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)**

TRIzol (Invitrogen, Carlsbad, CA, USA) was applied for isolating cellular RNA, followed by determination of RNA concentrations using a NanoDrop ND-1000 spectrophotometer. Complementary deoxyribonucleic acids (cDNAs) were obtained using the miScript II RT kit and their mRNA levels were determined using the miScript SYBR Green PCR kit (Qiagen, Hilden, Germany). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference. The mRNA levels were calculated by the 2^(-ΔΔCt) method.

The primer sequences are as follows: microRNA-132: 5′-CGCGTAACAGTCTACAGCCA-3′ (forward), 5′-AGTGCAGGGTCCGAGGTATT-3′ (reverse); U6: 5′-CTCGCTTCGGCAGCACA-3′ (forward), 5′-AACGCTTCACGAATTTGCGT-3′ (reverse); Bax: 5′-CCCGAGAGGTCTTTTTCGAG-3′ (forward), 5′-CCAGCCCATGATGGTTCTGAT-3′ (reverse), Bcl-2: 5′-GGTGGGGTCATGTGTGTGG-3′ (forward), 5′-CGGTTCAGGTCAGTCCAC-3′ (reverse); GAPDH: 5′-GCAAGGATACTGAGAGCAAGAG-3′ (forward), 5′-GGATGGAATTGTGAGGGAGATG-3′ (reverse), DAPK-1: 5′-ACGTGGATGTACCTATGCACACC-3′ (forward), 5′-TGCTTTTCTGATTCTTCATTTCT-3′ (reverse).

**Western Blot**

Cells were lysed in radioimmunoprecipitation assay (RIPA) for extracting proteins (Beyotime, Shanghai, China). After concentration determination, the protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 h. The membranes were then incubated with primary and secondary antibodies. Finally, band exposure and grey value analysis were finally conducted.

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

Cells were inoculated into a 96-well plate. At the appointed time points, 10 μL of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added in each well. Then, the absorbance at 450 nm of each sample was recorded.

**Dual-Luciferase Reporter Assay**

Cells were inoculated in a 24-well plate with 5×10⁵ cells per well. After co-transfection with DAPK-1 WT/DAPK-1 MUT and miRNA-132 mimic/NC for 48 h, relative Luciferase activity was finally measured (Promega, Madison, WI, USA).
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Transwell
A total of 2.5×10^4 cells were applied on the upper of a transwell insert pre-coated with Matrigel (Corning, Corning, NY, USA), and 750 μL of complete medium was added in the bottom. After 36-h cell culture, the transwell insert was taken out and fixed in 95% methanol for 20 min. Through 10-min violet crystal staining and phosphate-buffered saline (PBS) washing, the cells retained on the upper chamber were wiped off, while those invading to the bottom were captured and counted in 6 randomly selected fields (200×) (Nikon, Tokyo, Japan).

Statistical Analysis
GraphPad software Version 6.0 (La Jolla, CA, USA) was used for data analysis. All data were expressed as mean ± SD (standard deviation). The paired two-tailed t-test was used for comparing differences between two groups. p<0.05 considered as statistically significant.

Results
MicroRNA-132 was Downregulated in Placenta of PE Pregnancies
Compared with placenta tissues of healthy pregnancies, microRNA-132 was downregulated in those of PE pregnancies (Figure 1A). To uncover the potential influence of microRNA-132 on the development of PE, the transfection efficacy of microRNA-132 mimic and inhibitor was tested (Figure 1B, 1C). In addition, the overexpression of microRNA-132 markedly stimulated invasiveness in HTR-8/SVneo cells (Figure 1D).

MicroRNA-132 Stimulated Proliferative Ability and Inhibited Apoptosis in Trophoblasts
CCK-8 assay revealed that overexpression of microRNA-132 mimic markedly elevated viability, while the knockdown of microRNA-132 yielded the opposite trend (Figure 2A). Subsequently, the expression levels of apoptosis-associated genes were determined. It was found that microRNA-132 negatively regulated Bax level, and positively regulated Bcl-2 level, suggesting the inhibitory effect of microRNA-132 on trophoblast apoptosis (Figure 2B-2D).

DAPK-1 was the Target Gene Binding MicroRNA-132
Through bioinformatics analysis, the binding sequences in the 3’UTR of microRNA-132 and DAPK-1 were identified (Figure 3A). Based on the binding sequences, wild-type and mutant-type DAPK-1 vectors were constructed. Luciferase activity in wild-type DAPK-1 was negatively regulated by microRNA-132, verifying the binding

![Figure 1](image-url)  

Figure 3. DAPK-1 is the target gene binding to microRNA-132. A, Binding sequences in 3'UTR of microRNA-132 and DAPK-1. B, C, Luciferase activity in HTR-8/SVneo cells co-transfected with DAPK-1 WT/DAPK-1 MUT and NC/microRNA-132 inhibitor (B)/microRNA-132 mimic (C). D, MicroRNA-132 levels in placenta tissues of healthy pregnancies (n=24) and PE pregnancies (n=24). E, A negative correlation between expression levels of microRNA-132 and DAPK-1.
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Regulatory Effects of DAPK-1 on Trophoblasts

To elucidate the involvement of DAPK-1 in the development of PE, the transfection efficacy of overexpression plasmid of DAPK-1 was first tested (Figure 4A). It was shown that the overexpression of DAPK-1 inhibited cell viability (Figure 4B) and invasiveness (Figure 4C). On the contrary, the overexpression of DAPK-1 stimulated the apoptosis in trophoblasts (Figure 4D).

Discussion

PE is featured by proteinuria and hypertension, which seriously affects the health of pregnant women and infants. MiRNAs are extensively involved in the development of PE by mediating trophoblast homeostasis. MiRNA-132 was reported to regulate ovarian cancer progression and induce caspase-dependent cell apoptosis in glioma. Beside the deregulation of miRNA-132 is closely linked to a poor prognosis of colorectal cancer, MicroRNA-132 can stimulate proliferative ability and inhibit apoptosis in pancreatic cancer by activating the Hh signaling pathway.

Bioinformatics analysis proposes that the expression level of DAPK-1 in the placenta is 3.5 times that of other tissues. DAPK-1 is considered to be a vital regulator for cell death and apoptosis. A relevant trail uncovered that DAPK-1 increases in the blood circulation of PE pregnancies. Moreover, it is highly expressed in placenta tissues of PE pregnancies, suggesting that the expression of DAPK-1 in the blood circulation may be derived from the placenta.

DAPK-1 is an important enzyme that controls cell growth through the calcium ion/serine/threonine kinase pathways. It is also involved in IFN-γ-induced apoptosis. Unphysically silenced DAPK-1 is observed in tumors and hematological malignancies. Apoptosis is a physiological activity being strictly regulated, whose characteristics include membrane blistering, decreased potential difference of mitochondrial depolarization membrane, cytochrome C release, and activation of caspase-3. As a positive regulator for apoptosis, DAPK-1 can be activated by multiple factors including TGF-β, Fas, INF-γ, Ceramide, c-Myc, and p-53. Relevant studies have demonstrated that insufficient proliferation and metastasis of trophoblasts, as well as over-apoptosis, are the fundamental reasons of the trophoblasts. The trophoderm is the core organ during embryo implantation and placenta formation. Trophoblast differentiation is of significance to maintain the healthy pregnancy. The proliferative progression occurs in the cytotrophoblasts, which is the major mechanism responsible for the formation of villus structure in the first trimester. Moreover, miRNAs have been identified to be involved in this progression. For example, miR-376c stimulates trophoblasts to proliferate and invade through the Nodal and TGF-β pathways.

In this work, microRNA-132 was remarkably downregulated, while DAPK-1 was upregulated in the placenta tissues of PE pregnancies compared to those of healthy pregnancies. MicroRNA-132 was able to stimulate viability and in-

Table 1. Correlation between miR-132, DAPK-1 and clinical features and pregnancy outcomes.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Systolic blood pressure</th>
<th>Diastolic blood pressure</th>
<th>24-hour urine protein</th>
<th>Onset of gestational week</th>
<th>Neonatal weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPK-1</td>
<td>0.359 0.012</td>
<td>-0.710 0.001</td>
<td>0.685 &lt; 0.001</td>
<td>-0.256 0.007</td>
<td>-0.413 &lt; 0.001</td>
</tr>
<tr>
<td>miR-132</td>
<td>-0.525 0.003</td>
<td>-0.432 0.002</td>
<td>-0.428 0.001</td>
<td>0.615 0.034</td>
<td>0.498 0.017</td>
</tr>
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vasiveness and inhibits apoptosis in trophoblasts. In addition, DAPK-1 was the target gene to be negatively regulated by microRNA-132, and it was responsible for the regulatory effects of microRNA-132 on viability, invasiveness, and apoptosis in trophoblasts. The findings of this study provide a pathological basis for elucidating the role of microRNA-132 in the trophoblast development. Currently, only microRNA-132 level in the placenta was detected, and its expressions in plasma, decidua, and maternal-fetal interface require to be further detected.

Conclusions

Shortly, microRNA-132 stimulates proliferative and invasive capacities and inhibits apoptosis in trophoblasts by targeting DAPK-1.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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