European Review for Medical and Pharmacological Sciences 2020; 24: 5856-5862

MiR-507 inhibits the growth and invasion of trophoblasts by targeting CAMK4

H.-Q. LI[1], J.-J. FAN[2], X.-H. LI[3], D. BAO[4]

[1]Department of Obstetrics and Gynecology, Guangrao County People's Hospital Dongying, China
[2]Department of Clinical Laboratory, the Second Children & Women's Healthcare of Jinan City, Jinan, China
[3]Pharmacy Intravenous Admixture, Weifang People's Hospital, Weifang, China
[4]Department of Obstetrics and Gynecology, PLA Rocket Force Characteristic Medical Center, Beijing, China

Abstract. – OBJECTIVE: To elucidate the potential influences of miR-507 and CAMK4 on the progression of preeclampsia (PE).

PATIENTS AND METHODS: Placental tissues were collected from 24 PE pregnancies and 24 healthy pregnancies. The relative levels of miR-507 and CAMK4 in placental tissues were detected. In addition, expressions of apoptosis-associated genes in collected tissues were examined by both quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA). The influences of miR-507 and CAMK4 on proliferative and migratory abilities in HTR-8/SVneo cells were assessed by CCK-8 and transwell assay, respectively. The target relationship between miR-507 and CAMK4 was detected by Luciferase assay.

RESULTS: MiR-507 was upregulated in placental tissues collected from PE pregnancies. Overexpression of miR-507 suppressed proliferative and migratory abilities, and stimulated apoptosis in HTR-8/SVneo cells. CAMK4 was the target gene of miR-507, which was downregulated in placental tissues collected from PE pregnancies and negatively correlated to miR-507 level. The knockdown of CAMK4 suppressed proliferative and migratory abilities, and stimulated apoptosis in HTR-8/SVneo cells, and these trends were abolished by silence of miR-507.

CONCLUSIONS: Highly expressed miR-507 in PE pregnancies inhibits proliferative and migratory potentials, and induces apoptosis in trophoblasts by targeting CAMK4.

Key Words:
Preeclampsia, MiR-507, CAMK4, Proliferation, Migration.

Introduction

Preeclampsia (PE) is a gestational disease that is clinically characterized by hypertension and proteinuria during the gestation, which increases both maternal and perinatal mortality[1,2]. It is estimated that PE globally causes 500,000 infant deaths and 70,000 maternal deaths each year. In addition, PE pregnancies have higher risks of kidney disease, chronic hypertension, and cardiovascular disease than healthy population[3]. However, to date, the pathophysiology of PE has not been well clarified, which has greatly limited the efficacy of diagnostic and treatment strategies for PE patients[4].

The trophoblast is an extraembryonic tissue that exerts a critical role in embryonic development, such as placental implantation and formation[5]. Pathological infiltration and invasion of placental trophoblasts are responsible for the pathogenesis of PE. Once the function of placental trophoblasts is impaired, insufficient remodeling of spiral arteries results in ischemia and hypoxia of placenta. Insufficient trophoblast infiltration in the placenta and angiogenesis disorders can lead to PE. The expressions of invasion-associated genes and angiogenesis genes are remarkably changed during the course of PE[6].

MicroRNAs (miRNAs) are a class of highly conserved, non-coding, single-stranded RNAs that post-transcriptionally regulate gene expressions[7]. They are vital regulators in various diseases[8,9]. Differentially expressed miRNAs in the placenta and peripheral blood of PE patients may have an impact on the incidence of eclampsia[10]. Notably, miR-499a-5p, which is associated with

Corresponding Author: Xuhong Li, BM; e-mail: 4799410142qq.com

cardiovascular and cerebrovascular diseases, has been reported to be upregulated in placental tissues of PE patients, whereas miR-26a-5p, miR-103a-3p, and miR-145-5p are downregulated[11]. MiRNAs also influence trophoblast growth by mediating cell phenotypes[12]. MiR-507 has been identified to be abnormally expressed in colorectal cancer[13]. In addition, it is able to suppress the growth and metastasis of non-small-cell lung cancer *via* targeting ZEB2[14].

Our findings first uncovered that miR-507 was upregulated in placental tissues of PE pregnancies, suggesting a potential role of miR-507 in the progression of PE. We thereafter focused on the molecular mechanism of miR-507 on regulating trophoblast phenotypes.

Patients and Methods

Placental Samples

Placental tissues were collected from 24 PE pregnancies and 24 healthy pregnancies in Weifang People's Hospital from May 2017 to June 2019. Subjects with gestational diabetes, placenta previa, placental abruption, and other complications during pregnancy were excluded. Women of pre-eclampsia placental origin meet the diagnostic criteria: systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg for the first time after the 20th week, and proteinuria, or urinary protein ≥0.3 g/24 h, exclusion criteria: patients with multiple pregnancies, or with a history of labor induction at 16 weeks of gestational age >; gestational diabetes mellitus, idiopathic thrombocytopenia, antiphospholipid antibody syndrome, and other severe complications during pregnancy. This study was approved by the Ethics Committee of Weifang People's Hospital and conducted after informed consent of each subject.

Cell Culture

The human trophoblast cell line HTR-8/SV-neo was provided by American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) in a 5% CO_2 incubator at 37°C. 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin were applied in the culture medium.

Transfection

Transfection plasmids were constructed by GenePharma (Shanghai, China). Cells inoculated in 6-well plates were transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

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

TRIzol (Invitrogen, Carlsbad, CA, USA) was applied for lysing cells or tissues and extracting total RNAs. Reverse transcription of RNAs was performed by the PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan) and complementary deoxyribose nucleic acid (cDNA) was sent for qRT-PCR. Relative level of the target was calculated using $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows. MiR-507: 5′-GCATTTTGCACCTTTTGGA-3′ (forward), 5′-GTGCAGGGTCCGAGGT-3′ (reverse); U6: 5′-CTCGCTTCGGCAGCACA-3′ (forward), 5′-AACGCTTCACGAATTTGCGT-3′ (reverse); Bax: 5′-CCCGAGAGGTCTTTTTCCGAG-3′ (forward), 5′-CCAGCCCATGATGGTTCTGAT-3′ (reverse); Bcl-2: 5′-GGTGGGGTCATGTGTGTGG-3′ (forward), 5′-CGGTTCAGGTACTCAGTCATCC-3′ (reverse); CAMK4: 5′-AATCATATGCTCAAAGTCACGGTGCCC-3′ (forward), 5′-TACATCTCGAGTTAGTACTCTGGCAGGATC-3′ (reverse); GAPDH: 5′-GCAAGGATACTGAGAGCAAGAG-3′ (forward), 5′-GGATGGAATTGTGAGGGAGATG-3′ (reverse).

Enzyme-Linked Immunosorbent Assay (ELISA)

The protein levels of Bax and Bcl-2 were detected using the commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) following the strict instruction.

Cell Counting Kit-8 (CCK-8) Assay

Cells were inoculated into 96-well plates with 1×10^3 cells per well. At the appointed time points, 10 μL of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added in each well. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Luciferase Assay

CAMK4 3'-UTR containing the seed sequence of miR-507 (wild-type luciferase vector) and the mutant 3'-UTR fragment (mutant-type one) after site-directed mutation were inserted into the

pGL3-basic vectors, respectively. The cells were co-transfected with plasmids and Luciferase vectors for 48 h, followed by Luciferase activity measurement (Promega, Madison, WI, USA).

Transwell Assay

100 µL of suspension (1×10^5 cells/mL) was inoculated in the upper insert of a transwell chamber (Millipore, Billerica, MA, USA), which was inserted in a 24-well plate with 500 µL of medium containing 10% FBS in the bottom. 48 hours later, the bottom cells were reacted with 15-min methanol, 20-min crystal violet, and captured using a microscope. The number of migratory cells was counted in 10 random fields per sample (magnification 200×).

Statistical Analysis

Data were expressed as mean ± standard deviation (SD) and processed by GraphPad software Version 6.0 (GraphPad Software, Inc. La Jolla, CA, USA). The differences between the two groups were analyzed by the *t*-test. The relationship between expression levels of two genes was analyzed by Pearson correlation test. $p<0.05$ was considered as statistically significant.

Results

MiR-507 Inhibited Proliferative and Migratory Abilities in Trophoblasts

We collected placental tissues of PE pregnancies and healthy pregnancies, and miR-507 was detected to be highly expressed in the former group (Figure 1A). It is suggested that miR-507 may be involved in the progression of PE. To further elucidate the biological function of miR-507 in PE, we constructed miR-507 mimics and inhibitor. Their transfection efficacy was tested in HTR-8/SVneo cells (Figure 1B, 1C). CCK-8 and transwell assay showed that overexpression of miR-507 reduced viability in HTR-8/SVneo cells and the number of migratory cells, respectively (Figure 1D, 1E).

MiR-507 Induced Apoptosis in Trophoblasts

Relative levels of apoptosis-associated genes were examined. Both mRNA and protein levels of Bax were upregulated in HTR-8/SVneo cells overexpressing miR-507 (Figure 2A, 2B). On the contrary, Bcl-2 was downregulated by overexpression of miR-507 detected by both qRT-PCR and ELISA (Figure 2C, 2D).

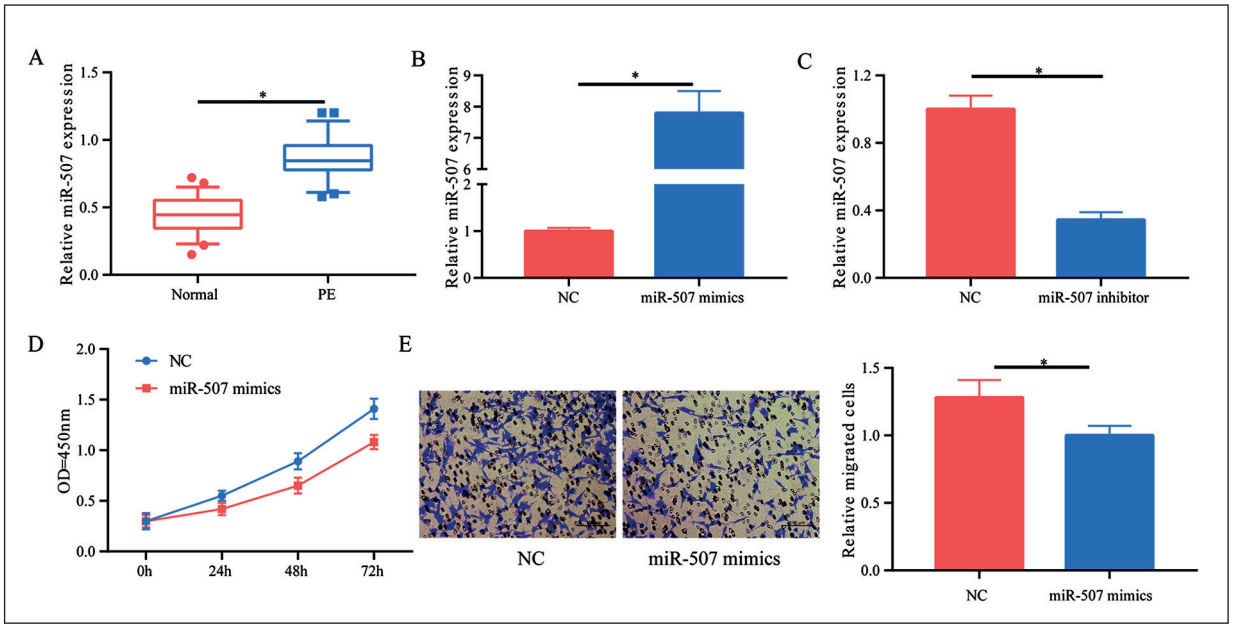

Figure 1. MiR-507 inhibited proliferative and migratory abilities in trophoblasts. **A,** MiR-507 level in placental tissues collected from PE pregnancies and healthy pregnancies. **B,** MiR-507 level in HTR-8/SVneo cells transfected with NC or miR-507 mimics. **C,** MiR-507 level in HTR-8/SVneo cells transfected with NC or miR-507 inhibitor. **D,** Viability in HTR-8/SVneo cells transfected with NC or miR-507 mimics. **E,** Migration in HTR-8/SVneo cells transfected with NC or miR-507 mimics (magnification: 200×).

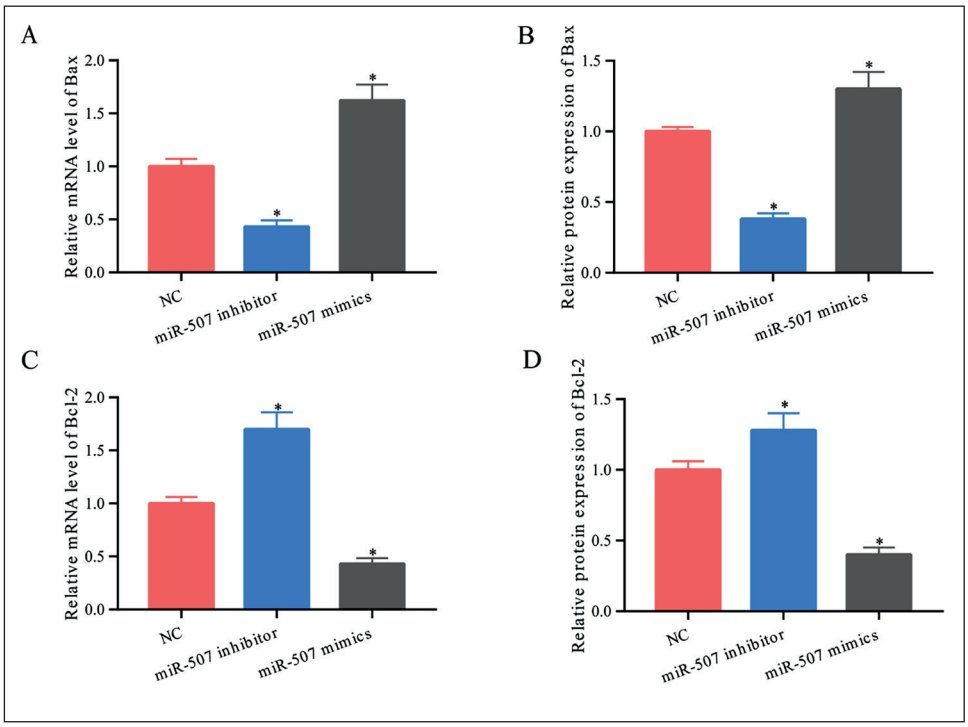

Figure 2. MiR-507 induced apoptosis in trophoblasts. **A,** Relative mRNA level of Bax in HTR-8/SVneo cells regulated by miR-507 detected by qRT-PCR. **B,** Relative protein level of Bax in HTR-8/SVneo cells regulated by miR-507 detected by ELISA. **C,** Relative mRNA level of Bcl-2 in HTR-8/SVneo cells regulated by miR-507 detected by qRT-PCR. **D,** Relative protein level of Bcl-2 in HTR-8/SVneo cells regulated by miR-507 detected by ELISA.

CAMK4 Was the Target Gene of MiR-507

Bioinformatics analysis predicted that CAMK4 was the potential target of miR-507. It is found that CAMK4 was downregulated in placental tissues of PE pregnancies than controls (Figure 3A). A negative correlation was identified between the expression levels of miR-507 and CAMK4 in the placental tissues of PE pregnancies (Figure 3B). As expected, CAMK4 was upregulated in HTR-8/SVneo cells transfected with miR-507 inhibitor, which was downregulated by the overexpression of miR-507 (Figure 3C). According to the binding sites in the 3'UTR of CAMK4 and miR-507 (Figure 3D), wild-type and mutant-type CAMK4 vectors were constructed. MiR-507 was able to negatively regulate Luciferase activity in wild-type CAMK4 vector, rather than the mutant-type one (Figure 3E, 3F). It is confirmed that CAMK4 was the downstream gene targeting miR-507.

CAMK4 Promoted Proliferative and Migratory Abilities, and Inhibited Apoptosis in Trophoblasts

Transfection of si-CAMK4 effectively downregulated CAMK4 level in HTR-8/SVneo cells (Figure 4A). The knockdown of CAMK4 in trophoblasts markedly reduced viability and the number of migratory cells. However, the suppressed trends were abolished by silence of miR-507 (Figure 4B, 4C). In addition, qRT-PCR and ELISA results showed upregulated Bax (Figure 4D, 4F) and downregulated Bcl-2 (Figure 4E, 4G) in HTR-8/SVneo cells transfected with si-CAMK4 than those of controls.

Discussion

The pathogenesis of PE remains largely unknown. Currently, most of the hypotheses believed that impaired function of the placenta during its development in early pregnancy attributes to the pathogenesis of PE. Stenosis and atresia of placental spiral artery in PE patients induce the release of a large number of toxic factors and inflammatory mediators, as well as the formation of placental microthrombus. These pathological changes eventually lead to a reduction in placental blood perfusion and symptoms of hypertensive disorder complicating pregnancy[15].

In recent years, the relationship between miRNAs and pregnancy-related diseases has been well concerned. The growth of the placenta and the outcome of pregnancy are inseparable. By detecting miRNA expressions in mouse pla-

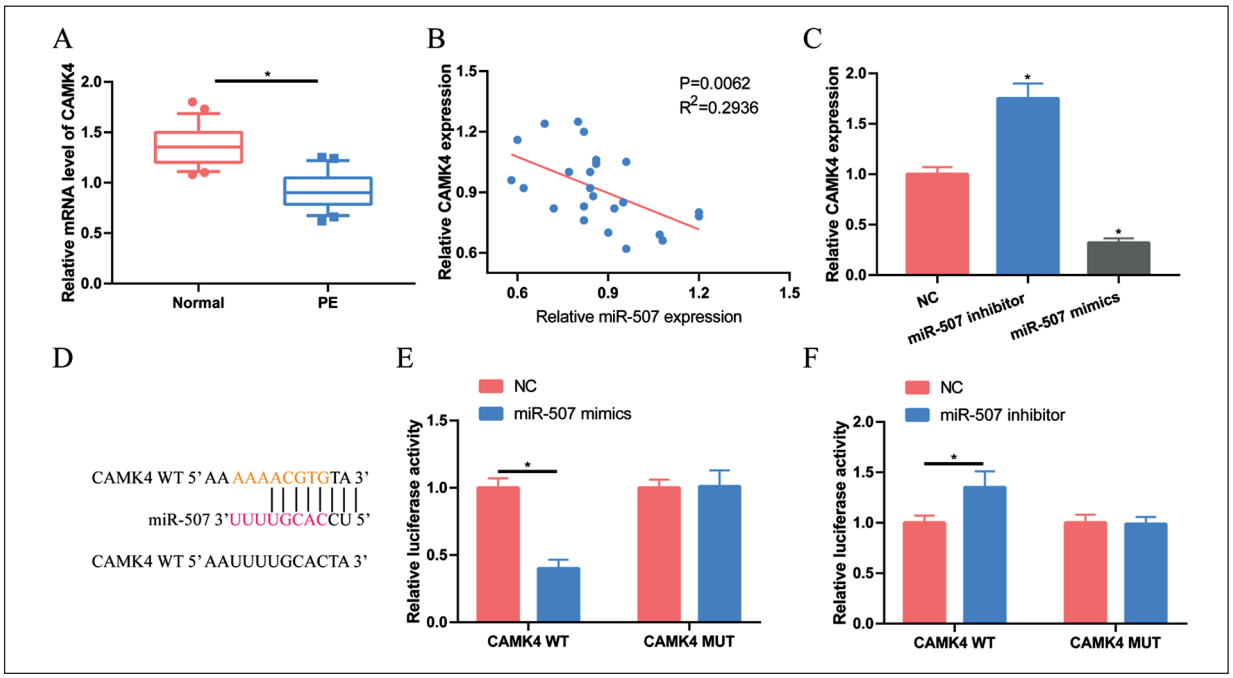

Figure 3. CAMK4 was the target gene of miR-507. **A,** CAMK4 level in placental tissues collected from PE pregnancies and healthy pregnancies. **B,** A negative correlation between miR-507 and CAMK4 level in placental tissues collected from PE pregnancies. **C,** CAMK4 level in HTR-8/SVneo cells regulated by miR-507. **D,** Binding sites in the 3'UTR of CAMK4 and miR-507. **E,** Luciferase activity in HTR-8/SVneo cells co-transfected with NC/miR-507 mimics and CAMK4 WT/CAMK4 MUT. **F,** Luciferase activity in HTR-8/SVneo cells co-transfected with NC/miR-507 inhibitor and CAMK4 WT/CAMK4 MUT.

cental tissues *via* Northern blot, it is speculated that miRNAs pose a certain impact on the growth and development of human placenta[16]. A microarray analysis uncovered 8 upregulated miRNAs in placental tissues of patients with severe PE (i.e., miR-16, miR-195). MiR-141-5p promotes the occurrence of PE by regulating ATF2, which is dependent on the MAPK1/

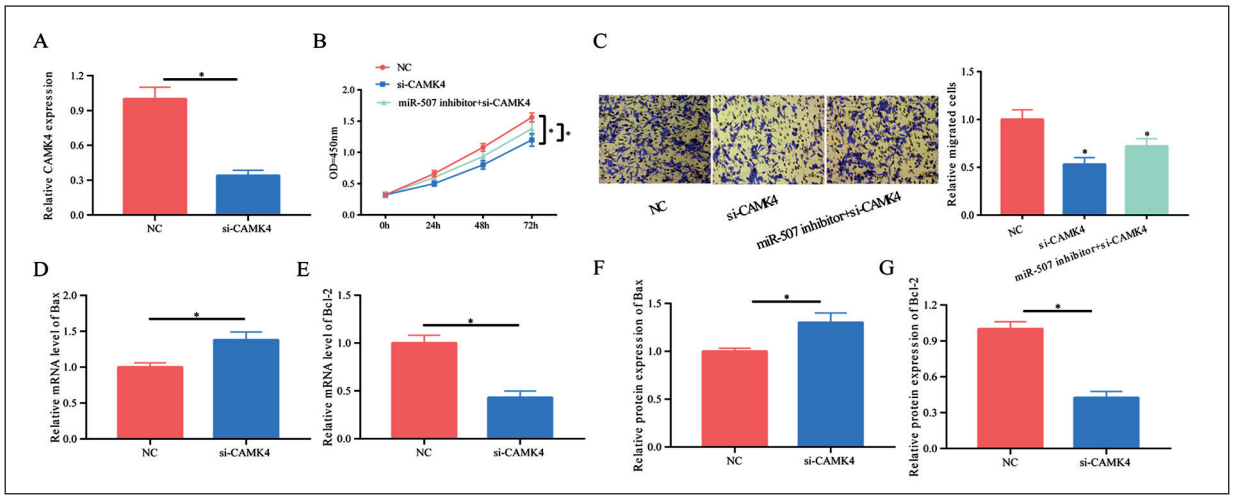

Figure 4. CAMK4 promoted proliferative and migratory abilities, and inhibited apoptosis in trophoblasts. **A,** CAMK4 level in HTR-8/SVneo cells transfected with NC or si-CAMK4. **B,** Viability in HTR-8/SVneo cells transfected with NC, si-CAMK4 or miR-507+si-CAMK4. **C,** Migration in HTR-8/SVneo cells transfected with NC, si-CAMK4 or miR-507+si-CAMK4 (magnification: 200×). **D,** Relative mRNA level of Bax in HTR-8/SVneo cells transfected with NC or si-CAMK4 detected by qRT-PCR. **E,** Relative mRNA level of Bcl-2 in HTR-8/SVneo cells transfected with NC or si-CAMK4 detected by qRT-PCR. **F,** Relative protein level of Bax in HTR-8/SVneo cells transfected with NC or si-CAMK4 detected by ELISA. **G,** Relative protein level of Bcl-2 in HTR-8/SVneo cells transfected with NC or si-CAMK4 detected by ELISA.

ERK2 signaling[17]. We collected placental tissues from both PE pregnancies and healthy pregnancies. MiR-507 was detected to be upregulated in the former tissues. As a tumor-suppressor gene, miR-507 inhibits the proliferative and invasive potentials of tumor cells. We therefore speculated whether miR-507 can promote the occurrence of PE by regulating trophoblast phenotypes. *In vitro* studies have proven that the overexpression of miR-507 suppressed proliferative and migratory abilities, and stimulated apoptosis in HTR-8/SVneo cells.

MiRNAs exert their biological functions by targeting their downstream genes. Using the bioinformatics tool, Ca^{2+}/calmodulin kinase IV (CAMK4) was predicted to be the target of miR-507, and this prediction was further confirmed by Luciferase assay results. CAMK4 is a multi-functional protein, which is extensively studied in the nervous system owing to its effect on triggering nerve cell growth[18]. The knockdown of CAMK4 in NIH 3T3 cells markedly induces cell apoptosis[19]. Moreover, CAMK4 expression in epithelial ovarian cancer tissues is closely related to tumor staging, pathological grade, and prognosis, predicting a poor clinical outcome of affected people[20]. Here, CAMK4 was found to be downregulated in placental tissues of PE pregnancies, and displayed a negative correlation to miR-507 level. The knockdown of CAMK4 inhibited the proliferative and migratory abilities, and induced apoptosis in trophoblasts. It is suggested that CAMK4 exerted an opposite role to that of miR-507 in regulating trophoblast phenotypes.

Conclusions

In this study, it was found, for the first time, that the expression of miR-507 in placental tissues of PE patients was significantly increased, and it was confirmed firstly that miR-507 may be involved in inhibiting the proliferation and invasion of trophoblast cells by targeting CAMK4. This study not only provides more theoretical basis for in-depth study on the role and molecular mechanism of miRNA in trophoblastic cell development, but also provides a new potential target for prevention and treatment of preeclampsia, and lays a research foundation for further exploration of the pathogenesis, disease outcome, clinical diagnosis, treatment, and prevention of gestational hypertension.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Amaral LM, Cunningham MJ, Cornelius DC, LaMarca B. Preeclampsia: long-term consequences for vascular health. Vasc Health Risk Manag 2015; 11: 403-415.
2) Mol B, Roberts CT, Thangaratinam S, Magee LA, de Groot C, Hofmeyr GJ. Pre-eclampsia. Lancet 2016; 387: 999-1011.
3) Paauw ND, Luijken K, Franx A, Verhaar MC, Lely AT. Long-term renal and cardiovascular risk after preeclampsia: towards screening and prevention. Clin Sci (Lond) 2016; 130: 239-246.
4) Pimentel C, Solene D, Frederique J, Guillaume B, Jean L, Maela LL. What are the predictive factors for preeclampsia in oocyte recipients? J Hum Reprod Sci 2019; 12: 327-333.
5) Lunghi L, Ferretti ME, Medici S, Biondi C, Vesce F. Control of human trophoblast function. Reprod Biol Endocrinol 2007; 5: 6.
6) Ilekis JV, Reddy UM, Roberts JM. Preeclampsia--a pressing problem: an executive summary of a National Institute of Child Health and Human Development workshop. Reprod Sci 2007; 14: 508-523.
7) Cheng M, Wang B, Yang M, Ma J, Ye Z, Xie L, Zhou M, Chen W. MicroRNAs expression in relation to particulate matter exposure: a systematic review. Environ Pollut 2020; 260: 113961.
8) Kalhori MR, Arefian E, Fallah AF, Kavousi K, Soleimani M. MiR-548x and miR-4698 controlled cell proliferation by affecting the PI3K/AKT signaling pathway in glioblastoma cell lines. Sci Rep 2020; 10: 1558.
9) Foinquinos A, Batkai S, Genschel C, Viereck J, Rump S, Gyongyosi M, Traxler D, Riesenhuber M, Spannbauer A, Lukovic D, Weber N, Zlabinger K, Hasimbegovic E, Winkler J, Fiedler J, Dangwal S, Fischer M, de la Roche J, Wojciechowski D, Kraft T, Garamvolgyi R, Neitzel S, Chatterjee S, Yin X, Bar C, Mayr M, Xiao K, Thum T. Preclinical development of a miR-132 inhibitor for heart failure treatment. Nat Commun 2020; 11: 633.
10) Zhou X, Li Q, Xu J, Zhang X, Zhang H, Xiang Y, Fang C, Wang T, Xia S, Zhang Q, Xing Q, He L, Wang L, Xu M, Zhao X. The aberrantly expressed miR-193b-3p contributes to preeclampsia through regulating transforming growth factor-beta signaling. Sci Rep 2016; 6: 19910.
11) Hromadnikova I, Kotlabova K, Hympanova L, Krofta L. Cardiovascular and cerebrovascular disease associated microRNAs are dysregulated in placental tissues affected with gestational hypertension, preeclampsia and intrauterine growth restriction. PLoS One 2015; 10: e138383.
12) Novakovic B, Mansell T, Saffery R. Micromanaging human placental function: differential microRNA

expression in feto-placental endothelial cells of gestational diabetes pregnancies. Clin Sci (Lond) 2019; 133: 315-319.

13) Wang S, Li J, Yang X. Long non-coding RNA LINC00525 promotes the stemness and chemoresistance of colorectal cancer by targeting miR-507/ELK3 axis. Int J Stem Cells 2019; 12: 347-359.

14) Lv HL, Li ZL, Song YB. MicroRNA-507 represses the malignant behaviors of non-small cell lung cancer via targeting zinc finger E-box binding homeobox 2. Eur Rev Med Pharmacol Sci 2019; 23: 9955-9964.

15) Flint EJ, Cerdeira AS, Redman CW, Vatish M. The role of angiogenic factors in the management of preeclampsia. Acta Obstet Gynecol Scand 2019; 98: 700-707.

16) Seitz H, Royo H, Bortolin ML, Lin SP, Ferguson-Smith AC, Cavaille J. A large imprinted microRNA gene cluster at the mouse Dlk1-Gtl2 domain. Genome Res 2004; 14: 1741-1748.

17) Wang Y, Cheng K, Zhou W, Liu H, Yang T, Hou P, Li X. MiR-141-5p regulate ATF2 via effecting MAPK1/ERK2 signaling to promote preeclampsia. Biomed Pharmacother 2019; 115: 108953.

18) Redmond L, Kashani AH, Ghosh A. Calcium regulation of dendritic growth via CaM kinase IV and CREB-mediated transcription. Neuron 2002; 34: 999-1010.

19) Tombes RM, Grant S, Westin EH, Krystal G. G1 cell cycle arrest and apoptosis are induced in NIH 3T3 cells by KN-93, an inhibitor of CaMK-II (the multifunctional Ca2+/CaM kinase). Cell Growth Differ 1995; 6: 1063-1070.

20) Takai N, Miyazaki T, Nishida M, Nasu K, Miyakawa I. Ca(2+)/calmodulin-dependent protein kinase IV expression in epithelial ovarian cancer. Cancer Lett 2002; 183: 185-193.