European Review for Medical and Pharmacological Sciences 2018; 22: 2199-2206

MiR-134 inhibits infiltration of trophoblast cells in placenta of patients with preeclampsia by decreasing ITGB1 expression

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Abstract. – OBJECTIVE: Preeclampsia (PE) is an idiopathic disorder of pregnancy. The specific regulatory mechanisms of microRNAs (miRs) in the placenta of PE patients have not yet been completely revealed. This study mainly explored the mechanism of miR-134 in preeclampsia.

PATIENTS AND METHODS: Real-time PCR and Western blot were used to detect the expression of miR-134 and ITGB1 in the placenta of patients with preeclampsia and normal pregnant women. Dual luciferase reporter assay was performed to detect luciferase activity in miR-134 and NC groups, respectively. Cell proliferation ability after transfection was evaluated by MTS colorimetric assay, and the effect of miR-134 on the infiltration of trophoblast cells was explored by cell invasion experiment. In addition, co-transfection of miR-134 and ITGB1 expression plasmids was carried out, and then changes in the cell invasiveness were also detected by cell invasion experiment.

RESULTS: Compared with placenta of normal pregnant women, miR-134 was significantly up-regulated in the placenta of patients with preeclampsia and negatively correlated with the expression of ITGB1. MiR-134 suppressed the infiltration of trophoblast cells by targeting ITGB1. When ITGB1 was overexpressed, the suppression of invasiveness of trophoblast cells by miR-134 was almost abolished. Meanwhile, we found that miR-134 inhibitor could promote the invasiveness of trophoblast cells. In addition, tumor necrosis factor-α (TNF-α) was found to enhance miR-134 expression as well as inhibit ITGB1 expression.

CONCLUSIONS: MiR-134 inhibited the infiltration of trophoblast cells in preeclampsia by down-regulating ITGB1 expression.

Key Words
MicroRNA-134, Preeclampsia, Cell invasion, ITGB1.

Introduction

Preeclampsia (PE) is an idiopathic disease during pregnancy characterized by newly developed hypertension and/or proteinuria after 20 weeks gestation. Its basic pathological changes are systemic microvascular constriction, causing tissues ischemia along with hypoxia, which may finally lead to systemic organ failure in some serious cases. Meanwhile, adverse pregnancy outcomes may occur, such as premature birth, placental abruption and fetal growth restriction, which make preeclampsia a serious threat to the health of expectant mothers and infants[1-3]. Researches have reported that preeclampsia affects 7-10% of pregnant women worldwide[4]. There are many theories about the pathogenesis of PE, but the specific mechanism has not been fully elucidated[5,6]. Pathological studies have shown that ischemia, hypoxia, and hypoperfusion in uterus and placenta often occur in the process of placental vascular remodeling[7]. Maternal-fetal interface plays an important role in the maintenance of pregnancy. The good balance between pro-angiogenic factors and anti-angiogenic factors at the maternal-fetal interface determines the development of the blood vessels, the process of placental vascular invasion and formation and the eventual success of pregnancy[8,9]. An imbalance between the two, however, can lead to undesirable pregnancy outcomes, such as PE, limit fetal growth, and preterm delivery[10]. MicroRNAs (miRNAs) are small non-coding RNAs that are evolutionarily conserved and ubiquitous. They can regulate various biological characteristics and activation of signaling pathways such as cell proliferation, cycle, differentiation and apoptosis through direct degradation

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of target gene or inhibition of target gene[11,12]. Studies have shown that in the pathological process of pregnancy-related diseases, expression profile of miRNAs has some abnormal changes[13-15]. Pineles et al[16] reported for the first time in 2007 that miRNAs were differently expressed in placenta of patients with preeclampsia compared with that of normal pregnant women, and the occurrence of preeclampsia was associated with changes in the expression of miRNAs in the placenta. Enquobahrie et al[17] found that miR-134 and miR-377 were highly expressed in the placenta of patients with preeclampsia. In addition, results of gene chips showed that the expression levels of miR-10b and miR-200c were significantly decreased in placental tissue of PE patients compared to normal placenta[18]. The pathological process of preeclampsia involves abnormal expression of multiple miRNAs. Each miRNA can target one or several mRNAs, and alternatively each mRNA may also be regulated by multiple miRNAs[19]. It has been found that miR-126 and miR-378 can upregulate pro-angiogenic factors by targeting FUS1, which causes remodeling of uterine spiral arteries and ultimately results in ischemia, hypoxia and hypoperfusion of PE placenta[20]. In addition, miR-30a has been reported to accelerate the apoptosis of trophoblastic cells, causing a superficial infiltration of the uterine artery and endometrium, which can lead to a superficial placental implantation and weakened functions of trophoblast secretion as well as material exchange. As a result, placental ischemia and hypoxia caused by above pathological process can potentially induce PE occurrence[21]. However, the specific regulation mechanism of miRNA in the placenta of PE patients has not yet been completely revealed. Therefore, it is of great significance to investigate the function and mechanism of miRNAs in PE.

Patients and Methods

Specimen Collection and Processing

Sterile placenta was delivered from uterus by cesarean section. Several placenta tissue pieces (1 cm × 1 cm × 1 cm) were taken from maternal surface of placenta near the root of umbilical cord (avoiding the machine stove, calcification, hemorrhage, etc.). The tissues were washed with phosphate-buffered saline (PBS) liquid repeatedly and were put in liquid nitrogen. After that, they were transferred to -80°C refrigerator for later use. This investigation was approved by the Ethics Committee of Linyi Hedong District People's Hospital. Signed written informed consents were obtained from all participants before the study.

Cell culture

Trophoblast HTR8/SVneo cells were cultured in RPMI-1640 (Roswell Park Memorial Institute-1640) (Vicente, Canada) containing 5% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) and placed in a 37°C, 5% CO_2 incubator. The medium was changed every other day until cells reached 80% confluence. The cells were then seeded in a 6-well plate (2 × 105/well) and cultured for 24 h. Subsequently, they were transfected with NC, miR-134 ITGB1, pcDNA-NC and pcDNA-miR-134, respectively, when they reached over 70% confluence.

RNA Isolation and Quantitative Polymerase Chain Reaction (PCR)

Total RNA was extracted from cells or tissue samples (Normal and PE placental tissue) with TRIzol (Invitrogen, Carlsbad, CA, USA) and RNA extraction kit. cDNA was synthesized by reverse transcription and Real-time PCR was performed to detect the mRNA expression of miR-134 and ITGB1. The sequences of the primers are as follows: miR-134 (Forward) 5'-CCTAAAGGCACGCTTCTTTG-3', miR-134 (Reverse) 5'-TGCAGGCTGGCGATCCTACT-3'; ITGB1 (Forward) 5'- ATCCCAGAGGCTCCAAAGAT-3'; 5'-CCCCTGATCTTAATCGCAAA-3'; GAPDH (Forward) 5'-AGGAGCGAGATCCCGCCAACA-3', GAPDH (Reverse) 5'-CGGCCGTCACGCCACATCTT-3'.

Cell Proliferation Assay

The four groups of cells mentioned in above methods were taken to analyze their ability of cell proliferation. After transfection for 24-72 h, the cells were seeded in a 96-well plate with 5000 cells per well and five replicates per treatment. 20 μL MTS were added to each well at 6, 24, 48, 72 and 96 h after the seeding, respectively. After incubation for 2 h, the absorbance (A) of each well was measured at 450 nm using Universal Microplate Spectrophotometer. The experiment was repeated three times.

Trophoblast Cells Infiltration Experiment

200 mg/mL Matrigel were paved in advance and 6xl04HTR8/SVneo cells in 0.1 mL medium were seeded in each chamber. Next, 0.6 mL of RPMI 1640 medium containing 10% FBS were added to the lower 24-well culture plate. After 24-48 h, the culture chamber was removed, washed with PBS, fixed with 0.25% pentylene glycol and stained with hematoxylin. At last, non-invading

cells were removed with cotton swabs and cells invading through the filter were counted and photographed in three randomly chosen fields.

Western Blot Assay

Radioimmunoprecipitation assay (RIPA) lysate was used to lyse cells to extract the total protein after transfection for 48 hours. Each protein sample was loaded in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in electrophoresis system. The gel was transferred to polyvinylidene difluoride (PVDF) membrane, the membrane was blocked and incubated with primary antibodies of miR-134 and ITGB1 (CST, Danvers, MA, USA 1:1000) at 4°C overnight. On the following day, protein bands were incubated with horseradish peroxidase (HRP)-labeled secondary antibody (Cell Signaling, Danvers, MA, USA, goat anti-rabbit IgG 1:5000) for 2 h at room temperature, and developed by enhanced chemiluminescence (ECL) (Biotech Co., Ltd. Encinitas, CA, USA). The integral optical density (IOD) value of each band was measured by a gel imaging analysis system and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. The relative expression of protein was calculated by the ratio of IOD in each band to GAPDH IOD.

Statistical Analysis

Statistical product and service solutions (SPSS 22.0, Armonk, NY, USA) statistical software was used for data analysis, and GraphPadPrism5.0 (La Jolla, CA, USA) for image editing. The measurement data were presented as mean ± standard deviation ($\bar{x}\pm s$), and *t*-test was used to analyze the difference between groups. $p<0.01$ was considered statistically significant; * $p<0.01$.

Results

Differential Expression of Mir-134 and Itgb1 In Placenta of Patients with Preeclampsia and Normal Pregnant Women

Real-time PCR and Western blotting were respectively used to detect the expression levels of miR-134 and ITGB1 in the placenta of patients with preeclampsia and normal pregnant women. The experimental results showed that the expression of miR-134 in placental maternal-fetal interface of patients with PE was significantly higher than that of normal pregnant women (Figure 1A), while the ITGB1 expression level presented the opposite tendency (Figure 1B). Western blotting revealed significant down-regulation of ITGB1 in the maternal surface of placenta in preeclampsia patients (Figure 1C, 1D).

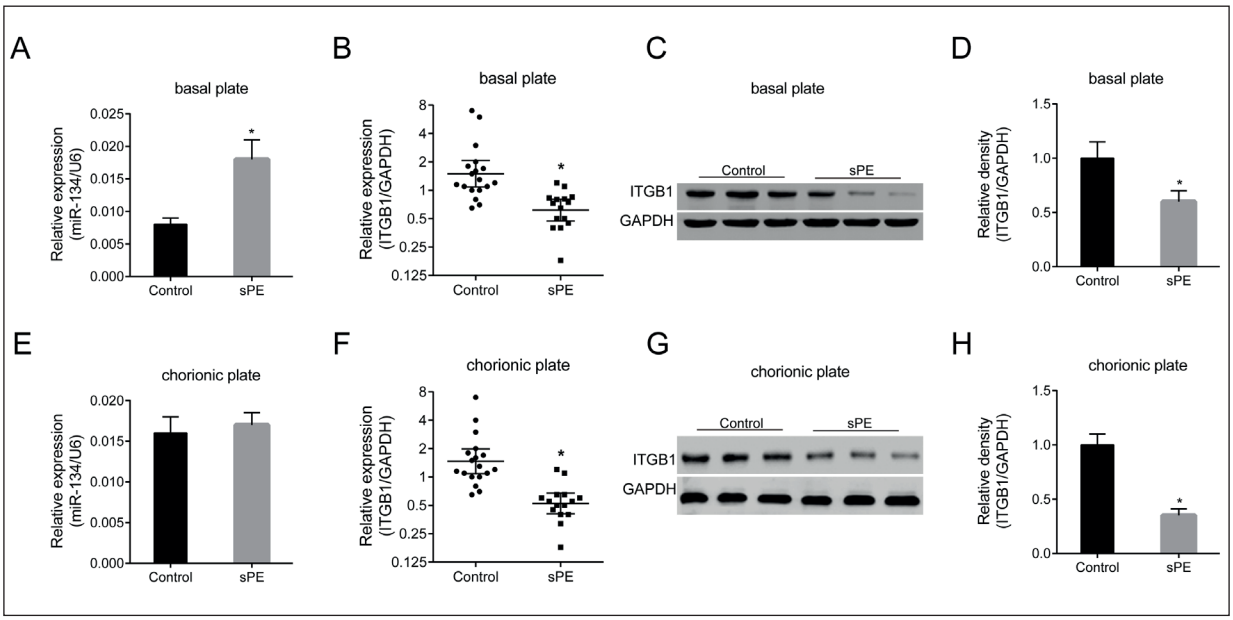

Figure 1. Expression levels of ITGB1 and miRNA-210 in placenta of normal pregnant women and patients with preeclampsia are shown. Real-time quantitative PCR (**A**, **B**, **E**, **F**) and Western blotting (**C**, **D**, **G**, **H**) were used to detect miR-134 and ITGB1 expression in placental maternal surface (**A**, **B**, **C**, **D**) and fetal surface (**E**, **F**, **G**, **H**) of normal pregnant women (n=18) and patients with severe preeclampsia (n=15). The expression levels of miR-134 and ITGB1 were corrected using U6 and GAPDH, respectively, as internal controls.

However, Western blotting assay demonstrated that compared with the placenta of normal pregnant women, the level of miR-134 in the fetal surface of PE placenta was not significantly changed (Figure 1E), while ITGB1 level was significantly down-regulated (Figure 1F-H).

MiR-134 Targets itgb1 Gene in Trophoblast Cells

Immunohistochemistry (IHC) results showed that ITGB1 was mainly located in the trophoblast cells of placenta, including trophoblastic cubic cells, villous trophoblast cells and endovascular trophoblast cells invaded into the uterine decidua matrix (Figure 2A-D). Its localization pattern in placenta was consistent with that of miR-134 in placenta. Therefore, we further investigated whether ITGB1 was a target gene of miR-134. MiR-134 was overexpressed in HTR8/SVneo by transfection, and then the mRNA and protein levels of ITGB1 were detected. The results showed that after miR-134 overexpression, the mRNA and protein levels of ITGB1 were significantly decreased compared with the NC group (Figure 2E-G).

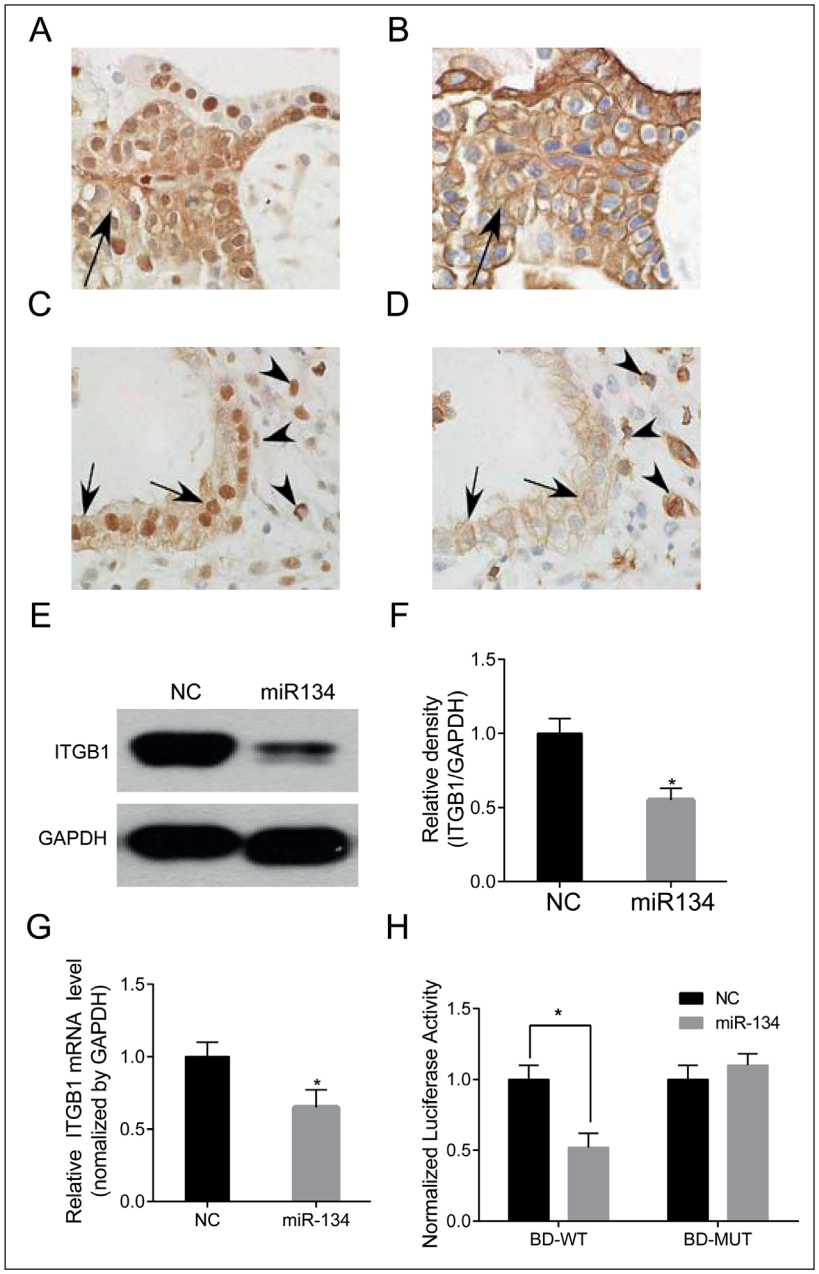

Figure 2. MiR-134 targeted ITGB1 gene in trophoblast cells. **A**-**D**, ITGB1 localization in the placenta is shown. Paraffin sections of early normal villus (**A** and **B**) and decidua (**C** and **D**) tissues were performed immunohistochemical staining. Arrows indicates the trophoblast cubic cells (**A** and **B**) and Arrowheads indicates the interstitial trophoblast cells (**C** and **D**). Figure scales are 200 nm for A and B, 50 nm for **C** and **D**. **E**, After transfection of NC and miR-134 mimic, Western blotting was used to detect the protein expression level of ITGB1 in trophoblast cells. **F**, After miR-134 mimics were over-expressed in HTR8/SVneo, Real-time PCR was used to detect the mRNA level of ITGB1. **G**, The ITGB1 BD-WT/BD-MUT luciferase reporter vector and NC or miR-134 were co-transfected into HTR8/SVneo cells, and luciferase activity was detected after 48 h with pRL-TK as an internal control.

To further validate our hypothesis, we performed a dual luciferase report assay. The results showed that compared with NC transfection group, transfection of miR-134 mimic significantly inhibited the luciferase activity of ITGB1I BD-WT but did not affect that of ITGB1 BD-MUT (Figure 2H). The above results indicate that ITGB1 was the target gene of miR-134 in trophoblast cells.

MiR-134 Significantly Inhibited Infiltration of Trophoblast Cells

MiR-134 mimic and inhibitor were respectively transfected into HTR8/SVneo cells, and Real-time PCR was employed to verify the transfection efficiency (Figure 3A-B). MTS assay was used to evaluate the influence of miR-134 on cell proliferation. As shown in Figure 3C-D, miR-134 didn't affect the proliferation of trophoblast cells. Subsequently, cell invasion assay was performed to confirm the effect of miR-134 on trophoblast cell invasiveness. As shown in Figure 3E, miR-134 could significantly inhibit the infiltration of trophoblast cells compared with the control group. In contrast, transfection of miR-134 inhibitor was found to dramatically promote the infiltration of trophoblast cells (Figure 3E).

To further investigate whether ITGB1 is a functional target gene of miR-134 in HTR8/SVneo cells, miR-134 and ITGB1 expression plasmids were co-transfected. Then, cell invasion ability results showed that the inhibitory effect of miR-134 on the invasive ability of trophoblast cells could be abolished by overexpression of ITGB1 (Figure 3F). It is further demonstrated that ITGB1 was involved in the process of miR-134 inhibiting trophoblast invasion.

The Inhibitory Effect of MiR-134 on ITGB1 was Regulated by TNF-α

miR-134 and ITGB1 expression levels were examined after HTR8/SVneo cells were treated with synthetic TNF-α. The results showed that

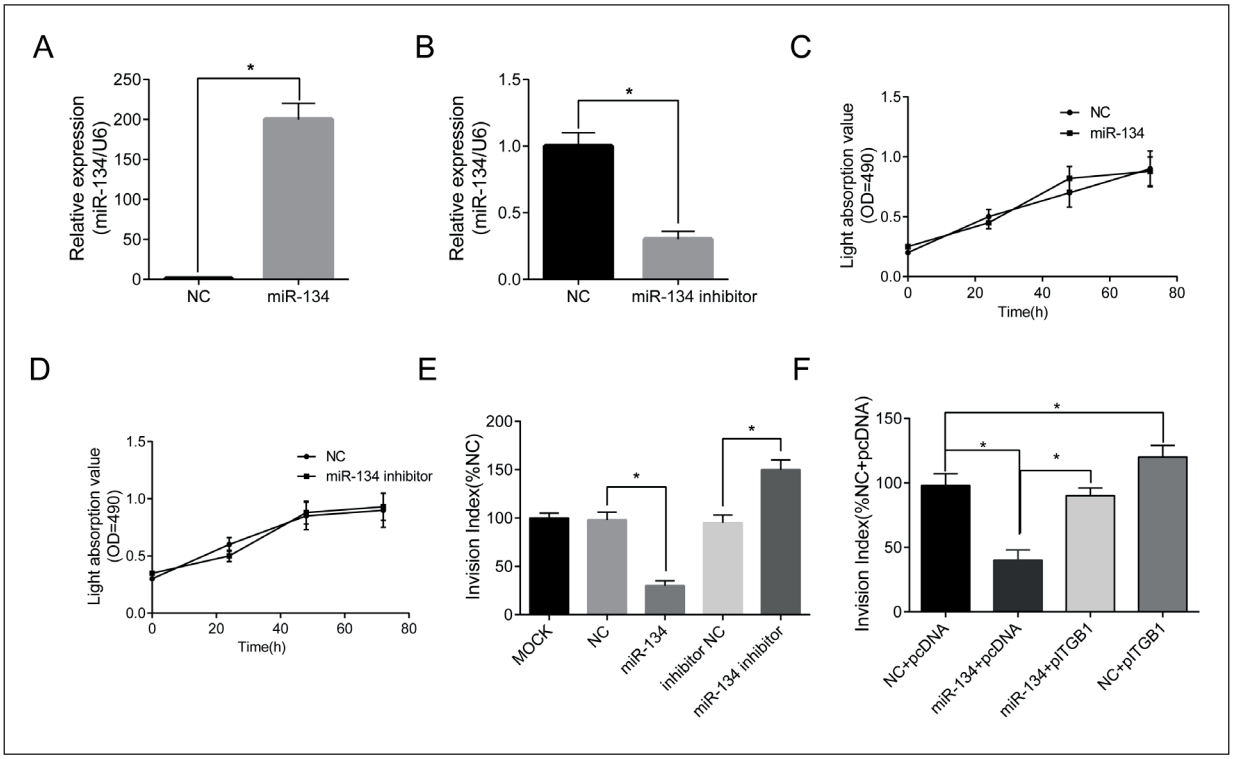

Figure 3. The effect of miR-134 on trophoblast cell proliferation and invasion is shown. **A-B**, After transfected miR-134 mimics or negative control or miR-134 inhibitor or inhibitor negative control into HTR8/SVneo cells, miR-134 expression was detected by Real-time quantitative PCR. The relative expression of miR-134 was corrected using U6 as the internal control. **C-D**, The effect of overexpression or inhibition of miR-134 on HTR8/SVneo cell proliferation was evaluated by MTS assay. **E**, The HTR8/Svneo cells were pre-treated with mitomycin **C** in order to inhibit their own proliferation, and followed by cell invasiveness detection. **F**, HTR8/SVneo cells were transfected with miR-134 and ITGB1, respectively, or co-transfected with negative control small RNA (NC) or empty plasmid control vector (PCDNA), respectively. The same cell invasion assay was used to detect the ability of cells infiltration.

miR-134 expression was enhanced dramatically in the TNF-α-treated cells (Figure 4A-B), while the level of ITGB1 dropped to 60-70% of that in the control group (Figure 4C-D). These findings suggested that TNF-α can improve the expression of miR-134 in placenta of patients with preeclampsia.

Discussion

MiRNAs have a high degree of evolutionary conservation, time regularity of expression, tissue specificity and gene clustering. However, abnormalities of miRNA expression may lead to the occurrence and development of a variety of diseases. The specific pathogenesis of PE has not yet been completely revealed. It was found that lots of pathological processes are involved in the occurrence and development of PE such as placental inflammatory response, oxidative stress, changes in signal pathways and immune dysfunction and many other processes. MiRNAs, as the body's important physiological activator, participate in the regulation of many pathological processes of PE. Some miRNAs are aberrantly expressed in the placenta of patients with preeclampsia compared to that of normal pregnant women[22-24], among which miR-134 was found significantly highly expressed in the placenta of patients with PE[24,25]. MiR-134 has been found to be abnormally expressed in many malignancies and involved in tumorigenesis, differentiation, proliferation, invasion and metastasis[26-28]. In non-small cell lung cancer, miR-134 showed a low expression in tumor tissues and its upregulation can inhibit epithelial-mesenchymal transition (EMT) by regulating the target protein FOXM1[29]. However, in head and neck neoplasm, miR-134 was found to have a high expression in tumor tissues and function as a tumorigenic agent to promote tumor metastasis by regulating WWOX (WW domain containing oxidoreductase) gene[30]. Therefore, miR-134 may have many different target genes and exert different biological effects through the regulation of its various target genes.

ITGB1 is a member of integrin beta subunit[31]. ITGB1 binds to fibronectin and laminin, two of

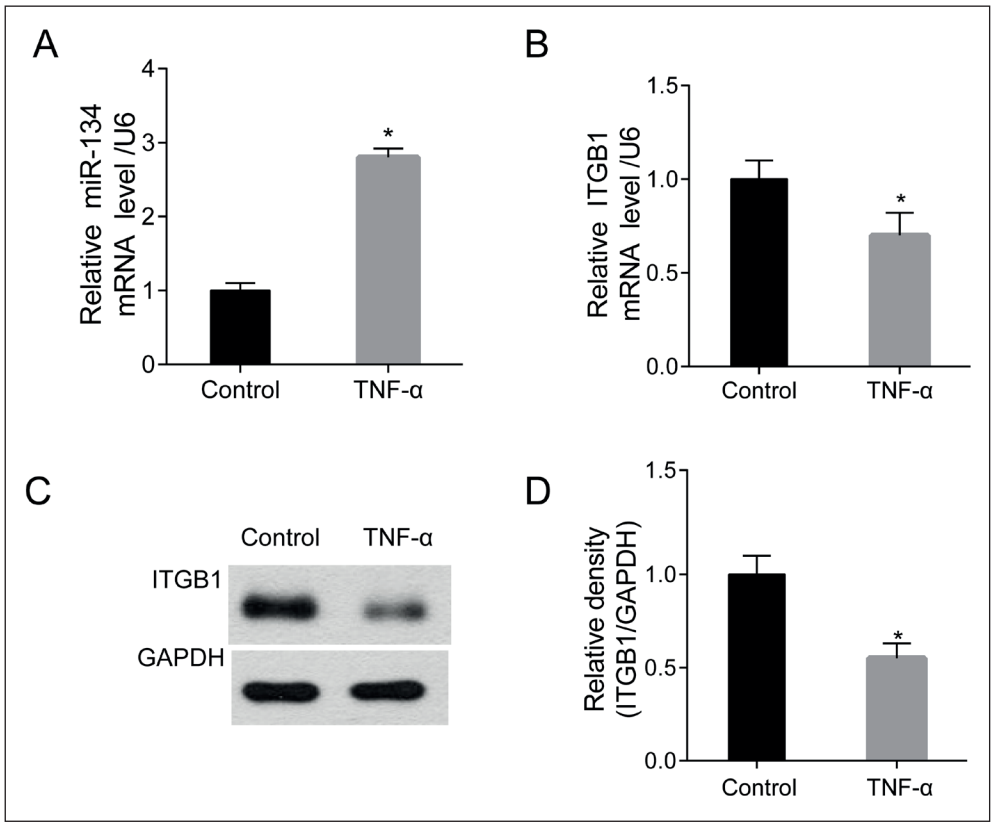

Figure 4. Inhibition of ITGB1 by MiR-134 was regulated by TNF-α. **A** and **B**, The levels of miR-134 **A**, and ITGB1 mRNA **B**, in trophoblast wells after TNF-α treatment were detected by Real-time quantitative PCR and the results are shown. **C**, ITGB1 protein expression in trophoblast cells treated with TNF-α was detected by Western blot and the result is presented.

extracellular matrix components, and is involved in the proliferation, apoptosis, migration, invasion and survival of tumor cells[32,33]. Studies have shown that the expression level of ITGB1 in tumor tissue was negatively correlated with the prognosis of patients[34] and ITGB1 was often overexpressed in malignant tumors. Although it has no kinase activity itself, ITGB1 can recruit and activate multiple intracellular signaling molecules such as FAK and c-Src, which can form a kinase complex to activate multiple downstream proteins such as pl30Cas and paxilline, in order to promote tumor cell proliferation, invasion and metastasis and chemoradiation resistance[33, 35]. In this work, the expression of ITGB1 and miRNA-210 in the placenta of preeclamptic patients was compared with their expression in the placenta of normal pregnant women. Furthermore, we studied miR-134 in human immortalized trophoblast cell line HTR8/SVneo and found that miR-134 affected the invasiveness of trophoblast cells by regulating the expression of ITGB1. MiR-134 was significantly upregulated in the placenta of patients with preeclampsia compared with normal pregnancy placenta and negatively correlated with the expression of ITGB1. In addition, we confirmed ITGB1 as miR-134 target in the trophoblast cell line HTR8/SVneo by luciferase reporter assay. MiR-134 inhibited the infiltration of trophoblast cells, whereas over-expression of ITGB1 could repress the inhibitory effect of miR-134. In contrast, miR-134 inhibitor contributed to the invasiveness of trophoblast cells. The mechanism of the upregulation of miR-134 in placenta of patients with PE is still poorly understood. TNF-α is known to be able to induce the expression of miR-134 in other cell lines, and this inflammatory cytokine has also been reported to have a significant increase in placental maternal-fetal interface of patients with PE[36, 37]. We indicated that tumor necrosis factor-α can promote the expression of miR-134 and inhibit the expression of ITGB1 in HTR8/SVneo cells. We demonstrated that abnormally high expression of miR-134 in human placenta may be involved in the occurrence and development of preeclampsia by interfering with ITGB1 and inhibiting infiltration of trophoblast cells. This is a new suggestion for further exploring the pathogenesis of preeclampsia.

Conclusions

We showed that MiR-134 can inhibit infiltration of trophoblast cells in the placenta of patients with preeclampsia by decreasing ITGB1 expression.

Acknowledgments

The authors declared no conflict of interest.

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