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MiR-4421 regulates the progression of preeclampsia by regulating CYP11B2

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Abstract. – OBJECTIVE: Preeclampsia is a serious disease that affects maternal and fetal health in pregnancy. Mechanism of miRNA in preeclampsia has gradually been explored. This study mainly investigated the mechanism of miR-4421 in preeclampsia.

PATIENTS AND METHODS: The expression of miR-4421 in 42 preeclampsia tissues and 42 normal pregnancy placentas tissues was detected by qRT-PCR. The relationship between the miR-4421 level and clinicopathological features of preeclampsia was analyzed. After miR-4421 was overexpressed, cell proliferation, cell cycle, and apoptosis were examined. The target gene CYP11B2 of miR-4421 was detected by luciferase reporter assay. The protein expressions were accessed by Western blot.

RESULTS: miR-4421 was highly expressed in the placenta of preeclampsia. Clinical data analysis revealed higher systolic blood pressure, diastolic blood pressure, and urinary protein level in preeclampsia patients with high expression of miR-4421 compared with those in low expression group. Birth weight of fetuses was significantly lower than those born from normal pregnant women. After overexpression of miR-4421, trophoblast proliferation was significantly inhibited and cell cycle was significantly blocked. Luciferase reporter assay and Western blot showed that CYP11B2 can be served as a target gene of miR-4421.

CONCLUSIONS: MiR-4421 was highly expressed in preeclampsia, which may promote the progression of preeclampsia by down-regulating the expression of CYP11B2.

Key Words:
microRNA, MiR-4421, Preeclampsia.

Introduction

Preeclampsia (PE) is a vasospastic disease causing significant maternal morbidity and mortality[1]. Its occurrence in primipara is about 3%-8%[2], and increases 3 times in some populations. PE occurs after 20 weeks of gestation and its clinical features are mainly hypertension and proteinuria, as well as different manifestations due to various terminal organs ischemia. PE is a multi-factor, multiple organ-impaired diseases, the etiology of which remains unknown[3,4]. It is not only the main cause of maternal mortality but also the most common cause of preterm birth due to iatrogenic treatment. PE leads to fetal growth restriction, placental abruption, and perinatal death, which is still one of the main causes of maternal and perinatal death[5]. Therefore, the early detection and prevention of risk factors of PE have important clinical significance.

With the further development of the human post-genomic project, non-coding sequence, which accounted for 99% of the human genome, has attracted more and more attention. Among them, the most notable one is the discovery of microRNAs (miRNAs). MiRNA is a kind of single-stranded RNA with a total length of 19-25 nt. It is processed by transcriptional precursors of stem-loop structure[6], which can cause the target mRNA to degrade or inhibit its translation through specific binding. Thereby it regulates the expression of the target gene after transcription[7-9]. Discovery of the microRNA regulatory function is an important supplement to the RNA intermediary role in the central rule. It will prompt biologists to rethink the important issues in cytogenetic regulation and development. Several studies have shown that some microRNAs had specific functions and played an important regulatory role in cell biology. Pineles et al[10] detected the expression of 157 miRNAs in PE and normal placenta by Real-time fluorescence quantitative PCR. It was found that there were significantly differential miRNAs in PE placenta compared with the normal placenta (miR-210, miR-182), suggesting that the dysregulation of miRNA expression may be involved in the pathogenesis of PE. Niu et al[11] found that microRNA-30a-3p was

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highly expressed in the placenta of PE patients and affected the invasion and apoptosis of trophoblasts *via* IGF-1. However, the underlying regulatory mechanism of miRNA in PE requires further exploration.

In our study, a novel miRNA miR-4421 was demonstrated to regulate the progression of PE by targeting CYP11B2. The discovery of this novel miRNA as well as the newly identified target will offer new insight of PE prevention and treatment.

Patients and Methods

Collection and Processing of Samples

After cesarean sterile placenta section was taken, and placental tissue from the proximal umbilical cord of the placental matrix was extracted (to avoid the organization, calcification, hemorrhage lesions, etc.). The extracted samples were repeatedly rinsed with phosphate-buffered saline (PBS) until no blood remained and placed in liquid nitrogen at -80°C refrigerator for further use. This study was approved by the Ethics Committee of Affiliated Hospital of Yan'an University. Signed written informed consents were obtained from the patients and/or guardians.

Cell Culture and Transfection

Trophoblastic HTR-8/SVneo, JEG-3, Wish and Bewo cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Rockville, MD, USA) supplemented with 5% fetal bovine serum (FBS), and maintained in an incubator at 37°C and 5% CO_2. Medium change was performed according to cell growth. Cells were passaged and digested by trypsin until cells confluency was over 80%. Cells were inoculated into 6-well plates (2×10^5/well) for 24 h incubation until the cells were grown to 70% confluent. The cells were transfected with NC and miR-4421 mimic, respectively, according to the instructions.

Quantitative Real-time PCR (qRT-PCR)

We used TRIzol (Invitrogen, Carlsbad, CA, USA) to extract RNA from tissues and cells, and the extracted RNA was reverse transcribed into cDNA using PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was performed on a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) with primers and RNA mixed with SYBR Premix Ex Taq II kit (TaKaRa, Otsu, Shiga, Japan). Each experiment was repeated for three times. The sequences of the primers were as follows: CYP11B2 (Forward) 5'-GGCAGAGGCAGAGATGCTG-3', CYP11B2 (Reverse) 5'-CTTGAGTTAGTGTCTCCACCAGGA-3'; GAPDH (Forward) 5'-CGGCGCGGTGCGCCAACA-3'.

CCK8 Assay for Cell Proliferation

The transfected JEG-3, Wish cells were seeded in 96-well plates. After cell culture for 24, 48, 72, and 96 h, the culture medium was replaced with serum-free medium, treated with 10 µL of CCK8 per well, incubated at 37°C, 5% CO_2 for 1 h. OD values at 450 nm were measured. Five replicates were set up for each group.

Cell Cycle Assay

Cells were seeded in 6-well plates and NC, miR-4421 mimic were transfected with lipofectamine following the instruction. After 24 h, 1×10^6 cells were harvested in each group, washed twice with PBS, and the supernatant was removed. 70% ethanol was added (precooling at -20°C) for fixation. After incubation with RNase for 15 min, 50 µg/mL of propidium iodide (PI) was added to each sample for 30 min. Lastly, the stained cells were detected by flow cytometry.

Apoptosis Assay

Cells were seeded in 6-well plates and divided into NC group and mirR-4421 mimic group. After 24 h of culture, 1×10^6 cells were harvested in each group and washed twice in cold PBS. The cells were resuspended in Annexin V-binding buffer with 5 µL of Annexin V-florescein isothiocyanate (FITC) and 5 µL of PI stain, and incubated in the dark for 15 minutes. Annexin-V and PI staining positive cells were detected by flow cytometry.

Luciferase Reporter Gene

Cells were seeded in 24-well plates with triplicates each group. Transfection was performed when the cell density was up to 80%-90%. Cells were then transfected with 80 ng of plasmid, 5 ng of Renilla luciferase, 50 nM of miR-4421 mimics and/or negative control respectively with Lipofectamine 2000. After 24 h of transfection, cells were collected and measured. The corrected enzyme activity per sample well was calculated as Firefly luciferase activity value/Renilla luciferase activity value.

Western Blotting

Radioimmunoprecipitation assay (RIPA) lysis solution was used to lyse cells to extract total proteins. After routine electrophoresis, CYP11B2 and CDK2 primary antibodies (CST, Danvers, MA, USA 1:1000) were electrophoresed and incubated overnight at 4°C. After washed with PBS, membranes were incubated with HRP-labeled secondary antibodies (Cell Signaling, Danvers, MA, USA, goat anti-rabbit IgG 1:5000) for 2 h at room temperature. At last, the membranes were developed with enhanced chemiluminescence (ECL) imaging (Shanghai Beyotime Biotechnology Co., Ltd. Shanghai, China). The integral optical density (IOD) value of each band was measured by a gel imaging analysis system using GAPDH as an internal reference.

Statistical Analysis

We used statistical product and service solutions (SPSS 22.0, IBM, Armonk, NY, USA) statistical software for data analysis, GraphPad Prism 5.0 (Version X; La Jolla, CA, USA) was used for image editing. Comparison between groups was analyzed using *t*-test, measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$), and classification data was analyzed using chi-square test. $p < 0.05$ was considered statistically significant; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Results

Detection of miR-4421 Expression and Analysis of Clinical Data

The miR-4421 level in 42 PE tissues and 42 normal pregnancy placentas tissues were evaluated by qRT-PCR. It showed that the miR-4421 level in placenta of PE was higher compared with that of normal pregnancy ($p < 0.05$) (Figure 1A). PE patients were further classified into miR-4421 high and low expression group. Results suggested that there was lower systolic blood pressure (Figure 1C) and diastolic blood pressure (Figure 1D) in patients from miR-4421 high expression group than those of low expression group. In addition, we observed a lower proteinuria in the high expression group than the low expression group (Figure 1E). The birth weight of neonates in high expression group was significantly higher than that of low expression group (Figure 1F). No significant differences in birth weight and childbearing age among PE patients were found (Table I). These data suggested that miR-4421 may function in the progression of PE.

MiR-4421 Promotes the Progression of PE

Firstly, the miR-4421 level in HTR-8/SVneo, JEG-3, Wish and Bewo cell lines was detected. The miR-4421 expression was lowest in HTR-8/SVneo and highest in Bewo (Figure 2A). JEG-3 and Wish cells were selected for overexpression experiments. The expression of miR-4421 was significantly increased in JEG-3 and Wish cell lines transfected with pcDNA-MIR-4421 comparing to control transfected with empty vector for (Figure 2B and 2C). Next, CCK-8 assay indicated that the proliferation of JEG-3 and Wish cells was significantly decreased after overexpression of miR-4421 (Figure 2D and 2G). Flow cytometry was used to detect cell cycle, and results showed that JEG-3 and Wish cells were significantly arrested in GO/G1 phase after overexpression of miR-4421 (Figure 2E and 2F). Furthermore, cell apoptosis results demonstrated that the apoptosis rate of JEG-3 and Wish cells were significantly increased after overexpression of miR-4421 (Figure 2H and 2I).

MiR-4421 Inhibits the Mechanism of PE

To explore the mechanism by which miR-4421 promotes PE, CYP11B2 was predicted and selected as target gene of miR-4421 for further study (DIANA, miRanda, PicTar) (Figure 3A). It was found that miR-4421 reduced relative luciferase activity of CYP11B2 (Figure 3B, C), indicating that CYP11B2 was the target gene of miR-4421. Figure 3D and 3E showed that the upregulation of miR-4421 can reduce the expression of CYP11B2, which further indicated that miR-4421 regulated the expression of its target gene CYP11B2. Western blot showed that after overexpression of miR-4421 the protein expression of CYP11B2 decreased, as well as the expression of cyclin CDK2 (Figure 3F, G). These studies showed that miR-4421 regulated the progression of PE by regulating CYP11B2.

Discussion

Polymorphisms of the transcriptional regulatory region of aldosterone synthase gene (CYP11B2) and the second intron have been recently reported[12]. Tamaki et al[13] showed that TC + CC genotype was significantly lower in hypertensive

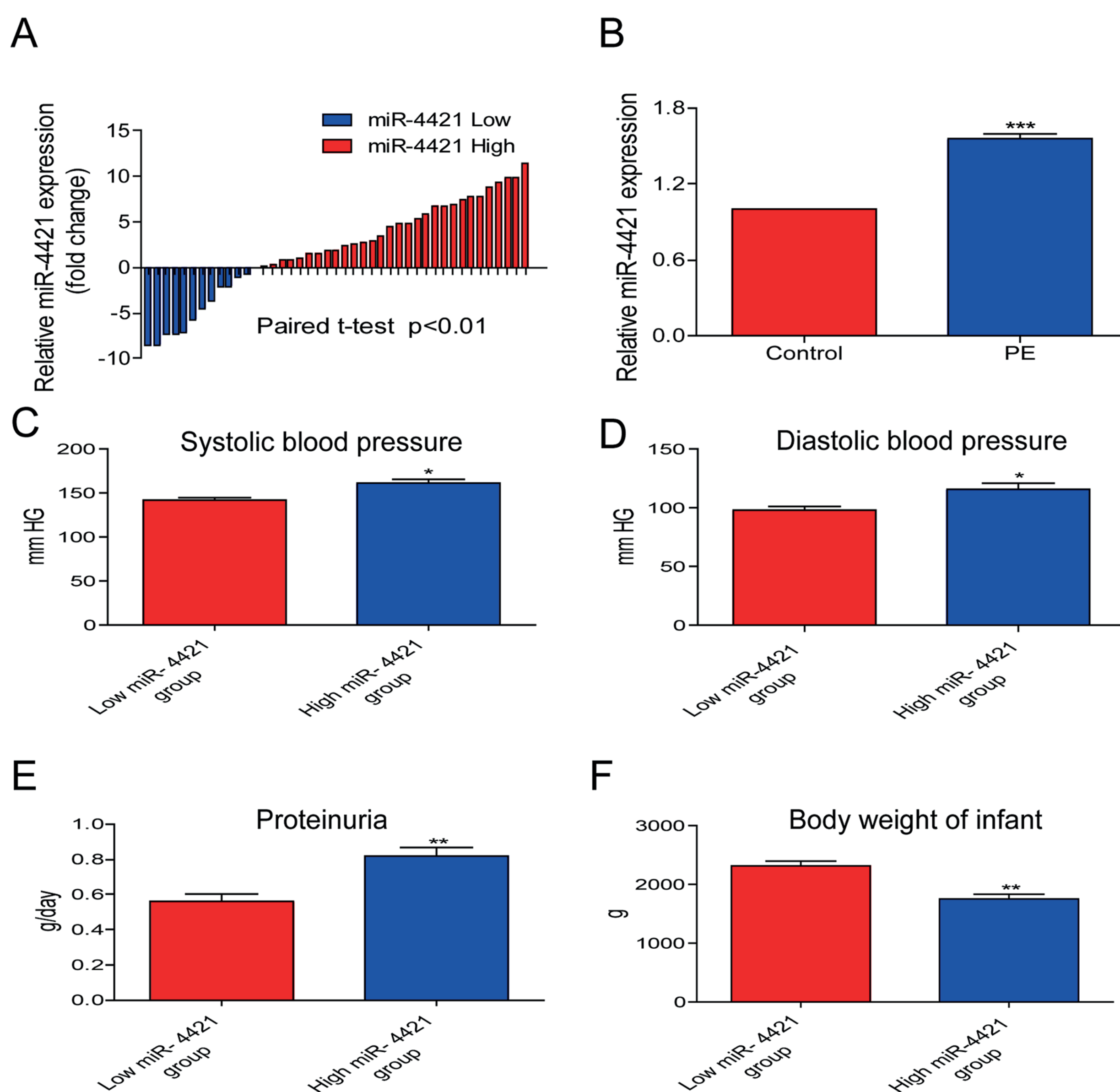

Figure 1. The relationship between overexpressed miR-4421 in PE patients and their clinical data. ***A,*** and ***B,*** Expression of miR-4421 in placenta of PE patients was significantly higher than that of normal group. ***C,*** Systolic blood pressure was significantly higher in patients from miR-4421 high expression group than those in low expression group. ***D,*** Diastolic blood pressure was significantly higher in patients from miR-4421 high expression group than in those in low expression group. ***E,*** Urine protein in patients from miR-4421 high expression group was significantly higher than those in low expression group. ***F,*** Newborn weight in patients from miR-4421 high expression group was significantly lower than those in low expression group.

Table I. Clinical characteristics of normal and pre-eclamptic pregnancies.

Variable	PE (n = 42)	Control (n = 42)	*p*-value
Maternal age (year)	30.12 ± 3.98	32.36 ± 4.87	> 0.05
Maternal weight (kg)	67.56 ± 9.84	69.71 ± 8.75	> 0.05
Proteinuria (g/day)	> 0.3	< 0.3	< 0.05
Systolic blood pressure (mm Hg)	155.44 ± 14.32	114.31 ± 16.47	< 0.05
Diastolic blood pressure (mm Hg)	108.68 ± 17.64	76.59 ± 18.25	< 0.05
Body weight of infant (g)	2015.78 ± 486.78	3495.67 ± 597.61	< 0.05

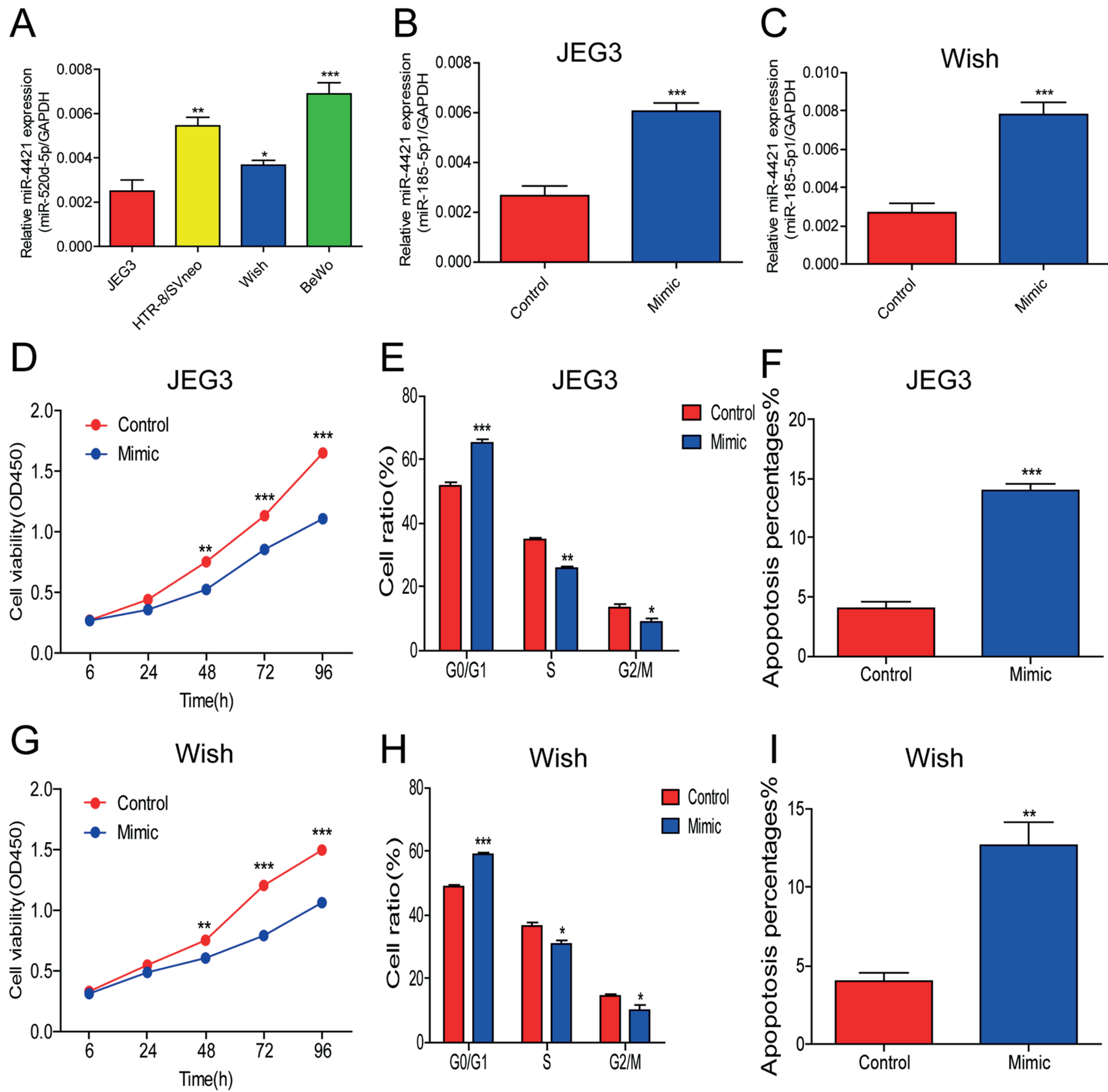

Figure 2. miR-4421 inhibits trophoblast cell proliferation. ***A,*** miR-4421 was lowly expressed in JEG3 and Wish cells. ***B,*** After transfection of mimic, miR-4421 expression was significantly increased in JEG3 cells. ***C,*** After transfection of mimic, miR-4421 expression in Wish cells was significantly increased. ***D,*** After overexpressing miR-4421, proliferation of JEG3 cells decreased significantly. ***E,*** After overexpressing miR-4421, cell cycle JEG3 was arrested. ***F,*** After overexpressing miR-4421, apoptosis of JEG3 cells increased. ***G,*** After overexpressing miR-4421, proliferation of Wish cells was significantly reduced. ***H,*** After overexpressing miR-4421, cell cycle of Wish was arrested. ***I,*** After overexpressing miR-4421, apoptosis of Wish cells increased.

patients than in normal people. The TC + CC genotype was significantly associated with high aldosterone plasma renin activity ratio (ALD/PRA) in patients with essential hypertension, and higher blood pressure. Therefore, aldosterone synthase gene (CYPIIB2) T (-344) C was considered as a genetic marker of low renin hypertension. Tsujita et al[14] reported that in a study conducted in another Japanese population, the frequency of the C allele in low renin hypertension was decreased, whereas other studies obtained different conclusions[6,15].

Recently, gene expression profiling and genome-wide sequencing have accelerated the iden-

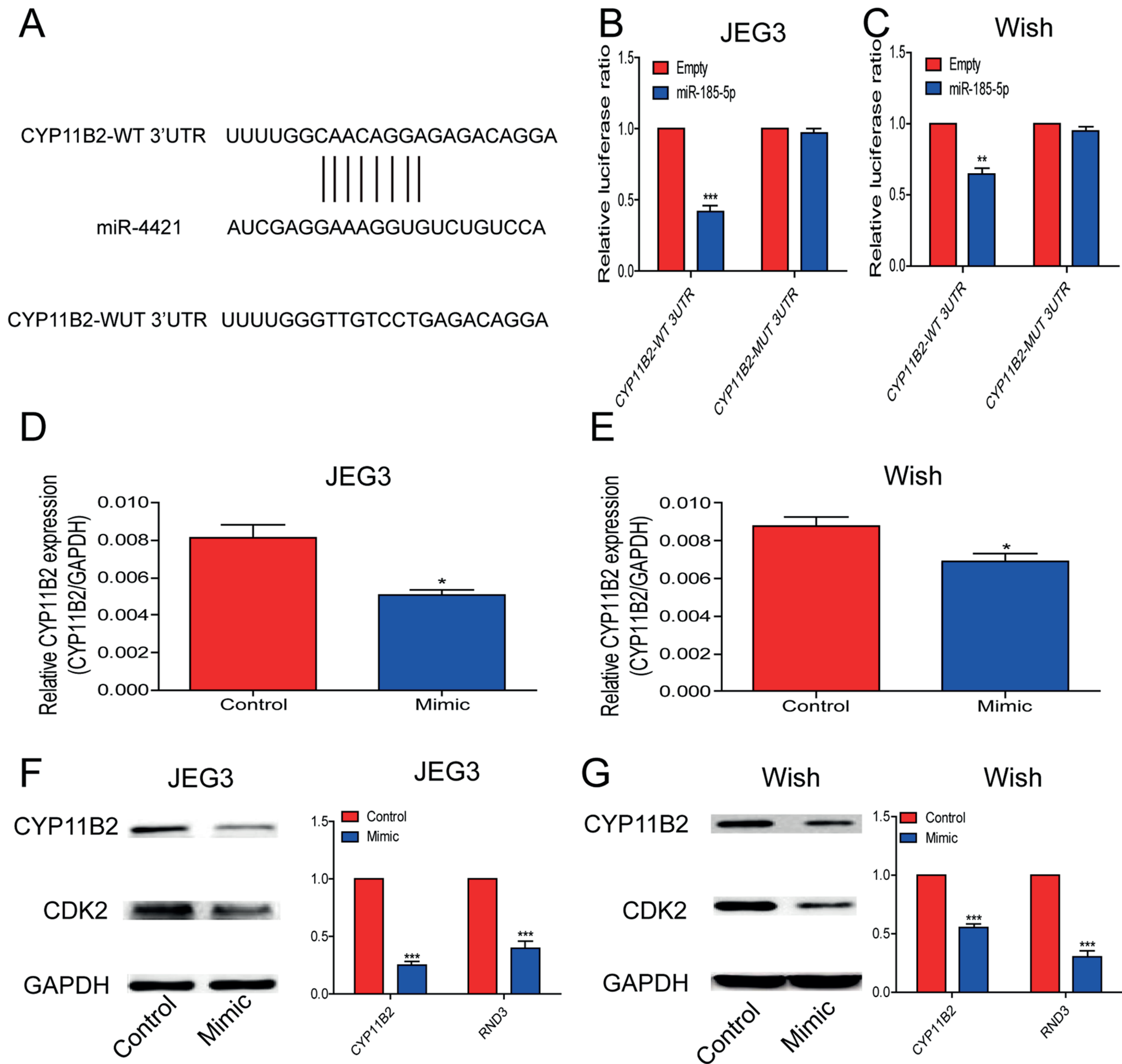

Figure 3. miR-4421 inhibits trophoblast cell proliferation. ***A,*** miR-4421 was lowly expressed in JEG3 and Wish cells. ***B,*** After transfection of mimic, miR-4421 expression was significantly increased in JEG3 cells. ***C,*** After transfection of mimic, miR-4421 expression in Wish cells was significantly increased. ***D,*** After overexpressing miR-4421, proliferation of JEG3 cells decreased significantly. ***E,*** After overexpressing miR-4421, cell cycle JEG3 was arrested. F, After overexpressing miR-4421, apoptosis of JEG3 cells increased. ***G,*** After overexpressing miR-4421, proliferation of Wish cells was significantly reduced. ***H,*** After overexpressing miR-4421, cell cycle of Wish was arrested. ***I,*** After overexpressing miR-4421, apoptosis of Wish cells increased.

tification of human genome transcription. MiRNAs are involved in the development of tumors by regulating the expression of target genes. Numerous studies have shown that overexpression of specific miRNAs in cancer cells may have potential antitumor effects[17], whereas others may have oncogenic effects[18]. Of note, they were widely involved in various pathological processes, such as invasion and metastasis of tumor cells[19-22]. The function of miR-4421, a newly discovered microRNA, has not been fully investigated.

This study found that miR-4421 expression in PE placenta tissues was higher than the normal group. Results suggested that miR-4421 may function in the pathogenesis of PE. To date, there have been many studies on the roles of miRNAs

in development and oncology, but there were relatively few studies on other areas, especially in pregnancy-related diseases, as well as association between miRNA and PE. We mainly investigated the differential expressions of miRNAs and their effects on trophoblast functions. Further experiments were carried out to explore its mechanism of regulating the pathogenesis of PE, which may enlighten early intervention of PE. We investigated the effect of miR-4421 on trophoblast function by overexpressing miR-4421. The results showed that up-regulated miR-4421 inhibited the growth of trophoblast and the cell cycle transformation. Some studies have reported that the genetic polymorphism of CYP11B2 (-344C> T) was involved in the progression of PE[2]. The luciferase reporter assay results showed that CYP11B2 had a binding site with miR-4421, and CYP11B2 was the target gene of miR-4421. Western blot further confirmed that after over-expression of miR-4421 CYP11B2 expression was significantly reduced. This result indicated that miR-4421 regulated the function of trophoblasts in PE placenta by regulating CYP11B2, which may influence the pathogenesis of PE. However, it is still unclear its specific regulatory role in the development of PE. Therefore, for the future work, it will be of significance to further explore the biological function of miR-4421 as well as its downstream regulatory mechanisms by expanding the sample size.

Conclusions

MiR-4421 was highly expressed in preeclampsia, which may promote the progression of preeclampsia by down-regulating the expression of CYP11B2.

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

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