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Influence of miR-34a on preeclampsia through the Notch signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to investigate the influence of micro-ribonucleic acid-34a (miR-34a) on preeclampsia through the Notch signaling pathway.

PATIENTS AND METHODS: The expressions of miR-34a, Notch-1, Notch-2, and Notch-3 in the placenta of 39 preeclampsia patients and 42 normal patients were detected by immunohistochemistry and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). The correlations between miR-34a expression with the expressions of Notch-1, Notch-2 and Notch-3 were analyzed, respectively. Besides, placental trophoblasts were isolated from preeclampsia patients and cultured *in vitro*. The expressions of miR-34a, Notch-1, Notch-2 and Notch-3 in placental trophoblasts were analyzed. Furthermore, the influences of miR-34a on the protein expressions of Notch-1, Notch-2, Notch-3, and hairy and enhancer of split-1 (Hes-1) in the Notch signaling pathway were analyzed by Luciferase reporter gene assay and Western blotting. The role of Notch in trophoblast invasion was investigated through the Notch inhibitors. In addition, its influence on the expression of urokinase-type plasminogen activator (uPA) was studied by miR-34a overexpression.

RESULTS: The expressions of miR-34a and Notch-1 were correlated with preeclampsia in the placentas of preeclampsia patients and normal patients to a certain degree. The expression of miR-34a in preeclamptic placenta was significantly higher than that of the normal placenta ($p<0.05$). However, Notch-1 expression was markedly lower in preeclamptic placenta ($p<0.05$). No significant differences were found in the expressions of Notch-2 and Notch-3 between the two types of placentas ($p>0.05$). MiR-34a had a remarkable negative correlation with Notch-1 expression in the Notch family ($p<0.001$, r=-0.5775). RT-PCR results revealed that the mRNA expression of miR-34a in placental trophoblasts of patients with preeclampsia was notably higher than that of normal people ($p<0.01$). However, Western blotting demonstrated that the protein expressions of Notch-1, Notch-2 and Notch-3 exhibited the opposite results. Additionally, the protein expression of Notch-1, Notch-2, Notch-3 and Hes-1 in trophoblasts transfected with pre-miR-34a was significantly decreased. The treatment with Notch inhibitors markedly reduced the trophoblast invasion. Furthermore, miR-34a overexpression or intracellular domain of Notch (ICN) overexpression regulated uPA expression.

CONCLUSIONS: MiR-34a regulates uPA system through the Notch signal transduction, thereby regulating the invasion of placental trophoblasts in patients with preeclampsia.

Key Words:
Preeclampsia, MiR-34a, Notch signaling pathway, Cell invasion.

Introduction

Preeclampsia is a complication that seriously affects pregnancy and threatens the health of pregnant mothers and fetuses[1-3]. It may cause changes in endothelial functions such as hypertension, proteinuria and edema in maternal body[4], and even impair fetal growth in severe cases[5,6]. In patients with preeclampsia, placental trophoblasts usually result in increased vascular resistance and decreased placental perfusion[7]. Preeclampsia syndromes reveal the importance of trophoblast differentiation and invasion[8,9]. Biopsy of uterine wall in patients with preeclampsia has manifested that invasive trophoblasts cannot be up-regulated to promote invasion[10]. However, the possible underlying mechanism remains unclear.

Micro-ribonucleic acid-34a (miR-34a) is closely correlated with cell proliferation, differentiation and invasion. However, its physiological function has not been fully elucidated. MiR-34a is one of the best studied cancer-associated miR-

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NAs. Previous studies[11-13] have shown that miR-34a is usually down-regulated in neuroblastoma, colon cancer and non-small cell lung cancer cell lines. MiR-34a is located on chromosome 1p36.23, where a variety of cancers are involved. The anti-tumor function of miR-34a can be p53- or p53-path-dependent[14]. Notch receptors have transcription factors on the cell surface, which regulate gene expression in the nucleus. After ligand binding, the Notch receptor protein is cleaved, releasing the intracellular domain of Notch (ICN)[15] and transferring to the nucleus. This may eventually control cell differentiation, proliferation, apoptosis, adhesion and angiogenesis16. In addition, abnormal Notch signaling is related to cell invasion[17,18].

The role of miR-34a in placental trophoblasts of preeclampsia remains unclear. Meanwhile, its regulatory effect on the Notch signaling pathway in preeclampsia of placenta still needs to be studied. Therefore, the aim of this work was to investigate the effect of miR-34a on the invasion ability of preeclamptic placental trophoblasts.

Patients and Methods

Main Reagents and Equipments

Antibodies against Notch1, Notch-2, Notch-3, hairy and enhancer of split-1 (Hes-1) and β-actin were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK); EasyPure® miRNA kit and complementary deoxyribonucleic acid (cDNA) reverse transcription kit from TransGen Biotech (Beijing, China); TaqMan MiRNA Assays and TaqMan® Gene Expression Assays from ABI (Foster City, CA, USA); Notch inhibitors from Abcam (Cambridge, MA, USA); and SpectraMax M5 microplate readers from Molecular Devices Co., Ltd. (Shanghai, China).

Data Source

Placenta samples of 39 clinical preeclampsia patients and 42 normal patients were obtained from the Nanjing Maternity and Child Health Care Hospital. Signed informed consent was obtained from each patient before the study. Collected placentas were rapidly frozen and fixed with paraformaldehyde for immunohistochemistry or preparation of trophoblast for isolation and culture, respectively. This study has been approved by the Ethics Committee of Nanjing Maternity and Child Health Care Hospital.

Methods

Immuno-Histochemical Detection of Notch 1, Notch 2, and Notch 3 in Placental Tissues

The sections were fixed in pre-cooled paraformaldehyde, washed with phosphate-buffered saline (PBS) and sealed with 0.3% bovine serum albumin (BSA)-PBS. Then, the sections were reacted with antibodies against members of the Notch family (Notch-1, Notch-2 and Notch-3) at 4°C for 12 h. Subsequently, the sections were rinsed in PBS and incubated with biotin-labeled second antibody for 1h. The results were observed using 3,3'-diaminobenzidine (DAB) reaction (R&D Systems, Minneapolis, MN, USA). Finally, the sections were photographed by a Leica DM5000B microscope.

Expressions of Notch-1, Notch-2, Notch-3, and Hes-1 Detected via Western Blotting

Cells were immersed in radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) and phenylmethanesulfonyl fluoride at 4°C for 30 min. The protein concentration was measured according to the instructions of the bicinchoninic acid (BCA) assay (Abcam, Cambridge, MA, USA). The protein expressions of Notch-1, Notch-2, Notch-3, Hes-1, and β-actin were detected by specific antibodies against Notch-1, Notch-2, Notch-3, Hes-1 and β-actin, respectively.

Expression of MiR-34a Detected via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted in strict accordance with EasyPure® miRNA Kit. The cDNA template was synthesized using a high-capacity cDNA reverse transcription kit. MiRNAs and messenger RNAs (mRNAs) were quantified by TaqMan MiRNA Assays and TaqMan Expression Assays, respectively. Real Time-PCR was performed in the Applied Biosystems 7500 detection system (Applied Biosystems, Foster City, CA, USA). For miRNA, Ct values were normalized to RNU6B. The expression of mRNA was quantified by the same method, and the relative level was normalized to 18S. Primer sequences used in this study were as follows: miR-34a, F: 5'-CATATGTAAACATCCTCGACTG-3', R: 5'-CCTTTGTGTCGCCCAGTCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Luciferase Reporter Gene Assay

According to the nucleotide sequence of the potential miR-34a binding region identified by Targets CAN 4.2 on Notch, oligonucleotides were synthesized. Luciferase reporter gene constructs and mutants in the three prime untranslated regions (3'UTRs) of Notch-1, Notch-2, and Notch-3 at the potential binding sites of miR-34a were constructed. Subsequently, constructed plasmids were co-transfected with pre-miR-34a or pre-miR scramble and β-gal expression vectors into trophoblasts. The digestion sites of HindIII and SpeI were added to the 5' and 3' ends of binding sites in the 3'UTR of predicted targets. Oligonucleotides were annealed and digested with HindIII and SpeI, respectively. Pre-miR-34a was applied to produce mature miR-34a in transfected cells. The control precursor (pre-miR scramble) was a random nucleotide sequence with similar size to pre-miR-34a. It has been widely verified in various human cell lines and tissues, and has no significant impact on known miRNA functions. After 48 h, the cells were lysed, and the Luciferase activity in the lysate was measured by a SPECTRA MAX M5 microplate reader.

Cell Invasion Assay

Cells were pretreated with dimethyl sulfoxide for 24 h and were then inoculated into the upper compartment of the serum-free medium intrusion chamber. The lower compartment was filled with conventional medium containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). To evaluate the effect of Notch on the cell invasion ability, the transfected cells were inoculated into the upper compartment of invasion chamber supplemented with Notch inhibitors. As mentioned above, the lower compartment was filled with normal culture medium. After 24 h, the remaining cells in the upper compartment were removed by cotton swabs, and the cells invading the matrix were stained. Then the stain in cells was dissolved in 10% acetic acid. Finally, the absorbance was measured by an enzyme-linked immunosorbent assay (ELISA) plate reader (R&D Systems, Minneapolis, MN, USA).

Overexpression of MiR-34a and Transfection of ICN Expression Plasmids

To examine the effect of the miR-34a overexpression, pre-miR-34a or pre-miR scramble was transfected into cells. The cells were transfected with ICN expression plasmids. 24 h before transfection, the cells were seeded into 12-well culture plates at a density of 5×105 with 50nM pre-miR-34a, pre-miR scramble and ICN expression plasmids. Subsequently, total protein was extracted from the transfected cells. Western blotting was used to detect the protein expression of urokinase-type plasminogen activator (uPA).

Statistical Analysis

GraphPad Prism 5.0 (La Jolla, CA, USA) was used for data analysis. Experimental data were expressed by mean ± standard deviation. The correlation between miR-34a expression and Notch-1 expression in the placenta of clinical preeclampsia patients was analyzed by linear correlation analysis. All other correlations were tested by the t-test. $p<0.05$ was considered statistically significant, and $p<0.01$ reflected that the difference was extremely significant.

Results

Expressions of Notch Family Proteins and MiR-34a in Placenta of Preeclampsia Patients

First, the expressions of Notch family proteins in normal placenta and preeclampsia placenta were detected and compared. The results revealed that the expression of miR-34a in the normal placenta was markedly lower than that of the preeclampsia placenta ($p<0.05$). Meanwhile, the expression of Notch-1 in the normal placenta was significantly higher than that of the preeclampsia placenta ($p<0.05$) (Table I). No significant differences were found in the expressions of Notch-2 and Notch-3 between normal and preeclampsia placentas ($p>0.05$).

Correlation Between MiR-34a Expression and Notch-1 Expression in Preeclampsia Patients

To further analyze the relationship between miR-34a and Notch family protein expressions in the placenta of clinical preeclampsia patients, a linear correlation analysis was conducted between miR-34a expression and the expressions of Notch-1, Notch-2, and Notch-3. The results manifested that the miR-34a expression was negatively correlated with Notch-1 expression in the Notch family ($p<0.001$, r=-0.5775) (Figure 1). However, no significant correlations were found between miR-34a expression and the expressions of Notch-2 and Notch-3.

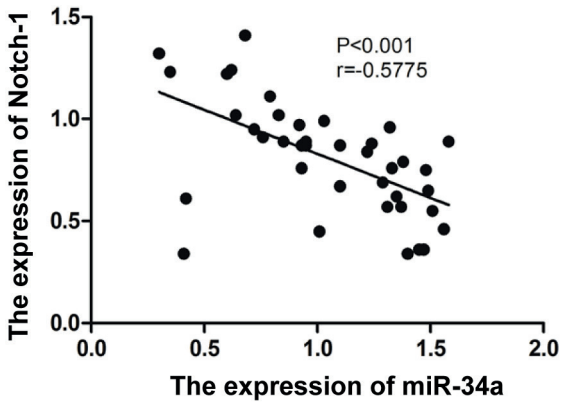

Figure 1. Correlation between miR-34a and Notch-1 expressions in preeclampsia patients. MiR-34a was negatively correlated with Notch-1 expression in Notch family (p<0.001, r=-0.5775).

Expressions of MiR-34a, Notch-1, Notch-2, and Notch-3 in Placenta Trophoblasts of Preeclampsia Patients

To study the expressions of miR-34a and Notch-1 in placental trophoblasts of preeclampsia, trophoblasts were isolated from placentas of preeclampsia patients and normal placenta, respectively. The expressions of miR-34a mRNA and Notch-1, Notch-2, and Notch-3 proteins were detected using quantitative RT-PCR (qRT-PCR) and Western blotting, respectively. It was found that the mRNA expression of miR-34a in placental trophoblasts of preeclampsia patients was notably higher than that of normal people (p<0.01) (Figure 2A). However, the protein expressions of Notch-1, Notch-2, and Notch-3 in placental trophoblasts of preeclampsia patients were significantly lower than those of normal placental trophoblasts (Figure 2B-2D).

MiR-34a Regulated the Expressions of Notch-1, Notch-2, and Notch-3

To demonstrate the regulatory effect of miR-34a on Notch-1, Notch-2, and Notch-3, Luciferase reporter gene constructs and mutants in the 3'UTRs of Notch-1, Notch-2, and Notch-3 at the potential binding sites were constructed. Subsequently, they were co-transfected with pre-miR-34a or pre-miR scramble and β-gal expression vectors into trophoblasts. Obtained Luciferase signal was normalized to respective β-gal activity. The results indicated that, compared with pre-miR scramble, Luciferase activities in the 3'UTRs of Notch-1, Notch-2 and Notch-3 were markedly reduced after transfection with pre-miR-34a (Figure 3A-3B). However, pre-miR scramble and pre-miR-34a did not affect the Luciferase activity of mutants. Meanwhile, no significant difference was found in the Luciferase activity between miR-34a mutant and control.

Western blotting analysis showed that the protein expression levels of Notch-1, Notch-2, and Notch-3 in pre-miR-34a transfected trophoblasts were decreased by 35%, 22% and 13%, respectively (Figure 4A-4D). The expression of Notch target gene Hes-1 was also affected. The results demonstrated that Hes-1 expression was significantly decreased in pre-miR-34a transfected

Table I. Expressions of miR-34a, Notch-1, Notch-2, and Notch-3 in the preeclampsia placenta.

		MiR-34a expression (%)			Notch-1 expression (%)		
Group		High expression	Low expression	p	High expression	Low expression	p
Normal placenta	42	15 (35.71)	27 (64.29)	0.032	30 (72.14)	12 (28.57)	0.002
Preeclampsia placenta	39	22 (56.41)	17 (43.59)		14 (35.90)	25 (64.10)	
		Notch-2 expression (%)			Notch-3 expression (%)		
Group		High expression	Low expression	p	High expression	Low expression	p
Normal placenta	42	22 (52.38)	20 (47.62)	0.887	18 (42.86)	24 (57.14)	0.212
Preeclampsia placenta	39	20 (51.28)	19 (48.72)		19 (49.72)	20 (51.28)	

Note: The expressions of miR-34a in the normal placenta and the preeclampsia placenta are significantly different (p<0.05). The difference in the expression of Notch-1 is significantly different (p<0.01). There are no significant differences in the expressions of Notch-2 and Notch-3 between the normal placenta and the preeclampsia placenta (p>0.05).

cells (Figure 4E). Therefore, changes in miR-34a expression not only affected the Notch 1 expression, but also the Notch signal transduction.

Notch Expression Regulated the Invasion of Trophoblasts

Cultured trophoblasts were then treated with Notch inhibitors. The influence of inhibiting Notch activation on the invasion of trophoblasts was evaluated. As shown in Figure 5, the results demonstrated that treatment with Notch inhibitors significantly reduced trophoblast invasion ($p<0.01$). This proved that Notch family members played a role in this process. In brief, these results confirmed that the Notch activity promoted trophoblast invasion.

Overexpression of MiR-34a Inhibited uPA Expression

ICN can activate transcription by interacting with CBF-1 through its RBP-Jκ/CBF1-associated module (RAM) and ankyrin (ANK) domains, thus relieving transcription inhibition. Besides, some studies have shown that the uPA system is related to the production of trophoblasts. In this work, qRT-PCR was adopted to determine the influences of pre-miR-34a and ICN plasmid transfected cells on uPA expression. The resul-

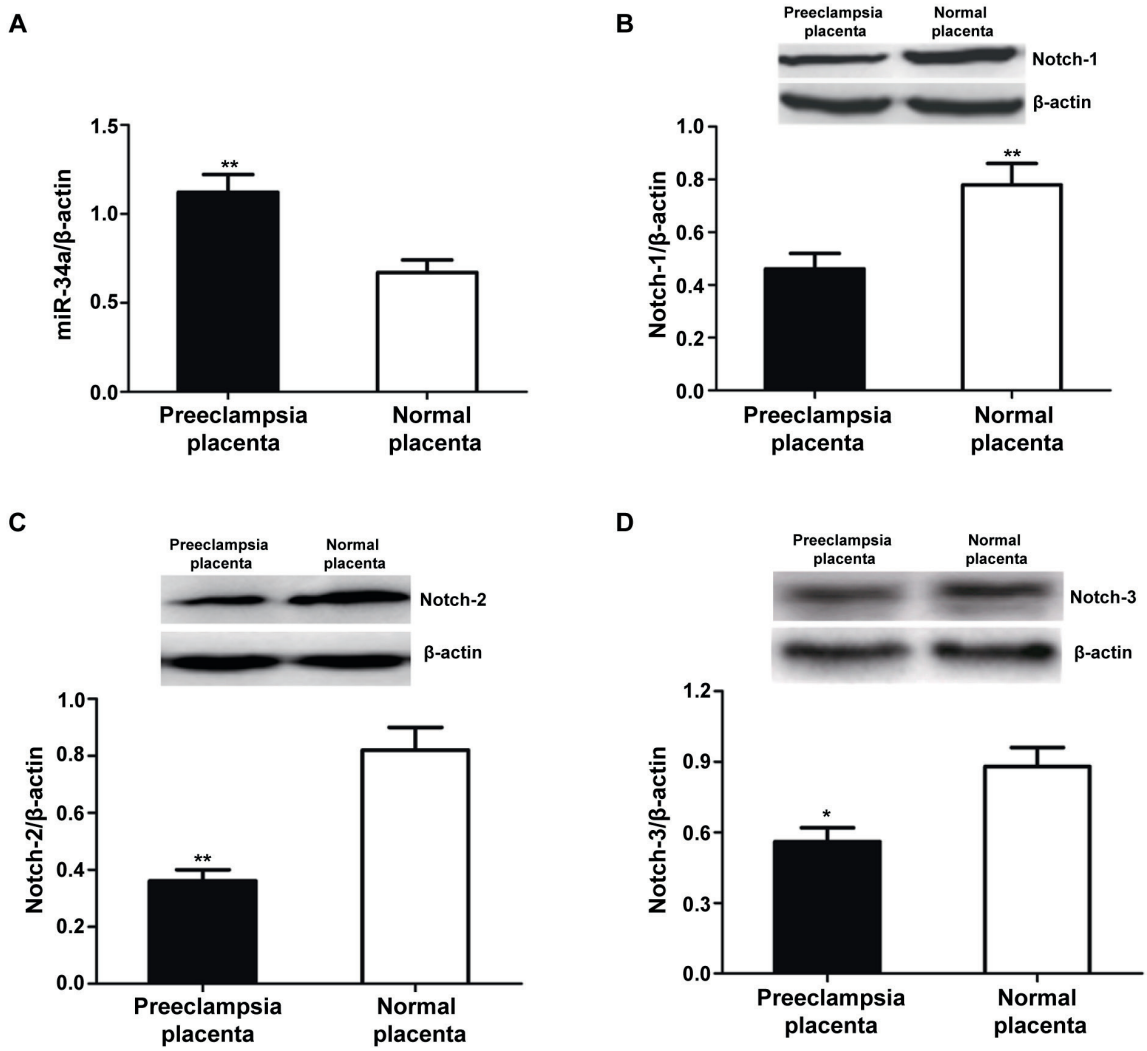

Figure 2. Expressions of miR-34a, Notch-1, Notch-2, and Notch-3 in trophoblasts. ***A,*** QRT-PCR results revealed that the expression of miR-34a in preeclampsia placenta was markedly increased compared with that of normal placenta ($p<0.01$). ***B-D***, Western blotting results showed that the expressions of Notch-1, Notch-2, and Notch-3 in preeclampsia placenta were significantly decreased compared with those of normal placenta (**$p<0.01$, *$p<0.05$).

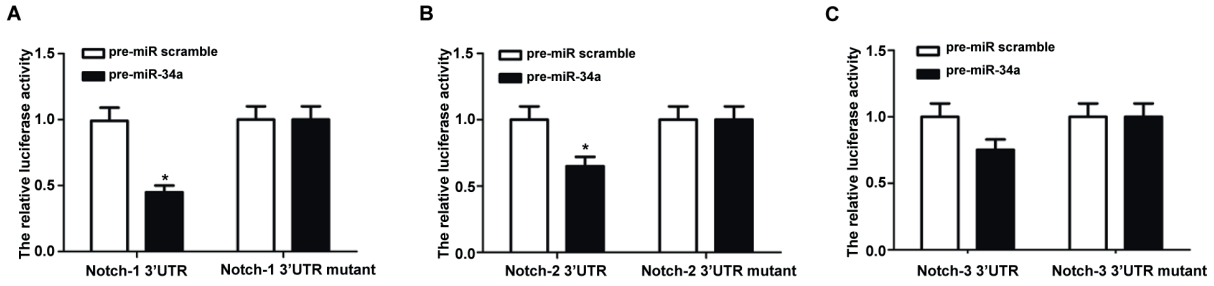

Figure 3. Determination and verification of the regulation of miR-34a on the expressions of Notch-1, Notch-2, Notch-3, and Hes-1 via Luciferase reporter gene assay. Luciferase activity assay was applied to determine the regulatory effect of miR-34a on Notch-1, Notch-2 and Notch-3 (*p<0.05).

ts showed that uPA expression was remarkably reduced after transfection with pre-miR-34a (Figure 6) (p<0.05). However, when cells were transfected with ICN expression plasmids, uPA expression was significantly increased (p<0.01). Therefore, miR-34a might regulate uPA expression through the Notch signal transduction, eventually regulating cell invasion.

Discussion

To study the influences of miR-34a and the Notch signaling pathway on preeclampsia, clinical preeclampsia cases were first investigated in this study. According to the results, miR-34a expression in the placenta of preeclampsia patients was significantly higher than that of the normal placenta. However, the expressions of the Notch family receptor proteins (Notch-1, Notch-2, and Notch-3) in the placenta of preeclampsia patients were markedly lower than those of the normal placenta. Meanwhile, the expression of miR-34a was significantly negatively correlated with the Notch-1 expression. The intrinsic relationship between miR-34a and Notch attracted our attention. Therefore, we assumed that miR-34a might affect the occurrence of preeclampsia through the Notch signaling pathway. Firstly, pre-miR-34a was transfected to induce overexpression of miR-34a. Subsequently, its influence on the expressions of Notch-1, Notch-2, Notch-3, and Hes-1 proteins was verified. Interestingly, the results manifested that the overexpression of miR-34a inhibited the expressions of Notch-1, Notch-2, Notch-3, and Hes-1 proteins in transfected cells. This confirmed that Notch receptors were targets of miR-34a. Previous studies have shown that Notch-1 is the target of miR-34a. However, there is no conclusive evidence of this relationship[19]. In this study, Luciferase activity detection provided supporting evidence for miR-34a binding to the potential 3'UTR binding sites of the Notch family proteins. Furthermore, it was found that the expression of Hes-1 in downstream molecules of Notch signal transduction was also affected by miR-34a induction. These findings indicated that miR-34a affected the Notch signaling pathway.

To further verify the impact of miR-34a on preeclampsia development through the Notch signaling pathway, the invasiveness of placental trophoblasts in preeclampsia was explored. The results illustrated that the overexpression of miR-34a remarkably decreased the invasion ability of trophoblasts by regulating the Notch signal transduction. The result is consistent with reported invasion of choriocarcinoma cells. Cell invasion occurs as a normal event in many physiological processes. During pregnancy, cytotrophoblasts differentiate into syncytiotrophoblasts and extravillous cytotrophoblasts. Inadequate invasion of uterine wall can lead to preeclampsia and intrauterine growth retardation. Notch-1 and its ligands are expressed in trophoblast cells and cytotrophoblasts[20,21]. Subsequently, our findings demonstrated that miR-34a reduced cell invasion by regulating Notch expression. Furthermore, treatment with Notch inhibitors significantly reduced trophoblast invasion, proving that Notch family members played a role in this process. In brief, these results revealed that the Notch activity promoted trophoblast invasion.

UPA exerts a crucial role in the degradation of the extracellular matrix[22]. The degradation of extracellular matrix is related to angiogenesis, matrix infiltration, tumor cell shedding, invasion and escape of the circulatory system,

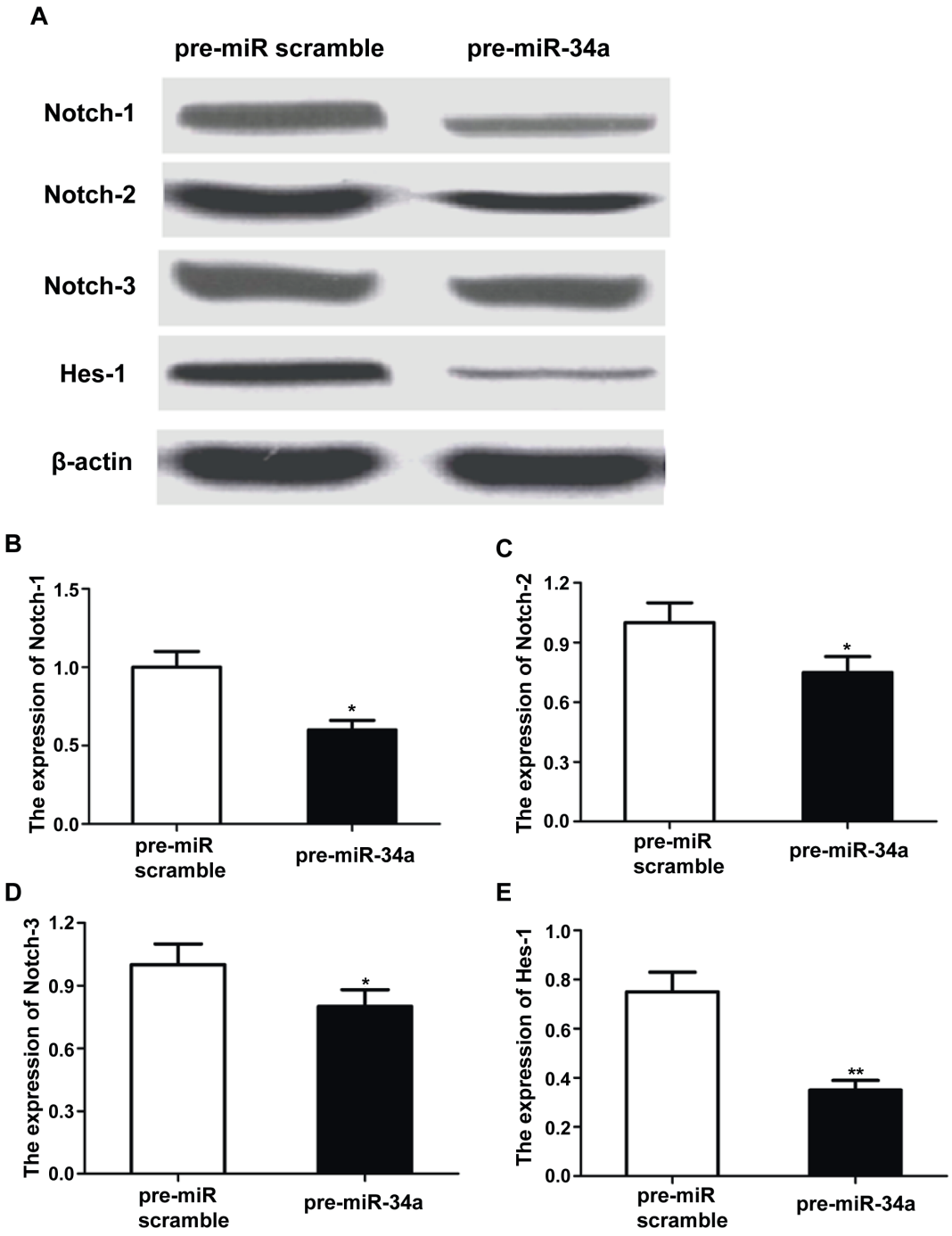

Figure 4. Regulation of miR-34a on the expressions of Notch-1, Notch-2, Notch-3, and Hes-1 verified by Western blotting (*p<0.05, **p<0.01).

secondary organ metastasis and environmental modification. In this study, changes in uPA level in cells transfected with pre-miR-34a and Notch ICN, or in cells with Notch-1 knockout were detected. The results indicated that the control of the Notch signal transduction on cell invasion was cell-dependent. In this work, transfection with Notch ICN expression plasmids eliminated the effect of miR-34a on cell invasion. However, miR-34a played a stronger role in inhibiting invasion than suppressing Notch-1 expression. This indicated that there were additional miR-

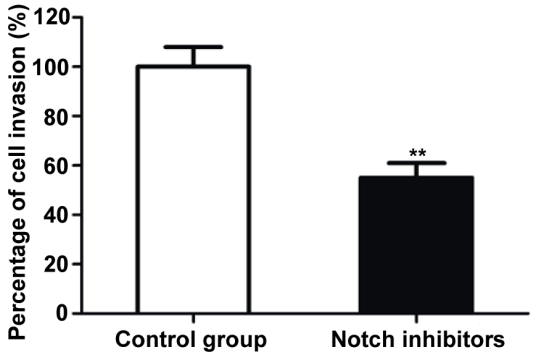

Figure 5. Influence of Notch expression on the regulation of trophoblast invasion. **$p<0.01$ *vs.* control group.

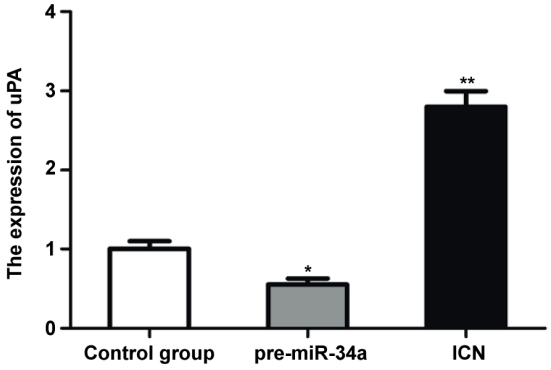

Figure 6. Influences of pre-miR-34a and ICN plasmid transfected cells on uPA expression. *$p<0.05$ vs. control group, **$p<0.01$ vs. control group.

34a targets controlling cell invasion. In fact, it has been proposed that single miRNA may regulate numerous target genes, and miRNA has the tendency to regulate gene families[23]. A recent study has also revealed that miR-34a inhibits the invasion of human hepatoma cell line HepG2 by down-regulating the expression of tyrosine kinase receptor c-Met[24,25]. The miR-34a gene targeted to control cell invasion needs to be further studied. Our findings showed that miR-34a regulated the invasion of placental trophoblasts in preeclampsia through Notch signal transduction, thereby regulating the uPA system.

Conclusions

We found that miR-34a regulated the uPA system through the Notch signal transduction to regulate the invasion of placental trophoblasts in patients with preeclampsia.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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