MEDICAL SCIENCE MONITOR

CLINICAL RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2019; 25: 8306-8314
DOI: 10.12659/MSM.916601

Received: 2019.03.29
Accepted: 2019.07.10
Published: 2019.11.05

Long Noncoding RNA VIM Antisense RNA 1 (VIM-AS1) Plays an Important Role in Development of Preeclampsia by Regulation of Epithelial Mesenchymal Transition

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Source of support: Departmental sources

Background: Long noncoding RNAs play important roles in the development of various diseases. This study aimed to evaluate the effects and mechanism of VIM antisense RNA 1 (VIM-AS1) in the development of preeclampsia.

Material/Methods: HTR-8/SVneo cells were divided into normal control (NC), Model, Blank, and VIM-AS1 groups. These groups were analyzed for their VIM-AS1 gene expressions by RT-PCR, HTR-8/SVneo cell invasion was assessed by transwell and migration by wound healing, cell morphology was assessed by microscopy examination, and E-cadherin, Snail, and Vimentin genes expressions were assessed by RT-PCR and WB assay.

Results: VIM-AS1 gene expression was significantly different among normal placenta tissue, mild preeclampsia tissues, and severe preeclampsia tissues ($P<0.001$ or $P<0.01$). VIM-AS1 gene expressions, cell invasions, and wound healing rates in the Model and Blank groups were significantly suppressed compared with that of NC group ($P<0.001$, all). With VIM-AS1 supplementation, VIM-AS1 gene expression, cell invasion, and wound healing rate in the VIM-AS1 group were significantly increased compared with that in the Model group ($P<0.001$). RT-PCR and WB assay showed that E-cadherin gene and protein expressions in Model and Blank groups were significantly upregulated compared with the NC group ($P<0.001$); Snail and Vimentin gene and protein expressions in the Model and Blank groups were significantly downregulated compared with the NC group ($P<0.001$). With VIM-AS1 supplementation, E-cadherin, Snail, and Vimentin gene and proteins expression levels in the VIM-AS1 group were significantly different compared with that in the Model group ($P<0.001$).

Conclusions: VIM-AS1 promotes preeclampsia via inducing epithelial-to-mesenchymal transition (EMT).

MeSH Keywords: Cadherins • Enoxaparin • Pre-Eclampsia • RNA, Long Noncoding • Snails • Vimentin

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/916601

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Background

Preeclampsia (PE) is a multisystem, pregnancy-specific disease. It is mainly manifested as high blood pressure (≥140/90 mmHg), proteinuria (≥0.3 g/24 h). Multisystem dysfunctions occur after 20 weeks of pregnancy, and it an important cause of maternal and fetal death. About 5% of pregnant women without complications are affected by preeclampsia, 25% of pregnant women with gestational hypertension are complicated by preeclampsia [1], and preeclampsia may develop into more severe diseases, such as eclampsia and HELLP syndrome. To date, the pathogenesis of preeclampsia has not yet been fully elucidated, and insufficient remodelling of endometrial spiral arteries has been widely recognised as one of the main predisposing factors of preeclampsia [2]. The process of spiral arterial remodelling mainly depends on the migration and invasion of trophoblast cells and apoptosis of vascular endothelial smooth muscle cells that it mediates. Therefore, trophoblast cells play a crucial role in the process of arterial remodelling, in which their proliferation, migration, invasion, differentiation, and apoptosis occur simultaneously and are regulated by many factors [3–5]. Therefore, exploring the functions that affect trophoblast cells will further elucidate the etiology of preeclampsia and make a significant contribution to defining its pathogenesis. Recent studies have shown that long noncoding RNA (lncRNA) is closely related to the function of trophoblast cells in preeclampsia [6–8]. lncRNA VIM antisense RNA 1 (VIM-AS1) is a recently discovered lncRNA that influences cellular invasion and migration by regulating the epithelial-to-mesenchymal transition (EMT) of cells [9,10]. The role of VIM-AS1 in the development of preeclampsia remains unclear. This study first detected the expression of VIM-AS1 in normal placenta, mild preeclampsia, and severe preeclampsia. Then, the mechanism of action of VIM-AS1 in preeclampsia was validated by *in vitro* experiments.

Material and Methods

Clinical data

A total of 30 pregnant women who had experienced normal delivery in the Department of Gynecology and Obstetrics of Baoji Maternal and Child Health Care Hospital from April 2013 to March 2017 were selected, among which 18 patients had mild PE and 9 patients had severe PE. The mean ages of the pregnant women with normal pregnancy, mild PE, and severe PE were 29.5±3.2, 30.4±3.4, and 31.5±3.5 years, respectively, and their mean gestational ages were 37.3±3.3, 33.5±4.2, and 30.7±3.7 weeks, respectively. The diagnostic criteria for PE were according to the international standards [11]. The subjects of this study were single-birth primiparas who underwent delivery by caesarean section and had no regular prenatal contractions and no history of other diseases. This study was approved by the Ethics Committee of Baoji Maternal and Child Health Care Hospital (No. 2013012102).

Specimen collection

Within 5 min after the delivery of placenta, tissues on its maternal surface (calcified area and bleeding point were avoided) with a size of about 1.0×1.0×1.0 cm were collected. The specimen was repeatedly rinsed with normal saline, and moisture was absorbed by dry gauze. The specimen was divided into 2 parts, one of which was placed in 10% paraformaldehyde and fixed, and the remaining one was stored in liquid nitrogen and stored in a –80°C freezer the next day until needed for future use.

HE staining

Placental tissues were fixed in 10% paraformaldehyde for 24 h, and then paraffin-embedded and serially sectioned at a 4-μm thickness. Routine HE staining was performed in strict accordance with the recommended procedure, and the placental villus and morphology of intervillous capillary were observed under a microscope (×40).

Cells and reagents

The HTR-8/SVneo cell line was purchased from the ATCC China Cell Bank. DMEM-F12 medium and fetal bovine serum (FBS) were purchased from Gibco. Phosphate-buffered saline (PBS) buffer for cell culture was purchased from HyClone. RNAiso was purchased from TaKaRa. PrimeScript™ RT reagent kits were purchased from TaKaRa. lncRNA VIM-AS1 and blank control were all synthesised by GenePharma. Opti-MEM culture medium was purchased from Gibco. Matrigel was purchased from BD Bioscience, and Transwell was purchased from Costar.

Cell grouping and culture

HTR-8/SVneo cells were divided into a normal control group (NC Group), a model group (Model Group), a blank control group (Blank Group), and VIM-AS1 transfection group (VIM-AS1 Group). The HTR-8/SVneo cells in the NC and Model groups were treated with normal medium, while the HTR-8/SVneo cells in the Blank and VIM-AS1 groups were transfected with empty vector and VIM-AS1, respectively.

The Model, Blank, and VIM-AS1 Groups were cultured at 37°C in a normoxic incubator (21% O_2). Normoxic (21% O_2) and anaerobic (1% O_2) treatments were performed when cells reached 70% to 80% confluency.

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Table 1. Primer sequences used for RT-qPCR.

Gene	Primer (forward) 5'-3'	Primer (reverse)5'-3'
VIM-AS1	ACTGTAATGGACTCGTGGTG	CGTCGTGTTGTCCTGATG
Vimentin	AGTTTCGTTGATAACCTGTCC	CTCTTCCAAACTTTTCCTCCC
E-cadherin	CTGAGAACGAGGCTAACG	TTCACATCCAGCACATCC
Snail	TGACCATGCAACTGGACT	AACCTGACCAATGACAGT
β-actin	CTCTTCCAGCCTTCCTTCCT	GACAGCACTGTGTTGGCGTA

RT-PCR detection

RNA was extracted from the placenta or cells by using a Trizol total RNA extraction kit (Thermo Fisher Scientific, Waltham, MA, USA) in strict accordance with the product instructions. Isolated RNA was dissolved in 30 mL of DEPC water, and then stored in a –80°C freezer. The absorbance of RNA at 260 nm and 280 nm were determined, and reverse transcription was performed by using RNA with an A260/A280 ratio of 1.8 to 2.2. Reverse transcription of cDNA was performed according to the following method: denatured 1.0 μL of RNA at 65°C for 5 min, and then a rapid cooling on ice; adding 2.0 μL of 5×RT buffer (Takara, Tokyo, Japan), 0.5 μL of Enzyme Mix (Takara, Tokyo, Japan), 0.5 μL of Primer Mix (Takara, Tokyo, Japan), and 6.0 μL of Nuclease-free water, and mixing; reverse-transcribing RNA into cDNA at 42°C for 20 min and at 98°C for 5 min, and adding DEPC water to dilute the reaction mix 3 times. Fluorescent quantitation of PCR was as follows: to each well we added 5.0 μL of SYBR Premix Ex TaqTM II (2×) (Takara, Tokyo, Japan), 0.5 μL of PCR Forward Primer (1 μmol/L), 0.5 μL of PCR Reverse Primer (1 μmol/L) (Keygene, Nanjing, China), 2.0 μL of cDNA template, 2.0 μL of Nuclease-free water, mixed well, and placed it in the quantitative PCR 7500. Reaction conditions were: 95°C for 3 min; 95°C for 15 s, 62°C for 1 min, 72°C for 30 s, 40 cycles; 72°C for 5 min. The relative expression of the relevant mRNA was calculated by using $2^{-\Delta\Delta C}$. See Table 1 for the primer sequences. β-actin was used as a housekeeping gene in this RT-PCR experiment.

Transwell assay

Transwell chambers and 24-well plates needed to be equilibrated before cell inoculation: we added 100 μl of serum-free medium to the upper chamber and 600 μl of FBS to the lower chamber, and stored it at a 37°C with 5% CO_2 overnight. We collected the cells of each group subjected to 24-h starvation after transfection, counted and inoculated them into the upper chamber at 2×10^4 cells/well, and then cultured them in at 37°C with 5% CO_2 for 36 h; we wiped away the cells of the upper chamber; and we fixed the cells in the lower chamber of the membrane with methanol and captured the images after Giemsa staining. Using 5 fields of view and high-power lens, we counted and obtained the average value; this was repeated 3 times for each group.

Wound healing test

The cells were starved for 12 h by changing the medium in the 6-well plate to serum-free medium. Then, a 200-μl pipette tip was used to form an '1'-shaped scratch along the culture plate, forming a central blank area, and then it was washed with PBS once. The scratch area was photographed under a microscope at 0 h, followed by culture for 24 h and observation and photography at 24 h. The relative migration distance of the cells was analyzed by Image J software. The experiment was repeated 3 times and the average value was obtained.

Western blot analysis

The cells of each group were collected and washed 3 times with pre-cooled PBS. Fifty microliters of protein lysate (containing 1: 100 protease inhibitor and phosphatase inhibitor) was added to obtain a protein solution, and the concentration was determined by BCA method. The sample amount of protein was 80 μg, and 12% of sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) was prepared and used to electrophoretically separate the proteins, followed by transferring of the membranes. The membranes were confined with 5% skim milk powder at room temperature for 2 h and then the confining liquid was discarded. In the solution, the primary antibodies E-cadherin (1: 500), Vimentin (1: 500), Snail (1: 500), and GAPDH (1: 500) were added and incubated overnight at 4°C. A secondary antibody (1: 1000 dilution) was added and cells were incubated at room temperature in the dark for 2 h. The ODYSSEY Clx detection and imaging system was used to scan and capture images. The color gradation of the protein bands in the 4 groups was observed and compared. GAPDH was used as a housekeeping gene in Western blotting.

Statistical analysis

SPSS20. 0 statistical software was used for statistical analysis. The experimental data are all expressed as mean ± standard error of the mean. One-way analysis of variance was used for

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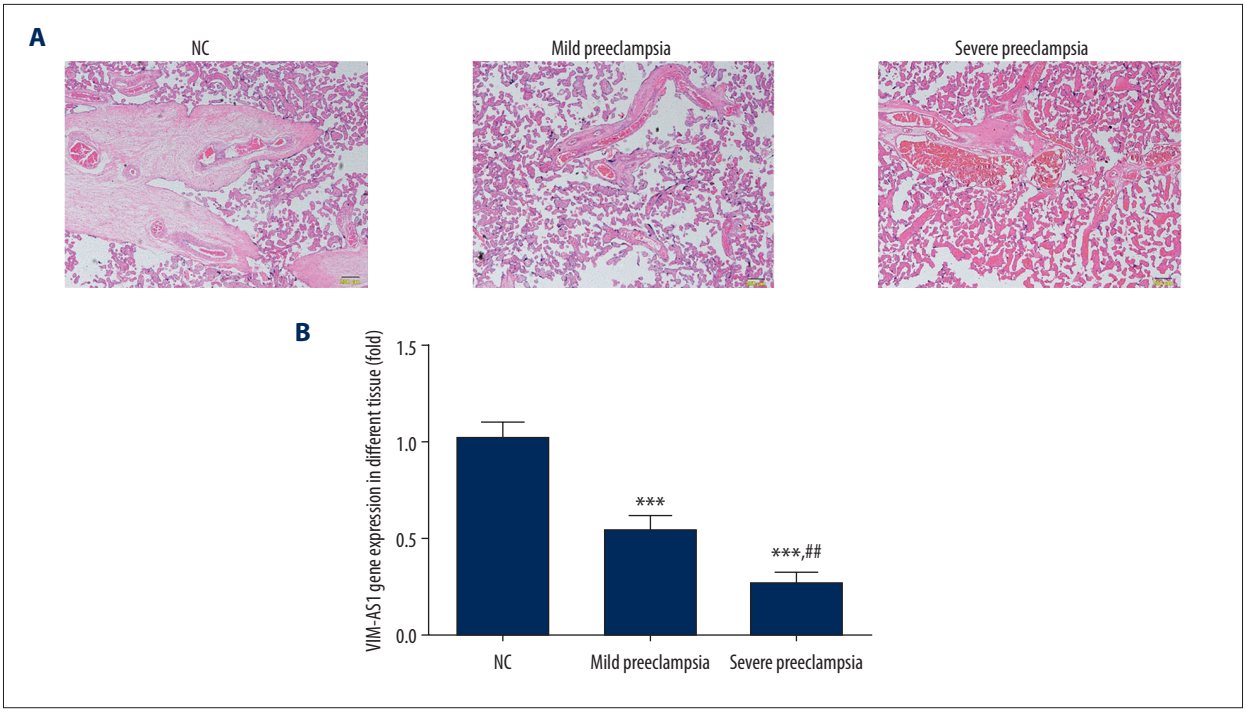

Figure 1. The pathological by HE staining (40×) and VIM-AS1 gene expression in different placenta tissues. (**A**) The pathological of difference tissues by HE staining (40×). NC: normal placenta tissue; Mild preeclampsia tissues; Severe preeclampsia tissues. (**B**) The VIM-AS1 gene expression in different placenta tissues by RT-qPCR. *** P<0.001 *vs.* NC; ## P<0.01 *vs.* Mild preeclampsia

comparison among multiple groups. The SNK-q test was used for multiple comparisons between groups. α=0. 05 was taken as the test standard.

Results

Clinical data and analysis

The normal placenta tissue (Figure 1A) had trophoblast cells, mainly consisting of syncytial trophoblast cells, and the trophoblast cells and syncytial trophoblasts were clear and intact. The mild preeclampsia placenta tissue (Figure 1A) had a trophoblast structure, the syncytial trophoblasts were unclear, and the morphology was incomplete. In the severe preeclampsia placenta tissue (Figure 1A), most of the placental villus were immature and villous, the cell trophoblast and syntrophoblasts were disorganised and incomplete, the cell trophoblast cells had obviously proliferated, the syncytial trophoblast nodules were increased, the villus capillaries were decreased, and there was blood stasis (Figure 1). RT-PCR revealed that the VIM-AS1 gene expression in mild and severe preeclampsia groups were significantly suppressed compared with that in the NC group (P<0.001). Meanwhile, there were significant differences in the VIM-AS1 gene expression between mild and severe preeclampsia groups (P<0.001, Figure 1B). The data are shown in Figure 1.

VIM-AS1 gene expression and invasion of different groups

Compared with the NC group, the VIM-AS1 gene expression in the Model and Blank groups was significantly downregulated (P<0.001, Figure 2A), and cellular invasion in the Model and Blank groups was significantly suppressed (P<0.001, Figure 2B). However, with VIM-AS1 supplementation, VIM-AS1 gene expression was significantly upregulated and the cellular invasion was significantly increased in the VIM-AS1 group compared with that in the Model group (P<0.001) (Figure 2).

Wound healing rate of different groups

Compared with the NC group, the wound healing rate in the Model and Blank groups was significantly depressed at 24 h and 48 h (P<0.01 and P<0.001, respectively). However, with VIM-AS1 transfection, the wound healing rate in the VIM-AS1 group was significantly higher at 24 h and 48 h (P<0.01 and P<0.001) (Figure 3).

VIM-AS1 affects cell morphology and related gene expression

Figure 4A shows that HTR-8/SVneo significantly changed from elongated stromal cells to round epithelioid cells in the eclampsia model in the Model and Blank groups. However, with VIM-AS1 supplementation, the HTR-8/SVneo cell morphology

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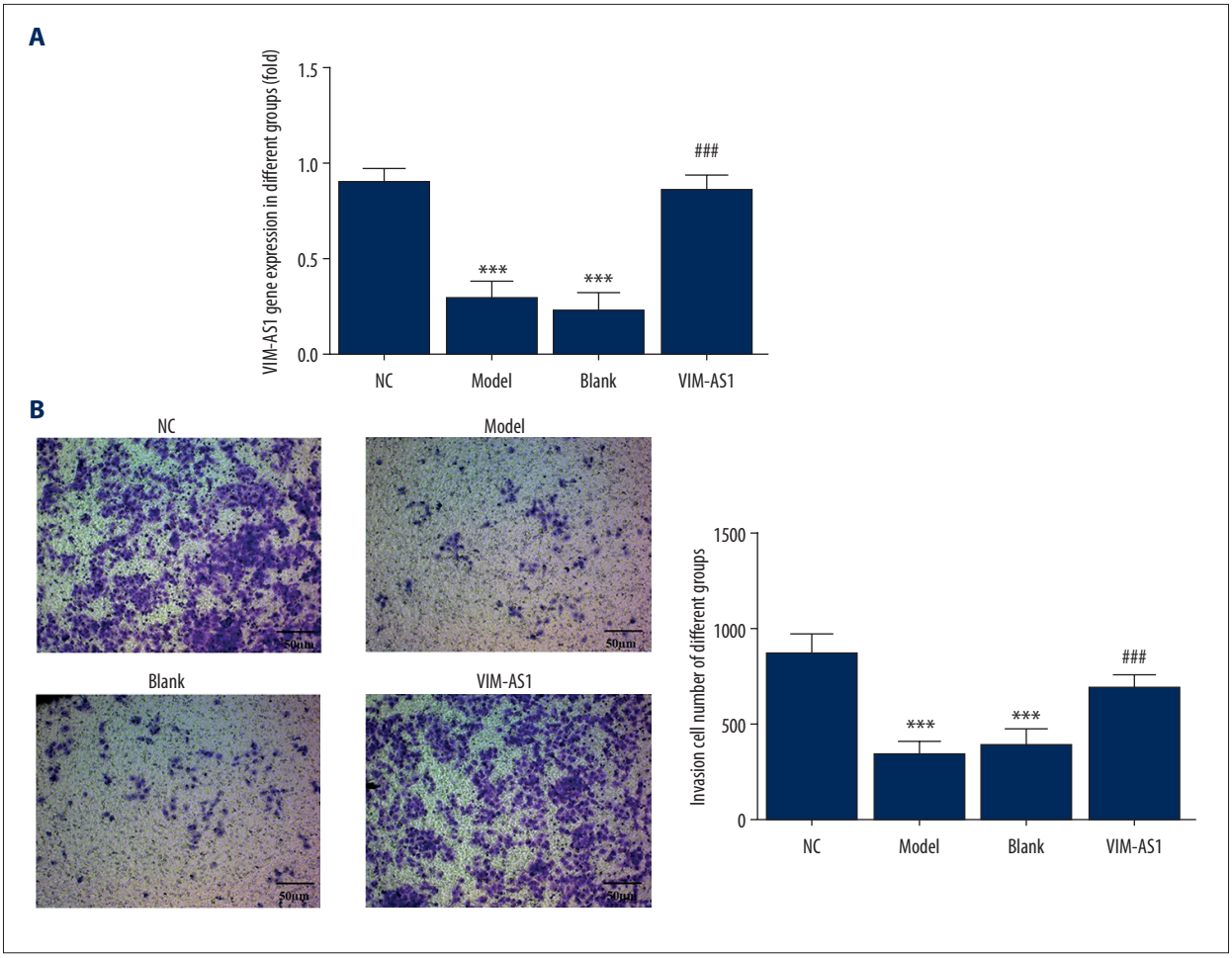

Figure 2. VIM-AS1 gene expression and invasion cell number in different groups (200×). NC – Normal Control group; Model – Hypoxia model group; Blank – HTR-8/SVneo cell transfected with empty vector based on hypoxia model treatment; VIM-AS1 – HTR-8/SVneo cell transfected with VIM-AS1 based on hypoxia model treatment. (**A**) VIM-AS1 gene expression of difference groups. (**B**) The invasion cell number of difference groups. *** P<0.001 *vs.* NC; ### P<0.001 *vs.* Model group.

restored the original cell morphology. RT-PCR analysis showed that E-cadherin gene expression was significantly upregulated and Snail and Vimentin gene expressions were significantly downregulated in the Model and Blank groups compared with that in the NC group ($P<0.001$, Figure 4B). With VIM-AS1 overexpression, E-cadherin gene expression was significantly downregulated and Snail and Vimentin gene expressions were significantly upregulated ($P<0.01$, Figure 4B).

EMT relative proteins expression in different groups

Western blot analysis showed that E-cadherin protein expression was significantly upregulated and Snail and Vimentin protein expressions were significantly downregulated in the Model and Blank groups compared with that in the NC group ($P<0.001$). With VIM-AS1 transfection, E-cadherin protein expression was significantly downregulated and Snail and Vimentin proteins expression were significantly upregulated ($P<0.01$) (Figure 5).

Discussion

Preeclampsia is a type of idiopathic hypertension syndrome in women with normal blood pressure who have hypertension, proteinuria, and other clinical manifestations after 20 weeks of gestation. It can cause severe maternal–fetal complications and it is also a cause of maternal and perinatal mortality [12]. At present, the incidence of preeclampsia in Europe and the United States is relatively low (about 2~5%), while preeclampsia is more common in the developing countries, with an incidence of about 10%. Maternal mortality caused by preeclampsia in Asia and Africa accounts for 9% of all maternal mortality [13]. Each year, out of 10 million pregnant women worldwide, there are an estimated 76 000 maternal deaths and 500 000 fetal or neonatal deaths caused by high blood pressure during pregnancy, especially preeclampsia [14]. The American College of Cardiology once included preeclampsia as a risk factor for cardiovascular disease and suggested that attention should be

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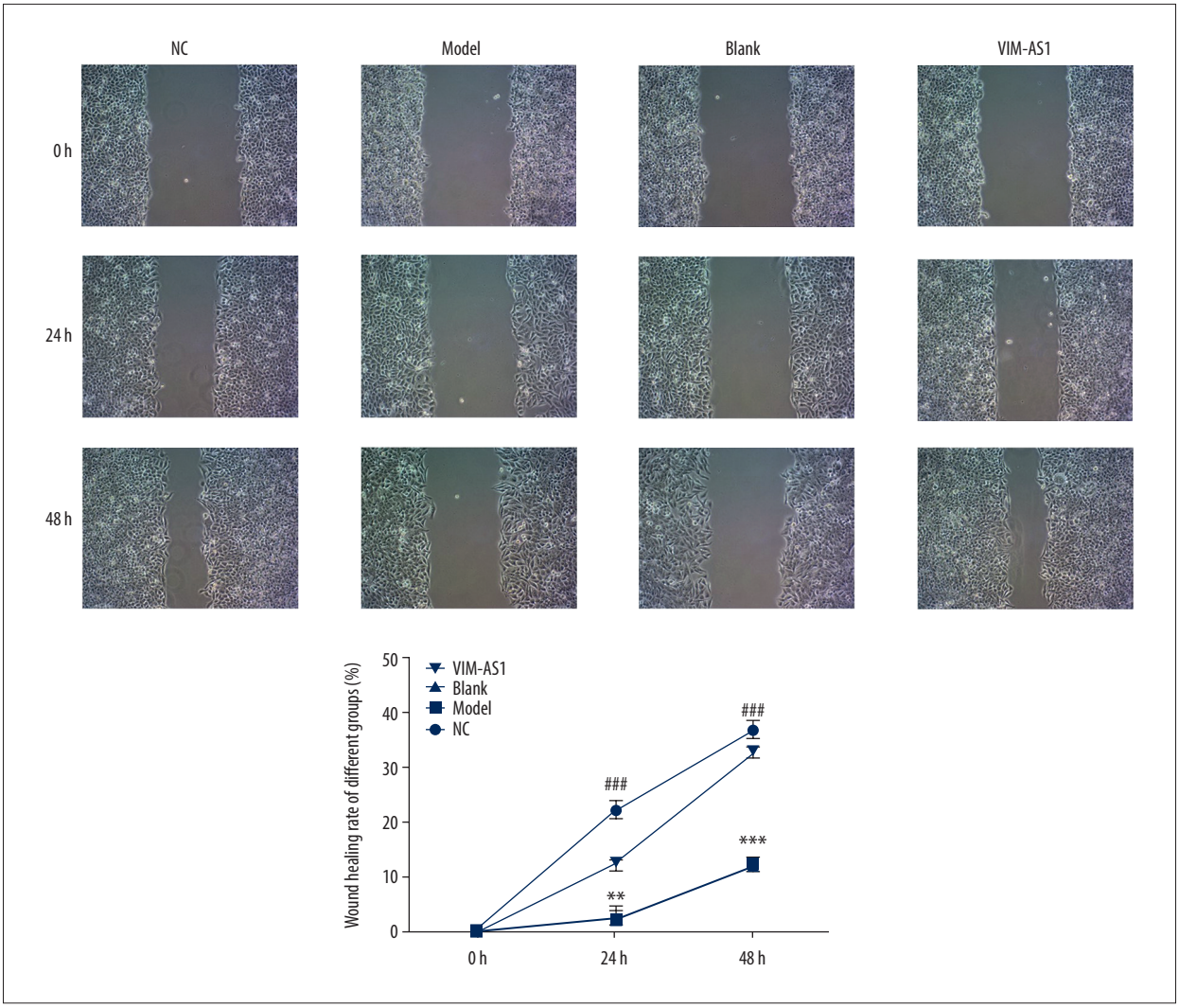

Figure 3. Wound healing rate of different groups (100×). NC – Normal Control group; Model – Hypoxia model group; Blank – HTR-8/SVneo cell transfected with empty vector based on hypoxia model treatment; VIM-AS1 – HTR-8/SVneo cell transfected with VIM-AS1 based on hypoxia model treatment. ** P<0.01, *** P<0.001 *vs.* NC; ## P<0.01, ### P<0.001 *vs.* Model.

paid to patients with a history of preeclampsia to improve their lifestyles [15,16]. However, the pathogenesis of PE is still unclear, and there are no effective treatments in clinical practice. In many cases, pregnancy has to be terminated prematurely, resulting in failed pregnancy. Many epidemiological studies also show that the occurrence of preeclampsia during pregnancy can have long-term adverse effects on the offspring [17,18]. The pathogenesis of PE may involve placental ischaemia and hypoxia, inflammatory reaction, changes in PE-related signalling pathways, abnormal trophoblastic infiltration, immune abnormality at the maternal–fetal interface, local coagulation and imbalanced anticoagulation mechanism in placenta, and abnormal ncRNA expression [19,20].

lncRNAs have been a focus of research in the past 5 years, and it has been reported that they play key roles in various cellular processes, such as the pluripotency of embryonic stem cells, embryonic development, and cell growth regulation. Many studies have shown that lncRNAs are closely involved in cell proliferation, migration, and invasion [21], such as MALAT1 [22], lncRNA-p21 [23], CCAT2 [24], and DQ786243 [25], which can induce abnormal behaviors of trophoblast cells. Zou et al. [26] reported that lncRNA SPRY4-IT1 is highly expressed in the placental tissues and can regulate the proliferation, migration, apoptosis, and cast formation of the HTR-8/SVneo cell line. The role of lncRNA in preeclampsia was demonstrated for the first time at the cellular level, expanding the study of preeclampsia at the cellular level.

Our study results confirmed that the placental expression of VIM-AS1 with preeclampsia was significantly reduced and gradually decreased with aggravation of preeclampsia. Therefore,

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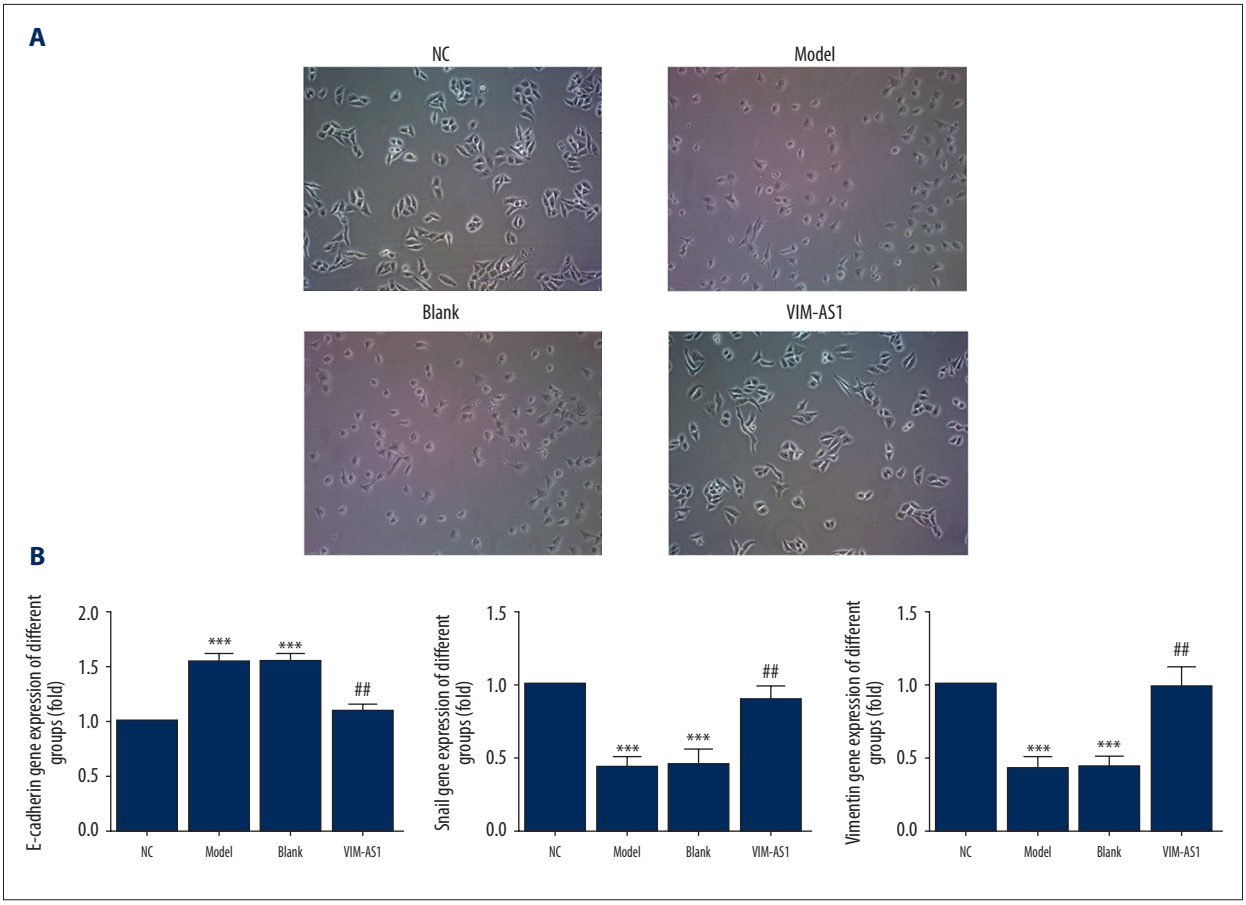

Figure 4. The cell morphology and relative genes expressions in difference groups (200×). NC – Normal Control group; Model – Hypoxia model group; Blank – HTR-8/SVneo cell transfected with empty vector based on hypoxia model treatment; VIM-AS1 – HTR-8/SVneo cell transfected with VIM-AS1 based on hypoxia model treatment. (**A**) Cell morphology in different groups. (**B**) The relative genes expressions in different groups by RT-PCR. *** P<0.001 *vs.* NC; ## P<0.01 *vs.* Model.

we speculate that overexpression of VIM-AS1 can effectively improve PE. In the *in vitro* experiment, we used a hypoxic model to simulate the pathological process of PE. Overexpression of VIM-AS1 can effectively improve the decreased cellular invasion and migration capacity of HTR-8/SVneo caused by hypoxia. Finally, we used Western blot analysis to detect the relevant proteins and explore their mechanism of action.

EMT refers to the conversion of epithelial cells that have polarity into mesenchymal cells that have activity, during which the expression of marker proteins of epithelial cells changes. E-cadherin is an adhesion molecule that can maintain normal morphology and structural integrity of epithelial cells. Its decrease or loss of expression can reduce the intercellular adhesion. At the same time, it can promote the entry of Vimentin and Snail into the cytoplasm, further activates EMT signalling, and promotes cellular invasion and migration [27,28]. Vimentin and Snail, as EMT marker molecules, play a key role in the occurrence of EMT [29,30]. The results of this study show that VIM-AS1, by regulating E-cadherin, Vimentin, and Snail, can effectively improve the decreased EMT capacity of placental trophoblast cells caused by hypoxia.

In the present study, we found the VIM-AS1 mRNA expression was significantly suppressed by increasing preeclampsia level, and we inferred that low expression level of lncRNA VIM-AS1 might lead to PE. To explain the effects and mechanism of VIM-AS1 in preeclampsia treatment, we transfected VIM-AS1 into HTR-8/SVneo in an anaerobic environment, showing that that VIM-AS1 overexpression restores HTR-8/SVneo cell biological activities and regulates EMT-related proteins and mRNA expressions.

Our study has some limitations. There is a dose- or time-dependent preeclampsia improvement effect after VIM-AS1 transfection and VIM-AS1 knockdown affects the HTR-8/SVneo cell biological activities based on anaerobic environment. We will continue to explore this in future research.

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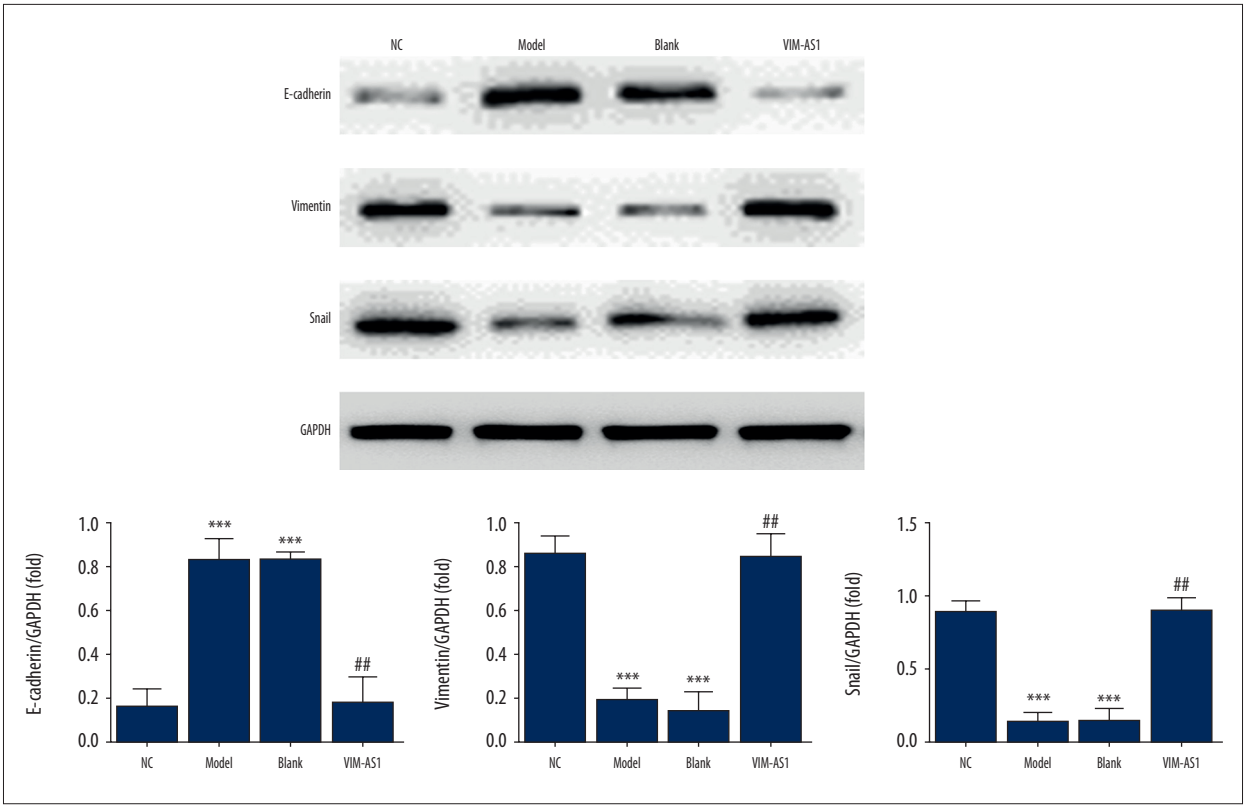

Figure 5. The relative proteins expressions of different groups by WB assay. NC – Normal Control group; Model – Hypoxia model group; Blank – HTR-8/SVneo cell transfected with empty vector based on hypoxia model treatment; VIM-AS1 – HTR-8/SVneo cell transfected with VIM-AS1 based on hypoxia model treatment. *** P<0.001 *vs.* NC; ## P<0.01 *vs.* Model.

Conclusions

Our results show that lncRNA VIM-AS1 play an important role in preeclampsia by regulation of the EMT.

Acknowledgements

We acknowledge the support received from Yanhong Huang from the Centre for Reproductive Medicine, Xijing Hospital, Air Force Medical University, and Li Wang, deputy director of Baoji Maternal and Child Health Care Hospital.

This research has been strongly supported by Yan-hong Huang (Reproductive Medicine Centre of Obstetrics and Gynecology, Xijing Hospital affiliated to the Military Medical University of the Air Force, Shaan Xi, Xi'an) and Li Wang (Baoji Maternal and Child Health Hospital Business Deputy Director, Shaan Xi, Xi'an), and we express our gratitude.

Conflict of onterests

None.

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