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miRNA-141-5p Affects the Levels of Neutrophil Elastase in Preeclampsia by Regulating MAPK1

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Abstract

Objective: The objective of this study was to investigate the expression levels of microRNA-141-5p(miRNA-141-5p), MAPK1 and neutrophil elastase in patients with and without preeclampsia (PE), and the relationship between miRNA-141-5p and MAPK1 with respect to the secretion of elastase by neutrophils in patients with PE.

Methods: Thirty patients with PE and 30 healthy pregnant (HP) women were recruited from The Second Hospital of Shanxi Medical University, Taiyuan, China, between February 2017 and July 2018. Neutrophils were isolated from 8 mL peripheral blood samples and cultured. We recorded neutrophil count and morphology during culture. Apoptosis was detected by flow cytometry in different groups at 0, 24, and 48 h. The expression levels of elastase were detected in neutrophils by enzyme-linked immunosorbent assay, whereas the expression levels of miRNA-141-5p in peripheral blood neutrophils were detected by real-time polymerase chain reaction. We used TargetScanHuman Release 7.2 to analyze the target genes of miRNA-141-5p. The expression of MAPK1 in peripheral blood neutrophils was detected by western blotting. Data were analyzed by SPSS version 21.0 software, and comparisons between groups were carried out with the Student *t* test.

Results: There was no significant difference between the PE and HP groups ($P > 0.050$) with regard to age or body mass index. The weight of newborns in the PE group (2846.00 ± 600.00 g) was significantly lower than that in the HP group (3055.00 ± 230.68 g). The number of neutrophilic granulocytes(NGs) in blood samples from the PE group was significantly higher than that in the HP group ($P = 0.003$). There was no significant difference between the groups with regard to morphology. Apoptosis in the PE group was delayed when compared with the HP group at different time points. The *P* value of apoptosis in the PE and HP groups were respectively 0.790, < 0.001 and 0.030 at 0 h, 24 h and 48 h. The expression levels of miRNA-141-5p in the PE group were significantly lower than those in the HP group ($P < 0.050$). The expression levels of MAPK1 in neutrophils from the PE group were significantly higher than those in the HP group ($P < 0.050$) by western blot. The expression levels of elastase in neutrophils from the PE group were significantly higher than those in the HP group ($P < 0.050$). Furthermore, the number of NGs in peripheral blood from the PE group was higher than that of the HP group; however, the levels of apoptosis were lower. The expression levels of miRNA-141-5p in NGs decreased, the expression of MAPK1 increased, and the secretion of neutrophil elastase in the NG medium increased in the PE group than those in the HP group.

Conclusion: Collectively, our analysis suggested that miRNA-141-5p may be involved in the pathogenesis of PE by regulating the MAPK1 signaling pathway to activate neutrophils and increase the secretion of elastase.

Keywords: Preeclampsia; Neutrophil; miRNA-141-5p; MAPK1; Neutrophil elastase

Introduction

Preeclampsia (PE) is a hypertensive disorder of pregnancy that is potentially associated with adverse maternal and neonatal outcomes.[1] The complications of PE include vasospasm, endothelial dysfunction, activation of the coagulation system, and enhanced platelet aggregation.[2] Furthermore, PE is a major global cause of morbidity and mortality for both the mother and the fetus.[3]

PE is closely related to vascular endothelial injury.[4] For example, neutrophilic granulocytes (NGs) become overactivated and adhere to vascular endothelial cells (VECs).[5] This leads to respiratory burst and the degranulation of NGs; this induces the significant release of oxygen free radicals and a variety of proteases and cytokines. Neutrophil elastase (NE) is a glycoprotein with a molecular weight of 30,000 kDa that is composed of 218 amino acids[6] and acts as a neutral proteolytic enzyme secreted by lysosomes in NGs. NE can be rapidly released into the extracellular matrix.[6] Subsequently, NGs can cause decomposition of the extracellular matrix via the action of NE, cross the blood-brain barrier, and then kill and dissolve pathogenic bacteria and the injured tissue.[7] Under pathological conditions, NG is overactivated, thus resulting in a "quantum explosion"[8] of aniline blue particles; this leads to its overenrichment and causes NG to undergo chemotaxis, exudate, and concentrate toward the inflammatory site.

The pathogenesis of PE, along with the key processes of immunity, angiogenesis, trophoblast proliferation and

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invasion, and placenta formation, is controlled by microRNAs (miRNAs).[9] Previous research has reported the aberrant expression of several miRNAs (eg, miRNA-210, miRNA-155, miRNA-141) in the placentae of patients with PE.[10,11] In a previous study, Wang *et al.*[12] constructed a model that could predict PE with high levels of accuracy by combining four peripheral leukocyte miRNAs that act as efficient biomarkers of PE. In another study, Jairajpuri *et al.*[13] discovered seven miRNAs that were differentially expressed in mild and severe PE patients by analyzing miRNAs in the plasma.

miRNA-141, located on human chromosome 12, is vulnerable to mutation,[14] translocation, or deletion.[15] Ozkan *et al.*[16] investigated the epigenetic effects of miRNA transmission and reported the expression patterns of miRNA-141-3p and its target gene *MAPK8* in ovarian and hepatic tissues. These authors also found that miRNA-141-3p targets many of the signaling pathways related to survival and metabolism, including the mitogen-activated protein kinase (MAPK) pathway. miRNA-141 is known to be up-regulated in placentae from patients with PE and plays a key role in regulating trophoblast invasion.[10] To illustrate the relationship between miRNA-141-5p and MAPK1 in the pathogenesis of PE, Wang *et al.*[17] reported that miRNA-141-5p up-regulated the transcription factor ATF2 to promote the expression of the phosphatase DUSP1. In addition, the expression of DUSP1 was shown to reduce the expression levels of p-MAPK1 and ERK1/2, thus promoting PE in JEG3 cells. Collectively, these results demonstrated the upstream and downstream relationships between miRNA-141-5p and MAPK1.

The MAPK signaling cascades include at least three protein kinases (MAPK1, MAPK2, MAPK3).[18] MAPK1 is one of the dominant members of the MAPK family. In a previous study, García-Hernández *et al.*[19] showed that activation of the MAPK1 pathway and its downstream target protein ERK1/2 was an important regulatory pathway in the activation of neutrophil. MAPK/ERK signaling is known to play an important role in the pathological process of PE.[20] Existing literature shows that the MAPK/ERK pathway is predominantly involved in the metabolic changes that occur during the metabolic syndrome that occurs in PE.[21] In another study, Armstrong *et al.*[22] found that neutrophil function including chemotaxis, activation, degranulation, and cell migration were all related to the p38 MAPK signal pathway.

In this study, we determined the expression levels of miRNA-141-5p, MAPK1, and NE in patients with and without PE and investigated the relationships between miRNA-141-5p, MAPK1, and NE with regard to PE.

Materials and methods

Study samples

We recruited 30 PE patients and 30 healthy pregnant (HP) women from the Second Hospital of Shanxi Medical University between February 2017 and July 2018. The 30 patients with PE were selected by strict criteria[1]: elevated systolic blood pressure (≥160 mm Hg) and/or elevated diastolic blood pressure (≥110 mm Hg after 20 weeks of pregnancy), low platelet count ($<100 \times 10^9$/L), impaired liver function (a serum transaminase level twofold higher than the normal value), impaired renal function (serum creatinine > 97.2 μmol/L or more than twofold higher than the normal value), pulmonary edema, and emerging brain and visual impairment. Additional inclusion criteria were (1) first pregnancy and without hepatorenal disease, diabetes, and tumor-related complications and (2) pregnant women without liver and kidney diseases before pregnancy. The exclusion criteria were as follows: history of hypertension, multiple pregnancies, severe liver and kidney function damage, cardiovascular disease, mental disorders, and other pregnancy-related diseases. This protocol was approved by the Ethics Committee of the Second Hospital of Shanxi Medical University (reference: 201603D321038). All participants provided written and informed consent.

Tissue collection

In total, 8 mL of peripheral blood was collected from pregnant nontreated women with PE and women with healthy normal pregnancies. Next, we added 8 mL of lymphocyte separation solution followed by centrifugation at 1500 r/min at room temperature for 20 min. After centrifugation, NGs were separated into the third separation layer, which appeared turbid. This turbid layer was transferred to a 15-mL sterile centrifuge tube, mixed with 10 mL of phosphate-buffered saline and centrifuged at 1500 r/min for 10 min. The upper liquid was discarded, and 5 mL of red blood cell lysate was added, then centrifugation at 1500 r/min at room temperature for 5 min. After centrifugation, the supernatant was extracted; this fraction contained the NGs used for primary culture. We recorded neutrophil count and morphology during culture by microscope (LB100 plus, Guangdong, China). Representative images were obtained at 40× magnification. The other samples were preserved at –80 °C.

Annexin V-FITC apoptosis assay

Cells were collected at 0, 24, and 48 h after culture. Then, apoptotic cells were detected by flow cytometry using an Annexin V-FITC kit (Bestbio, Shanghai, China) according to the manufacturer's instructions.

Determination of NE in NG culture medium by enzyme-linked immunosorbent assay (ELISA)

For the NE ELISA, 40 μL of sample was mixed well with 10 μL of NE antibody labeled with biotin (1:500, ZSGB-BIO, Beijing, China) in a 96-well plate. Then, we added 50 μL of an enzyme standard reagent to each well of the plate except for the blank wells. The plate was then incubated at 37 °C for 30 min after sealing. Next, we added chromogenic agent A (50 μL) followed by chromogenic agent B (50 μL) to each well and mixed well at 37 °C for 10 min. Next, we added 50 μL of termination liquid into each well and measured the absorbance (optical density value) of each sample at a wavelength of 450 nm. Then, we took the concentration of the standards as the abscissa and optical density value as the ordinate to generate a standard curve. Finally, we used the standard curve to generate a linear regression equation.

Determination of the expression levels of miRNA-141-5p in peripheral blood NGs by quantitative real-time polymerase chain reaction

Frozen NGs were thawed and ground, and total RNA was extracted with a miRNeasy Mini Kit (QIAGEN, Dusseldorf, Germany) in accordance with the manufacturer's instructions. Total RNA was then reverse transcribed to complementary DNA (cDNA) using a miScript II RT kit (QIAGEN,

Dusseldorf, Germany) for reverse transcriptase-polymerase chain reaction. Each reverse transcriptase-polymerase chain reaction consisted of cDNA (1:10; 2 μL), forward primer (0.4 μL), reverse primer (0.4 μL), 2× QuantiTect SYBR Green PCR (10 μL), and dH_2O (5.6 μL). The primer sequences used to amplify miRNA-141-5p were as follows: RNA, 5′-CAUCUUCCAGUACAGUGUUGGA-3′; DNA, 5′-TTTAGCCACGTTAGTCAGGACT-3′. The sequences of GAPDH were forward, 5′-TGTGGGCATCAATGGATTTGG-3′, and reverse, 5′-ACACCATGTATTCCGGGTCAAT-3′.

Comparison of the expression levels of MAPK1 and the target genes of miRNA-141-5p in NGs between the PE and HP groups

We used TargetScanHuman Release 7.2 to analyze the target genes of miRNA-141-5p. And we detected MAPK1 expression in peripheral blood NGs by western blotting. For each sample, NGs were mixed with 200 μL of protein extraction reagent and protease inhibitor cocktail (Sigma, Missouri, USA) (mixed at a ratio of 100:1). Then, each protein sample was loaded onto a gel, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Subsequently, the membrane was blocked with 5% evaporated milk at room temperature for 1 h. Next, the membrane was incubated with rabbit anti-MAPK 1 (Abcam Co, Cambridge, UK) (diluted 1:1000) at 4 °C overnight. On the following day, the membranes were washed incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin G (ZSGB-BIO, Beijing, China) (diluted 1: 500) at room temperature for 1.5 h. The chemiluminescence method was then used to measure protein expression, using β-actin as an internal reference. Rabbit anti–β-actin antibody (1:500) (ZSGB-BIO, Beijing, China) was used as a loading control and was detected by an HRP-labeled goat anti-rabbit antibody (1:2000) (ZSGB-BIO, Beijing, China). Immunoreactive protein bands were analyzed by Image J version 1.8.0 (National Institutes of Health).

Statistical analysis

Data were analyzed with SPSS version 21.0 software (SSPS Inc, Chicago, IL, USA). Measurement data are shown as mean ± standard deviation (*SD*). Comparisons between two groups were performed with the Student *t* test. $P < 0.05$ was statistically significant.

Results

Clinical features

As presented in Table 1, a total of 60 women were recruited, consisting of 30 patients with PE (the PE group) and 30 HP women (the HP group). There was no significant difference between the two groups with regard to age or body mass index ($P > 0.050$); however, the weight of newborns in the PE group was significantly lower than that in the HP group ($P < 0.050$).

Table 1

Characteristics of the two groups (mean ± standard deviation).

Groups	Number	Age (year)	BMI (kg/m^2)	Neonatal weight (g)
PE	30	29.30 ± 4.95	29.23 ± 2.57	2846.00 ± 600.00
HP	30	29.10 ± 3.54	28.14 ± 1.01	3055.00 ± 230.68
t		0.10	1.24	3.63
P		0.920	0.230	<0.050

BMI: Body mass index; HP: Healthy pregnant; PE: Preeclampsia.

Comparisons of cell count and morphology between the PE and HP groups

Primary culture of neutrophils in the peripheral blood in the two groups was observed at 0, 24, 48, and 96 h of culture. As presented in SDC Figure 1, http://links.lww.com/MFM/A21, cells grew in suspension with a round and full appearance. There was no significant difference between the two groups with regard to cellular morphology.

Comparison of the number of NGs between the PE and HP groups

Next, the number of NGs was compared between two groups. There were (7.53 ± 1.54) × 10^9/L in the PE group and (6.15 ± 1.19) × 10^9/L in the HP group. The number of NGs in the PE group was significantly higher than that in the HP group ($P = 0.003$).

Comparison of apoptosis in NG cells between the PE and HP groups

As shown in Figure 1 and Table 2, the onset of apoptosis in the PE group was delayed when compared with the HP group, as determined by flow cytometry. After 24 h, the extent of apoptosis in NGs from the HP had reached 44.10 ± 1.98%; it was 11.94 ± 1.16% in the PE group. As culture time increased, a large number of NG cells had died in the HP group. Furthermore, the extent of apoptosis in NGs remained high (34.98 ± 7.35%) at 48 h; in contrast, the extent of apoptosis in NG cells in the PE group had increased to 57.80 ± 8.91%.

Comparison of NE levels in the culture medium of NGs between PE and HP groups

We used ELISA to detect the levels of NE in the culture media of NGs extracted from the peripheral blood from two groups. The expression levels of NE in the PE and HP groups were 0.82 ± 0.13 and 0.27 ± 0.10, respectively. As shown in Figure 2, the levels of NE in the culture medium of NGs extracted from the peripheral blood of patients in the PE group were significantly higher than those in blood from the HP group ($P < 0.050$).

Comparison of the expression levels of miRNA-141-5p in NGs between the PE and HP groups

We used polymerase chain reaction to determine the expression levels of miRNA-141-5p in NGs from the PE and HP groups. The expression levels of miRNA-141-5p were 0.34 ± 0.12 and 0.93 ± 0.19 in the PE and HP groups, respectively. The expression levels of miRNA-141-5p in NGs from the PE group were significantly lower than those in the HP group ($P < 0.050$).

Comparison of the expression levels of MAPK1 in NGs between the PE and HP groups

Finally, we used TargetScanHuman Release 7.2 to analyze the target genes of miRNA-141-5p. A schematic representation of the binding sites of miRNA-141-5p for MAPK1 is

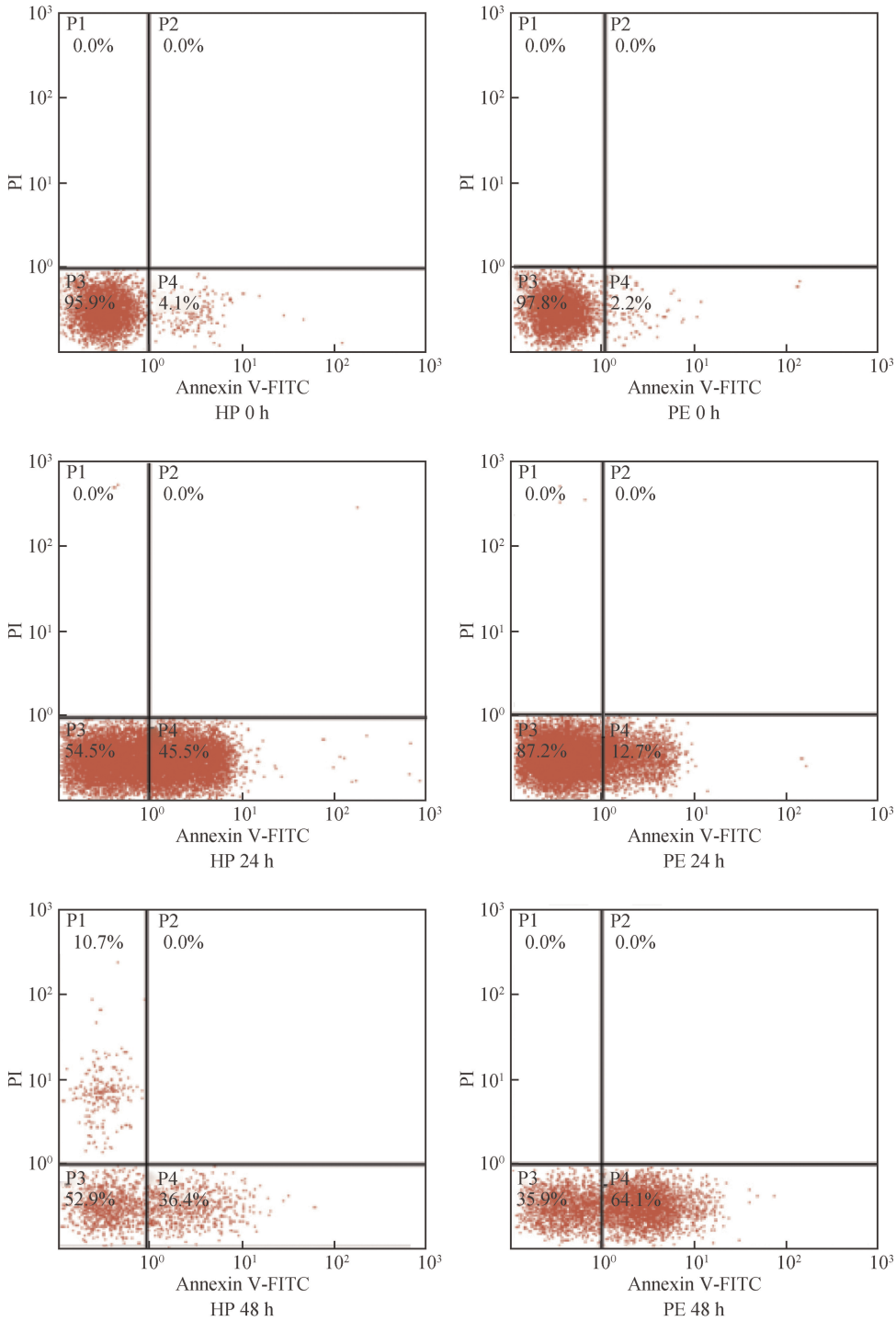

Figure 1. The extent of apoptosis in the PE and HP groups. HP: Healthy pregnant; PE: Preeclampsia.

shown in Figure 3A. We also used western blotting to investigate the expression of MAPK1 in NGs in peripheral blood from the PE and HP groups. We found that the expression levels of MAPK1 were significantly up-regulated in NGs in peripheral blood from the PE group when compared with the HP group (t = 4.33, P < 0.050) (Figs. 3B, C).

Discussion

In this study, we showed that miRNA-141-5p may be involved in the pathogenesis of PE by regulating MAPK1 to

Table 2

The extent of apoptosis in the PE and HP groups (mean ± standard deviation).

Groups	Number	0 h (%)	24 h (%)	48 h (%)
PE	30	0.09 ± 0.01	11.94 ± 1.16	57.80 ± 8.91
HP	30	0.06 ± 0.01	44.10 ± 1.98	34.98 ± 7.35
t		0.27	28.20	3.39
P		0.790	<0.001	0.030

HP: Healthy pregnant; PE: Preeclampsia.

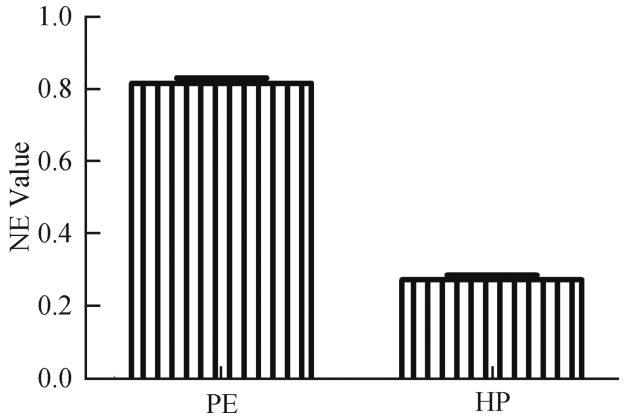

Figure 2. The expression of neutrophil elastase in the PE and HP groups. $P < 0.05$ by Student t test, $n = 30$ per group. HP: Healthy pregnant; PE: Preeclampsia.

activate neutrophils and increase the secretion of elastase. The reduced expression of miRNA-141-5p led to an increase in the expression of MAPK1; this overactivated NGs, increased the production and secretion of NE, damaged the VECs, and finally led to the occurrence of PE.

PE is a common and specific disease in pregnancy.[23] Increasing lines of evidence suggest that the mechanisms underlying PE and endothelial disease do not only affect pregnancy; these mechanisms can also increase cardiovascular risk.[24] The pathogenesis of PE may be related to VEC damage, trophoblast invasion, and nutrition. Previous studies found that the activation of NGs and the inflammatory response induced by their adhesion to endothelial cells represent an important cause of vascular endothelial damage.[8] Kunder *et al.*[25] measured NE in the peripheral blood of PE patients and healthy patients by ELISA and reported that NE activity in PE patients was significantly increased; furthermore, activity was positively correlated with disease severity. We extracted NGs from the peripheral blood of a group of patients with PE and a group of women with healthy pregnancies; these NGs were then used in various culture experiments. Analysis showed that the levels of NE in PE patients were much higher than those in women with healthy pregnancies. These results showed that the activation of NGs in PE patients was much higher than that in HP women.

Neutrophils play a key role in the pathogenesis of many diseases, including inflammatory diseases, infectious diseases, and tumors.[26] NGs mature in the bone marrow and are then released into the blood. The apoptosis of neutrophils is accompanied by a decline in cellular function, including the reduction of antibacterial and inflammatory ability.[27] However, in inflammatory conditions, neutrophils become activated. In our study, we used flow cytometry to detect the levels of apoptosis in NGs from the peripheral blood of women with PE and women with healthy pregnancies. We found that the levels of apoptosis in NGs from PE patients were significantly delayed when compared with those of women with healthy pregnancies.

	Predicied cons equential pairing of target regiom (top) and mRNA (bottom)	Site type	Context++ score
Position 3665-3671 of MAPK1 3' UTR	5' …UUAAAAAAUGUUAAAGGAAGAUU…	7mer-m8	-0.25
hsa-miR-141-5p	3' AGGUUGUGACAUGACCUUCUAC		
Postion 5862-5868 of MAPK1 3' UTR	5' …CACAAAAUACCCAAUGGAAGAUG…	7mer-m8	-0.05
hsa-miR-141-5p	3' AGGUUGUGACAUGACCUUCUAC		
Position 7207-7213 of MAPK1 3' UTR	5'…GAACAAGGCACUGGA--GGAAGAUC…	7mer-m8	-0.05
hsa-miR-141-5p	3' AGGUUGUGACAUGACCUUCUAC		

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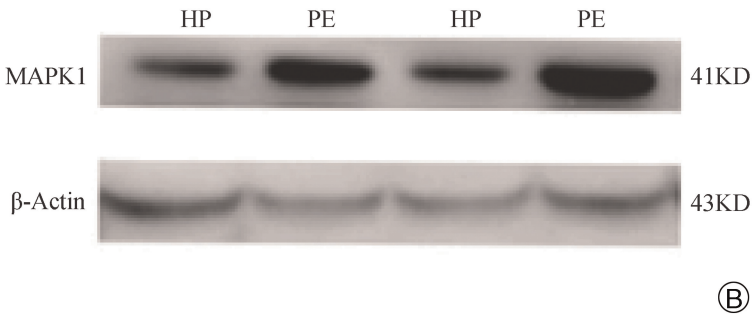

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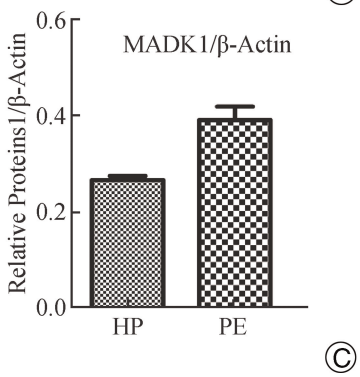

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Figure 3. The expression levels of MAPK1 in NGs in peripheral blood from the PE and HP groups. A, Schematic representation of the binding sites of miRNA-141-5p with MAPK1. B, Protein expression levels of MAPK1 in the PE and HP groups. C, Relative expression of MAPK1 proteins in the PE and HP groups ($n = 30$ per group). $P < 0.050$ by Student t test. HP: Healthy pregnant; NGs: Neutrophilic granulocytes; PE: Preeclampsia.

miRNA is known to be differentially expressed in the plasma of women with healthy pregnancies and women with both mild and severe PE.[13] Thus, miRNA can be used as a predictor of PE and disease severity. In a previous study, Luo *et al.*[28] identified 65 differentially expressed miRNAs in PE patients, including 32 up-regulated miRNAs and 33 down-regulated miRNAs. In another study, Ospina-Prieto *et al.*[10] showed that the expression levels of miRNA-141 were higher in the placentae of patients with PE. Yamaleyeva *et al.*[29] detected significant abnormal expression levels of miRNAs in the blood of patients with PE. In our study, we showed that the expression levels of miRNA-141-5p in NGs from the PE group were significantly lower than those from women with healthy pregnancies.

miRNAs are directly involved in the pathogenesis of PE, including immunity, angiogenesis, trophoblast proliferation and invasion, and formation of the placenta.[16] In a previous study, Larsen *et al.*[30] found that miRNA may participate in the regulation of NGs via differential expression. The expression levels and activation status of miRNAs in different parts of NGs are known to vary during different points in the cell developmental process. In this study, we showed that the number of NGs in the PE group was higher than that in the group of women with healthy pregnancies. Furthermore, compared with women with healthy pregnancies, the levels of NE in the culture medium of NGs extracted from the peripheral blood of women in the PE group were significantly higher. Therefore, miRNA may be closely related to the activation of NGs and the pathogenesis of PE.

Activation of the MAPK1 pathway and its downstream target protein ERK1/2 is known to be an important regulatory pathway in the process of neutrophil activation. Dai *et al.*[31] confirmed that abnormal expression of miRNA-141 was closely related to the MAPK signaling pathway. In another study, Kobayashi *et al.*[27] found that stimulatory signals such as tissue damage can increase the infiltration of NGs in the injured area and activate the p38 MAPK signaling pathway and the ERK1/2 target protein downstream. These events subsequently cause the activation degranulation of NGs, thus releasing NGs that can induce an inflammatory response; specific blockers can prevent this process. In this study, we found that the expression levels of MAPK1 protein in NGs from the peripheral blood patients with PE were higher than those in NGs from women with healthy pregnancies.

This study has some limitations that need to be considered; for example, the sample size was small; this may limit the generalizability of our findings to other populations. Further research is required to confirm our findings in a larger study population.

Conclusion

Herein, we characterized the relationship between miRNA-141-5p, MAPK1, and PE. Our data indicate that the reduced expression of miRNA-141-5p increased the expression levels of MAPK1; this overactivated NGs, increased the production and secretion of NE, caused damaged to the VECs, and finally led to the occurrence of PE. However, many factors are known to affect the occurrence of PE; further studies are needed to investigate these other factors.

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Author Contributions

Yonghong Wang conceived and supervised the study. Jialei Cui, Wenli Zhou, Huiqiang Liu, Keyan Cheng, and Taotao Yang performed experiments and analyzed data. Yonghong Wang and Keyan Cheng wrote the manuscript and manuscript revisions. All authors reviewed the results and approved the final version of the manuscript.

Conflicts of Interest

None.

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