

Original Article

Elevated expression of TNF- α and NF- κ B p65 in the placenta of patients with preeclampsia: association with disease severity and predictive value

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Abstract: Objective: This study aimed to investigate the expression, correlation, independent effects, and predictive value of tumor necrosis factor- α (TNF- α) and nuclear factor-kappa B (NF- κ B) p65 in the placental tissue of patients with preeclampsia (PE). Methods: Placental tissue was retrospectively collected from 96 pregnant women with PE who underwent cesarean section (51 with mild PE (mPE), 45 with severe PE (sPE)), and 40 healthy full-term controls (Ctrl). The expression levels of TNF- α and NF- κ B p65 were detected using immunohistochemistry, western blot, and fluorescence quantitative PCR (q-PCR). Their correlation with clinical symptoms and predictive efficacy for the severity of PE was analyzed. Results: The expression levels of TNF- α and NF- κ B p65 in the placental tissue of the mPE and sPE groups were significantly higher than those of the control group (all $P < 0.05$), and were higher in the sPE group than in the mPE group ($P < 0.05$). The expression of both proteins was positively correlated with systolic blood pressure, diastolic blood pressure, and 24-hour urinary protein quantification ($r = 0.458-0.671$, all $P < 0.05$). In addition, TNF- α expression was positively correlated with NF- κ B p65 expression ($r = 0.615$, $P < 0.001$). Multivariate analysis showed that TNF- α (OR = 2.84, 95% CI: 1.67-4.82, $P < 0.001$) and NF- κ B p65 (OR = 2.13, 95% CI: 1.28-3.54, $P = 0.004$) were independent influencing factors for the severity of PE. The ROC curve showed that the AUC for TNF- α alone was 0.84, for NF- κ B p65 alone was 0.79, and for the combination of the two it was 0.91 (95% CI: 0.85-0.96), with a sensitivity of 86.7% and a specificity of 84.3%. Conclusion: The expression levels of TNF- α and NF- κ B p65 in the placental tissue of patients with PE are elevated and closely correlated. Both are independent influencing factors for disease severity, and their combined detection has excellent predictive value for severe PE.

Keywords: Nuclear factor-kappa B p65, pre-eclampsia, placental tissues, tumor necrosis factor- α

Introduction

Preeclampsia (PE) is a common multisystem syndrome that occurs after 20 weeks of gestation and is clinically classified into mild PE (mPE) and severe PE (sPE). As the disease progresses, it can lead to complications such as preterm birth, fetal growth restriction, and fetal distress, and in severe cases, it poses a threat to both maternal and fetal life [1, 2]. According to the Global Preeclampsia Summit, PE accounts for 16% of maternal deaths and causes 500,000 fetal and neonatal deaths annually [3]. Although the exact pathogenesis of PE remains incompletely elucidated, it is widely accepted that abnormal trophoblast

invasion, immune dysregulation, endothelial dysfunction, genetic factors, oxidative stress, and insulin resistance are all involved in its onset and progression [4]. Among these, excessive inflammatory response is considered a core pathologic mechanism of PE: placental ischemia-reperfusion injury releases a large number of pro-inflammatory cytokines, leading to endothelial cell damage and systemic small vessel vasospasm, ultimately resulting in multiple organ dysfunction [5, 6].

Tumor necrosis factor- α (TNF- α) is a key pro-inflammatory cytokine that induces endothelial cell injury, increases vascular permeability, and inhibits trophoblast migration [7, 8]. Nuclear

factor-kappa B (NF- κ B) is a central transcription factor regulating the transcription of various inflammatory genes. Its activation upregulates the expression of inflammatory factors such as TNF- α , thereby promoting smooth muscle cell proliferation, vascular remodeling, and elevated blood pressure [9]. Previous studies have suggested increased expression levels of TNF- α and NF- κ B p65 in placental tissue and peripheral blood of patients with PE [10, 11]. However, most studies have examined only one factor at a time, or have had small sample sizes, and lack systematic correlation analyses between their expression and clinical indicators such as blood pressure and urinary protein. More importantly, it remains unclear whether TNF- α and NF- κ B p65 can serve as independent predictors to distinguish between mild and severe PE, and what the clinical predictive value of their combined detection might be.

To address this, the present study retrospectively analyzed placental tissues (PTs) from 96 PE patients and 40 normal full-term pregnant women. Immunohistochemistry and fluorescence quantitative PCR (q-PCR) were used to detect the expression levels of TNF- α and NF- κ B p65. The correlations between these factors and systolic blood pressure, diastolic blood pressure, and 24-hour urinary protein quantification were investigated. The synergistic relationship between the two factors was analyzed, and multivariate regression along with receiver operating characteristic curves was employed to assess their status as independent risk factors and their predictive value for severe PE. The innovations of this study are as follows: (1) It was the first to simultaneously detect the expression of both TNF- α and NF- κ B p65 in placental tissues from a relatively large sample of PE patients, demonstrating a significant positive correlation between them; (2) It was the first to analyze systematically the linear relationships between both factors and multiple clinical indicators; (3) It was the first to establish a multivariate regression model incorporating both TNF- α and NF- κ B p65, demonstrating that both are independent risk factors for severe PE; (4) It was the first to provide data on the area under the ROC curve, sensitivity, and specificity for the combined prediction of severe PE using both factors. The clinical significance lies in the poten-

tial use of TNF- α and NF- κ B p65 as auxiliary diagnostic markers for the severity of PE, which may facilitate early identification of patients with severe PE and timely intervention to improve maternal and neonatal outcomes.

Materials and methods

Clinical data

The clinical data of 96 pregnant women with PE who were hospitalized and delivered at Affiliated Jinhua Hospital, Zhejiang University School of Medicine from October 2022 to October 2023 were analyzed retrospectively. In accordance with the criteria set out in the 2015 *Diagnostic and Treatment Guidelines for Pregnant Hypertensive Disorders*, 51 parturients were determined as those who experienced mPE, while 45 parturients were assigned as suffering from sPE. In addition, 40 full-term pregnant women with no other complications were enrolled in the control group, who had received routine prenatal check-ups at the same time. Data, including age, baseline blood pressure, height, weight, 24-hour urinary protein (UPRO) levels, and C-reactive protein, were collected. Among these, systolic blood pressure, diastolic blood pressure, and UPRO are core clinical indicators for the diagnosis and severity classification of PE. Elevated blood pressure and proteinuria are the defining criteria for PE and directly reflect disease progression and the extent of target organ damage. Therefore, this study selected the above to measure for clinical correlation analysis.

Inclusion criteria: (1) Meeting the diagnostic criteria for PE as outlined in the "Guidelines for the Diagnosis and Management of Hypertensive Disorders in Pregnancy (2020)" [i.e., after two or more measurements (at least 4 hours apart), systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg, and 24-hour urinary protein quantification ≥ 0.3 g or random urine protein $\geq (+)$]; (2) Natural conception and singleton pregnancy; (3) Age between 25 and 35 years; (4) Gestational age ≥ 28 weeks and delivery by cesarean section.

Exclusion criteria: (1) Pre-existing hypertension, diabetes mellitus, chronic kidney disease, autoimmune diseases (e.g., systemic lupus erythematosus, antiphospholipid syndrome, etc.), or malignant tumors before pregnancy; (2)

Other obstetric complications, including placenta previa, placenta accreta, premature rupture of membranes, gestational diabetes mellitus, or fetal distress; (3) History of acute infection (e.g., upper respiratory tract infection, urinary tract infection, etc.) within 2 weeks before admission; (4) Long-term history of smoking, alcohol consumption, or drug abuse; (5) Use of immunomodulators, glucocorticoids, or anticoagulant drugs within the past 3 months; (6) Significant organ dysfunction in the heart, liver, and kidneys; (7) Stillbirth, fetal malformation, or abnormal fetal development; (8) Missing clinical data or laboratory data.

This study was approved by the Ethics Committee of the Affiliated Jinhua Hospital, Zhejiang University School of Medicine. The ethics committee waived the requirement for written informed consent.

Data collection

This study was a retrospective analysis, and all clinical data were obtained from medical records. The primary outcome measures of this study were the expression levels (including mRNA and protein) of tumor necrosis factor- α (TNF- α) and nuclear factor-kappa B (NF- κ B) p65 in placental tissue. Secondary outcome measures included systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), 24-hour urinary protein quantification (UPRO), and patient general information (age, gestational age, BMI, etc.).

Placental tissue samples were obtained immediately after placental delivery during the cesarean section. SBP, DBP, MAP, and 24-hour UPRO were based on the most recent valid records after admission and before the cesarean section (all obtained within 48 hours before surgery). Blood pressure values were taken as the average of two consecutive measurements. General information (age, gestational age, BMI) was based on records at the time of admission.

Sample collection and processing

All pregnant women underwent cesarean section. Immediately after the delivery of the placenta through cesarean section, tissue blocks measuring approximately 1 cm³ were excised from the central maternal side of the placenta,

avoiding areas of calcification and necrosis during excision. The tissue blocks were thoroughly washed with physiological saline (Shandong Hualu Pharmaceutical Co., Ltd., Liaocheng, China) and divided into two portions. Tissue slices were soaked in 4% paraformaldehyde solution (Biosharp, Hefei, China) for a whole day, then encapsulated in paraffin (RM2235, Leica, Wetzlar, Germany), and divided into 4 slices with a slicer (RM2235, Leica, Wetzlar, Germany). Simultaneously, the remaining tissues were stored at -80°C.

Immunohistochemical staining and scoring

The paraffin sections were placed in a slide warmer and baked at 37°C for 30 minutes, and then incubated for two hours at a constant temperature. During dewaxing, the slices were soaked in xylene II, xylene I (China National Pharmaceutical Group, Shanghai, China), 100%, 95%, 80% and 70% ethanol (China National Pharmaceutical Group, Shanghai, China) in sequence. After each immersion, these PTs were cleaned with phosphate-buffered saline (PBS, Solarbio, Beijing, China). Then, high-pressure antigen extraction (Sanyo, Osaka, Japan) was carried out in 0.01 M citric acid buffer (pH = 6.0, ZSGB-BIO, Beijing, China) for 2 minutes, and washing with PBS for 3 times for 5 minutes. Subsequent steps included the addition of 50 μ L of 3% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO, USA) to the sections at room temperature for a 10-minute incubation, and we used PBS to wash for five minutes, and repeated the washing three times. Next, at Room Temperature (RT), 50 μ L of sheep serum (ZSGB-BIO, Beijing, China) was added to the TP segment for 10 minutes, and then the solution was discarded. Subsequently, the slice was treated with 50 μ L TNF- α (rabbit polyclonal antibody, ab6671, Abcam, Cambridge, UK; dilution ratio 1:200) or NF- κ B p65 (rabbit monoclonal antibody, #8242, Cell Signaling Technology, Danvers, MA, USA; dilution ratio 1:400) main antibody and cultured at RT for 2 hours. After that, we used PBS to wash for 5 minutes, and repeated the washing three times. Then, we added 50 μ L goat anti-mouse IgG secondary antibody (ZSGB-BIO, Beijing, China) to the slice and cultured it at room temperature for 15 minutes. After that, we used PBS to wash for five minutes, and repeated the washing three times.

Subsequently, a fresh DAB chromogenic solution (Fuzhou Xinbio Co., Ltd., Fuzhou, China) of 100 μ l was added to the PT sections for 5 minutes of incubation at RT. Subsequently, we rinsed these PT tissues with tap water for 5 minutes. The slices were counterstained by hematoxylin (Solarbio, Beijing, China) for two minutes, and then blued under running tap water. The tissues were subjected to step-wise dehydration using 70%, 80%, 95%, and 100% ethanol (China National Pharmaceutical Group, Shanghai, China), and they were cleaned with xylene I and xylene II (China National Pharmaceutical Group, Shanghai, China). Finally, the PT sections were fixed with neutral glue. PBS was used instead of the first antibody for the negative control. Microscopic (BX53, Olympus, Tokyo, Japan) examination revealed the positive expression of TNF- α or NF- κ B p65 within the PT.

First, the evaluation was based on the positive staining intensity of positive cells on each slide, which was classified into four levels: no coloration (0 points), pale yellow (1 point), yellowish-brown (2 points), and brown (3 points). Second, assessment was made according to the proportion of positive cells to the total cells on each slide, with categorization as follows: 0-25% (1 point), 25-50% (2 points), and 50-100% (3 points). By summing the scores for positive cell staining intensity and proportion, the evaluation of TNF- α and NF- κ B p65 was conducted. Scores ranging from 0 to 1 were considered as negative (-), 2 to 3 points were regarded as weak positive (WP, +), 4 to 5 points were regarded as moderate positive (MP, ++), and 5 to 6 points were regarded as strong positive (SP, +++). Both negative and WP positive are low expression, while MP and ST are high expression.

Western blot detection

Frozen placental tissues were collected, and RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) was added to extract total protein. Protein concentrations were determined using a BCA protein quantification kit (Thermo Fisher Scientific, Waltham, MA, USA). For each group, 30 μ g of protein was separated by SDS-PAGE electrophoresis (Bio-Rad, Hercules, CA, USA) and then transferred onto a PVDF membrane (polyvinylidene difluoride

membrane, Millipore, Burlington, MA, USA). The membrane was blocked with 5% non-fat milk (BD Biosciences, Franklin Lakes, NJ, USA) for 1 hour at room temperature and subsequently incubated overnight at 4°C with primary antibodies: anti-TNF- α antibody (1:1000, Abcam, Cambridge, UK), anti-NF- κ B p65 antibody (1:800, Cell Signaling Technology, Danvers, MA, USA), and anti-GAPDH antibody (1:5000, Proteintech, Rosemont, IL, USA). The next day, the membrane was incubated with HRP-conjugated secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG, 1:5000, Cell Signaling Technology, Danvers, MA, USA) for 1 hour at room temperature. Protein bands were visualized using ECL chemiluminescent substrate (enhanced chemiluminescence solution, Millipore, Burlington, MA, USA) and imaged using a gel imaging system (ChemiDoc MP, Bio-Rad, Hercules, CA, USA). The grayscale values of the bands were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA), and the relative expression levels were expressed as the ratio of the target protein grayscale value to that of GAPDH. The experiment was independently repeated three times, and results were presented as mean $\pm$ standard deviation.

The fq-PCR detection

Liquid nitrogen (Zhejiang Zhonglong Gas Technology Co., Ltd., Zhejiang, China) was added to the frozen PT tissue to make it into powder. Then, the complete RNA of PT tissue was obtained by the Trizol method (Trizol reagent, Invitrogen, Carlsbad, CA, USA). First, the integrity of RNA was detected by 1% agarose gel electrophoresis (agarose, Biowest, Shanghai, China), and then its content was obtained by nucleic acid analysis equipment (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA, USA). According to the instructions of the reverse transcription kit (PrimeScript RT Master Mix, Takara, Dalian, China), reverse transcription of RNA to cDNA. Using this cDNA as a template, we used fluorescence quantitative PCR (fq-PCR) (LightCycler 480, Roche, Basel, Switzerland) to amplify TNF- α and NF- κ B p65, and verified their functions. The PCR amplification steps are as follows: pre-denaturation for 1 min at 95°C; Denaturation was carried out at the same temperature

Table 1. Primer sequences for fluorescence quantitative PCR

Gene	Primer direction	Sequence (5' $\rightarrow$ 3')
TNF- α	Forward	CCTCTCTCTAATCAGCCCTCTG
	Reverse	GAGGACCTGGGAGTAGATGAG
NF- κ B p65	Forward	GTGGGGACTACGACCTGAATG
	Reverse	GGGGCACGATTGTCAAAGATG
GAPDH	Forward	CTGGGCTACACTGAGCACC
	Reverse	AAGTGGTCGTTGAGGGCAATG

Note: TNF- α : tumor necrosis factor- α ; NF- κ B p65: nuclear factor-kappa B p65; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; PCR: polymerase chain reaction.

for 15 seconds, and annealing or extension was carried out at 60°C for 1 minute, totaling 40 times. Finally, using GAPDH (GAPDH antibody, Abcam, Cambridge, UK) as an internal standard, and using the $2^{-\Delta\Delta C_t}$ method obtain the contents of TNF- α and NF- κ B p65 in cells. The primer sequences are shown in **Table 1**.

Data processing

SPSS 22.0 (IBM, Armonk, NY, USA) was employed to handle and examine all collected data. The normality of the data was assessed using the Shapiro-Wilk test. Normally distributed continuous variables were expressed as mean $\pm$ standard deviation, while non-normally distributed variables were expressed as median (interquartile range). Comparisons between two groups were performed using the independent samples t-test or the Mann-Whitney U test (for non-normally distributed data), and comparisons among three groups were performed using one-way analysis of variance (ANOVA). When overall differences were significant, pairwise comparisons were further conducted using the least significant difference (LSD) method. Categorical variables were expressed as frequencies and percentages, and group comparisons were performed using the chi-square (χ^2) test. Pearson correlation analysis was used to evaluate the correlations between the mRNA expression levels of TNF- α and NF- κ B p65 and various clinical indicators. For logistic regression analysis, univariate analysis was first performed, and variables with $P < 0.1$ were included in the multivariate model to calculate the odds ratio (OR) and 95% confidence interval. The dependent variable was assigned as follows: sPE = 1, mPE = 0. The

diagnostic value was evaluated using receiver operating characteristic (ROC) curves. The optimal cutoff value was determined at the maximum Youden index, and the 95% confidence interval of the area under the curve (AUC) was calculated using the DeLong method. If $P < 0.05$, the data were considered to be significantly different.

Results

Patient screening process

During the study period, a total of 850 pregnant women delivered, including 120 with suspected PE and 180 as potential controls. After applying the inclusion/exclusion criteria, 24 PE cases were excluded, resulting in the final inclusion of 96 PE patients (51 with mild PE and 45 with severe PE). Among the control group, 140 cases were excluded, leaving 40 healthy full-term pregnant women (as depicted in the corresponding **Figure 1**).

Basic features of individuals

Differences among the basic features of individuals in the Ctrl group, mPE group, and sPE group were compared (**Table 2**). In age, gestational age, and BMI, no significant differences were observed across the mPE, sPE, and Ctrl groups ($P > 0.05$).

In the mPE, sPE, and Ctrl groups, age, gestational weeks, and BMI among patients showed no variation across groups ($P > 0.05$). Patients in the mPE and sPE groups exhibited sharply higher SBP, DBP, mean arterial pressure (MAP), and 24-hour UPRO levels relative to those in the Ctrl group (all $P < 0.05$). In addition, the levels of SBP, DBP, MAP, and 24-hour UPRO in sPE patients were higher than those in mPE (all $P < 0.05$).

Difference in TNF- α expression in PTs of patients with mPE and sPE

Immunohistochemical staining was conducted to observe the positive TNF- α expression in PT from patients in Ctrl, mPE, and sPE groups (**Figure 2A, 2B**). In the Ctrl group, 35 cases (87.5%) had a negative result (-), 5 cases (12.5%) had a WP result (+), 0 cases (0.0%) had an MP expression (++), and 0 cases (0.0%) had a SP expression (+++). These results signi-

TNF- α and NF- κ B p65 in Pre-eclampsia patients

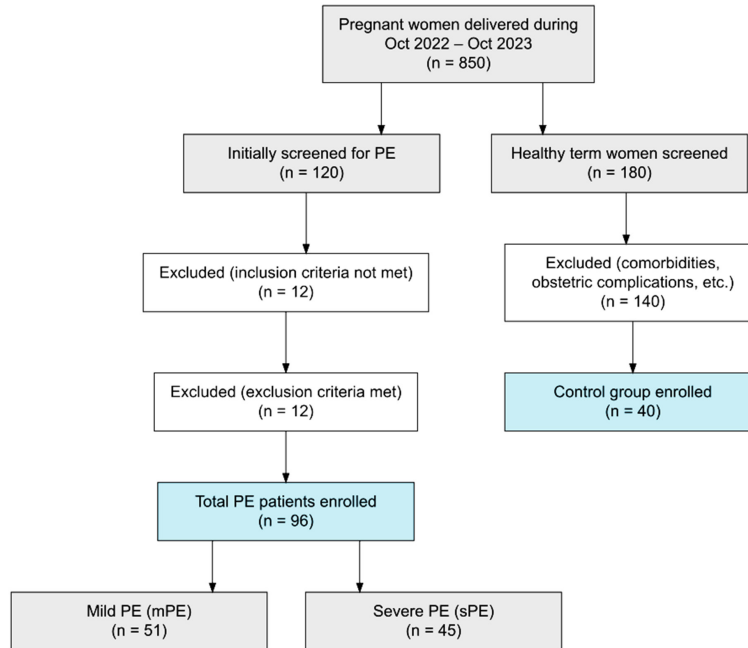


Figure 1. Flow chart of patient screening. Note: PE: preeclampsia; mPE: mild preeclampsia; sPE: severe preeclampsia; Ctrl: control.

fied that TNF- α exhibited low expression in 40 cases (100.0%), with no cases showing high expression (0.0%). In the mPE group, there were 9 cases (17.6%) with negative observation (-), 14 cases (27.5%) with WP expression (+), 28 cases (54.9%) with MP expression (++) and 0 cases (0.0%) with SP expression (+++). TNF- α was decreased in 23 cases (45.1%), while 28 cases (54.9%) exhibited elevated TNF- α . For the sPE group, 4 cases (8.9%) with negative result (-), 10 cases (22.2%) with WP expression (+), 28 cases (62.2%) with MP expression (++) and 3 cases (6.7%) with SP expression (+++) were observed. This suggested that TNF- α was expressed at low levels in 14 cases (31.1%), while 31 cases (68.9%) exhibited high expression. The rates of WP (+), MP (++) and SP (+++) expressions of TNF- α in mPE and sPE groups were higher in comparison to those of the Ctrl group. Additionally, the low-expression rate was lower than the high-expression rate in both mPE and sPE groups ($P < 0.05$) (Figure 2C).

The expression level of TNF- α mRNA in PC of different groups of patients was evaluated by fq-PCR (Figure 2D). In the mPE and sPE groups, the TNF- α mRNA expressions in PT from patients were sharply higher than in the

Ctrl group ($P < 0.05$). In addition, in the sPE group, the contents of TNF- α mRNA in the PT of the sPE group were markedly elevated compared to those of the mPE group ($P < 0.05$).

NF- κ B p65 expressions in PTs of patients with mPE and sPE

Similarly, the positive level of NF- κ B p65 was detected in each case by the immunohistochemical staining method (Figure 3A, 3B). In the Ctrl group, 36 cases (90.0%) with a negative result (-), 4 cases (10.0%) with WP expression (+), 0 cases (0.0%) with MP expression (++) and 0 cases (0.0%) with SP expression (+++) were identified. These observations suggested that NF- κ B p65 was lowly ex-

pressed in 40 cases (100.0%), with no cases showing high expression (0.0%). In the mPE group, there were 10 cases (19.6%) with negative observation (-), 12 cases (23.5%) with WP expression (+), 29 cases (56.9%) with MP expression (++) and 0 cases (0.0%) with SP expression (+++). The above data reflected that NF- κ B p65 had low expression in 22 cases (43.1%), while those with high expression in 29 cases (56.9%) were included in this group. The sPE group encompassed 6 cases (13.3%) with negative observation (-), 12 cases (26.7%) with WP expression (+), 26 cases (57.8%) with MP expression (++) and 1 case (2.2%) with SP expression (+++). Consequently, it was concluded that NF- κ B p65 was lowly expressed in 18 cases (40.0%) but highly expressed in 27 cases (60.0%). Therefore, WP (+), MP (++) and SP (+++) expression of NF- κ B p65 in patients suffering from mPE and sPE exhibited higher rates than those of the Ctrl group. Additionally, the rate of low expression was lower than that of high expression in both mPE and sPE groups (both $P < 0.05$) (Figure 3C).

The fq PCR was used to test the NF- κ B p65 mRNA levels in PT of different groups of patients (Figure 3D). The mRNA levels of mPE and sPE groups exceeded those of the Ctrl group

Table 2. Differences among the basic characteristics of patients in different groups

Characteristic	Ctrl group	mPE group	sPE group	F	P
Sample	40	51	45	-	-
Age (weeks)	26.12 $\pm$ 3.74	27.08 $\pm$ 3.41	28.14 $\pm$ 4.08	1.230	0.295
Gestational weeks	38.61 $\pm$ 1.53	38.16 $\pm$ 1.92	37.77 $\pm$ 2.29	1.670	0.192
BMI (kg/m ²)	24.73 $\pm$ 2.86	25.12 $\pm$ 2.08	24.42 $\pm$ 1.71	0.989	0.378
SBP (mmHg)	116.88 $\pm$ 8.14	144.39 $\pm$ 7.06*	166.29 $\pm$ 14.53*#	39.631	<0.001
DBP (mmHg)	72.54 $\pm$ 4.30	95.06 $\pm$ 6.29*	110.95 $\pm$ 8.87*#	17.352	<0.001
MAP (mmHg)	86.49 $\pm$ 5.71	105.17 $\pm$ 9.51*	118.25 $\pm$ 9.26*#	29.546	<0.001
UPRO (mg/24 h)	0.07 $\pm$ 0.03	2.01 $\pm$ 0.42*	4.62 $\pm$ 1.05*#	25.431	<0.001

Note: * and # suggested a substantial significance with $P < 0.05$ to Ctrl and MPE groups, respectively. Ctrl: control group; MPE: mild preeclampsia; SPE: severe preeclampsia; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; UPRO: 24-hour urinary protein.

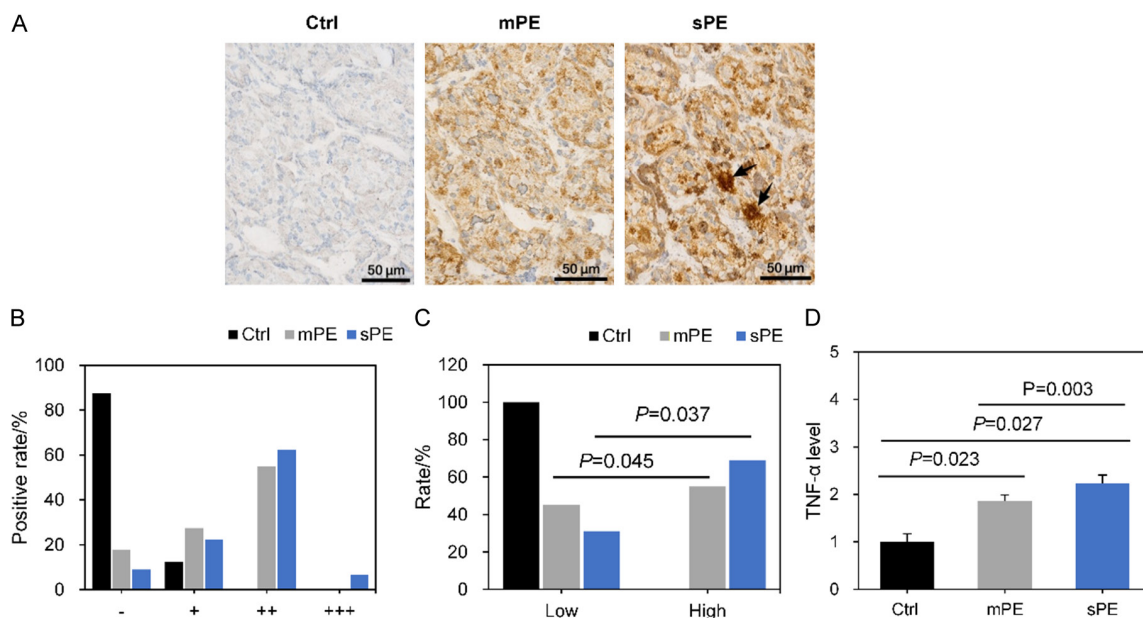


Figure 2. Expression levels of TNF- α in PTs of study subjects in each group. Note: (A) Representative immunohistochemical staining images (×200); (B) immunohistochemical score of TNF- α ; (C) TNF- α expression rate, with arrows indicating strongly positive cells; (D) Expression levels of TNF- α mRNA in placental tissues of each group; Ctrl: control group; mPE: mild preeclampsia; sPE: severe preeclampsia; TNF- α : tumor necrosis factor- α ; PTs: Placental tissues.

($P < 0.05$). The NF- κ B p65 mRNA level in the sPE group was higher than that of the mPE group ($P < 0.05$).

Western blot validation of TNF- α and NF- κ B p65 protein expression

To further validate the protein expression levels of TNF- α and NF- κ B p65 in placental tissue, western blot analysis was performed. The results (Figure 4A, 4B) showed that compared to the Ctrl group, the protein expression levels of TNF- α and NF- κ B p65 in the mPE and sPE groups were significantly elevated ($P < 0.05$).

Among these, the expression levels in the sPE group were higher than those in the mPE group ($P < 0.05$). Figure 4C shows representative blots, with the trend in each group consistent with the quantitative analysis.

Correlations of TNF- α and NF- κ B p65 expressions to clinical manifestations

PCA was implemented to understand the correlation between TNF- α mRNA in PT and the values of SBP, DBP, and 24-hour UPRO in patients with PE (Figure 5A-C). It was evident that the TNF- α mRNA expression in PT showed

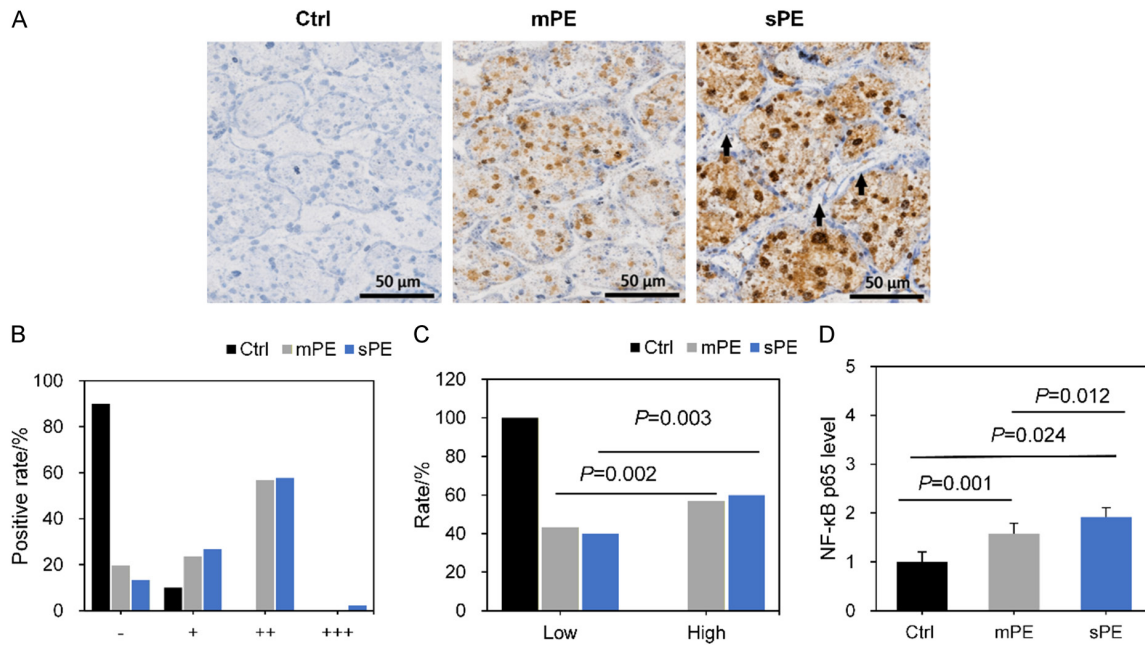


Figure 3. NF- κ B p65 level in PTs of patients in various groups. Note: (A). Representative immunohistochemical staining images ($\times 200$); (B) immunohistochemical score of NF- κ B p65; (C) NF- κ B p65 level rate, arrows indicated strongly positive cells; (D) Expression levels of NF- κ B p65 mRNA in placental tissues of each group; Ctrl: control group; mPE: mild preeclampsia; sPE: severe preeclampsia; NF- κ B p65: nuclear factor-kappa B p65; PTs: Placental tissues.

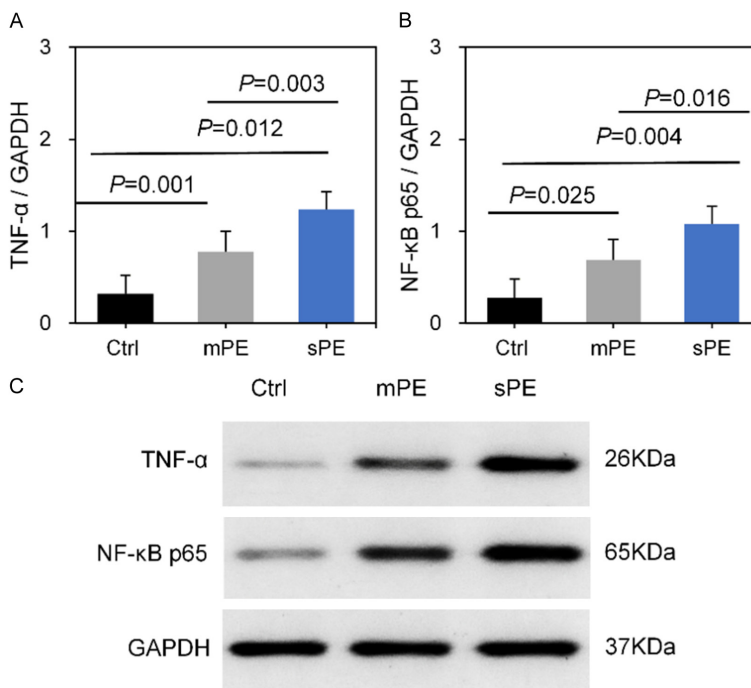


Figure 4. Protein expression levels of TNF- α and NF- κ B p65 in placental tissues of each group were detected by western blot. Note: (A) Quantitative analysis of TNF- α protein expression; (B) Quantitative analysis of NF- κ B p65 protein expression; (C) Representative western blot bands of TNF- α and NF- κ B p65 in each group. Ctrl: control group; mPE: mild preeclampsia; sPE: severe preeclampsia; TNF- α : tumor necrosis factor- α ; NF- κ B p65: nuclear factor-kappa B p65; PTs: Placental tissues; GAPDH: glyceraldehyde-3-phosphate dehydrogenase (loading control).

a positive link with SBP, DBP, and 24-hour UPRO levels in PE patients ($r = 0.671, 0.602$, and 0.654 , respectively; $P < 0.05$).

In parallel, PCA was performed to explore the relationship of NF- κ B p65 mRNA level in PT with SBP, DBP, and 24-h UPRO levels in patients with PE (Figure 6A-C). NF- κ B p65 mRNA expression in PT demonstrated a positive relation with SBP, DBP, and 24-hour UPRO levels in PE patients ($r = 0.530, 0.559$, and 0.458 , respectively; $P < 0.05$).

Correlation analysis of TNF- α and NF- κ B p65 expression in placental tissues of PE patients

The purpose of this experiment was to explore whether there is a synergistic regulatory mechanism between TNF- α and NF- κ B p65 in the process of inflammation caused by PE.

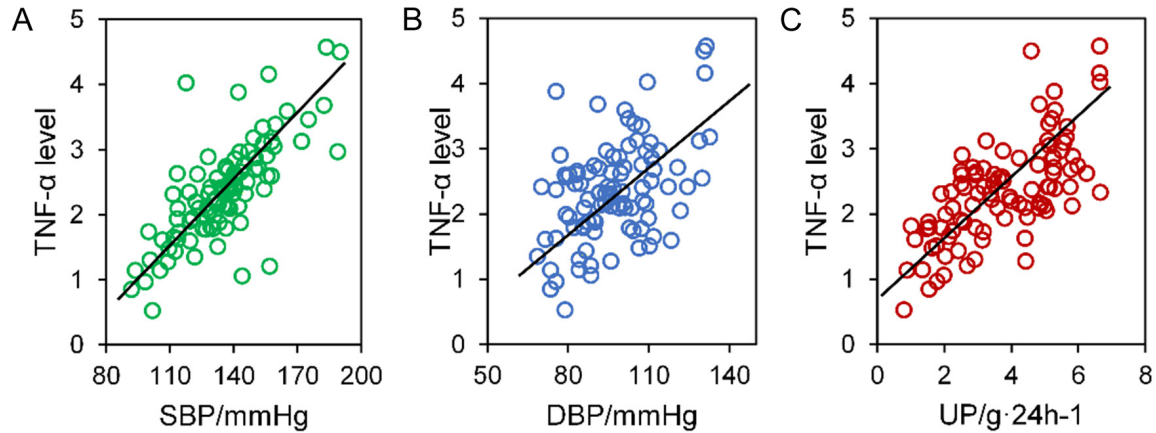


Figure 5. Correlation of TNF- α mRNA expression and manifestations. Note: (A-C) Scatter diagrams for the relationship to SBP, DBP, and 24-h UPRO, respectively. SBP: systolic blood pressure; DBP: diastolic blood pressure; UP: urinary protein; TNF- α : tumor necrosis factor- α .

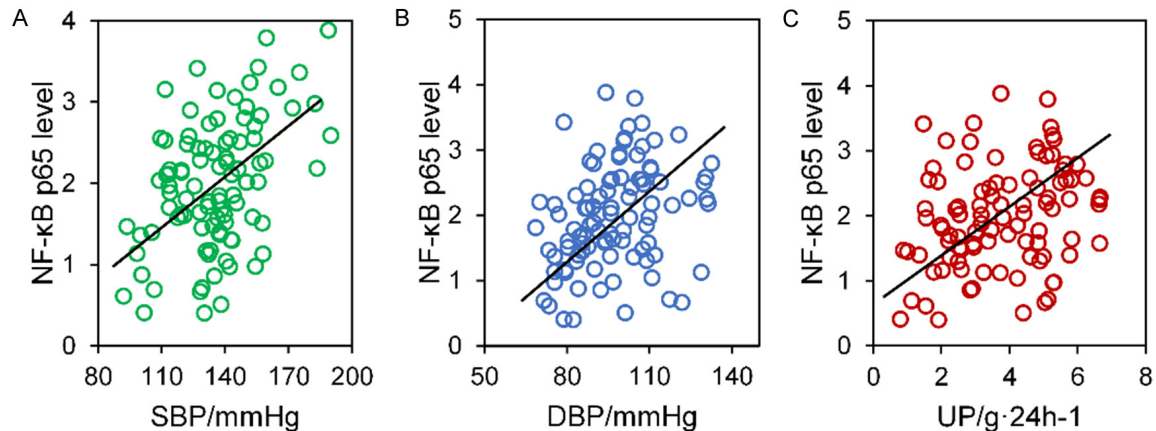


Figure 6. Correlation of NF- κ B p65 mRNA expression and manifestations. Note: (A-C) Scatter diagrams for the relationship to SBP, DBP, and 24-h UPRO, respectively. SBP: systolic blood pressure; DBP: diastolic blood pressure; UP: urinary protein; NF- κ B p65: nuclear factor-kappa B p65.

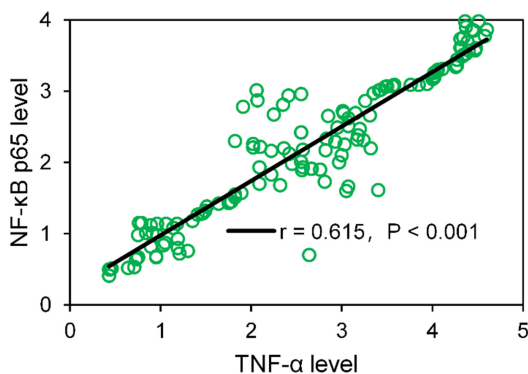


Figure 7. A scatter plot showing the correlation between TNF- α and NF- κ B p65 mRNA expression in the placental tissues of patients with PE. Note: PE: preeclampsia; TNF- α : tumor necrosis factor- α ; NF- κ B p65: nuclear factor-kappa B p65.

A correlation analysis was further conducted on their mRNA expression levels in placental tissues (**Figure 7**). A strong positive correlation ($r = 0.615, P < 0.001$) was detected between the mRNA expression levels of TNF- α and NF- κ B p65 in the PE group ($n = 96$) using Pearson correlation analysis.

Multivariate logistic regression analysis of PE severity

To further clarify whether TNF- α and NF- κ B p65 independently influence the severity of PE, univariate logistic regression analysis was first performed, with PE severity (sPE = 1, mPE = 0) as the dependent variable. All potential variables-including age, gestational age, BMI, sys-

Table 3. Univariate logistic regression analysis: Factors influencing the severity of PE (sPE vs. mPE)

Variable	OR (95% CI)	P
Age (years)	1.08 (0.96-1.21)	0.192
Gestational age (weeks)	0.95 (0.87-1.04)	0.276
BMI (kg/m ²)	1.02 (0.94-1.11)	0.631
SBP (mmHg)	1.12 (1.05-1.19)	<0.001
DBP (mmHg)	1.09 (1.03-1.16)	0.003
MAP (mmHg)	1.18 (1.09-1.28)	<0.001
UPRO (mg/24 h)	3.45 (2.01-5.92)	<0.001
TNF- α mRNA	3.12 (1.86-5.24)	<0.001
NF- κ B p65 mRNA	2.45 (1.51-3.98)	<0.001

Note: Variables with $P < 0.1$ in the univariate analysis were included in the multivariate model. PE: preeclampsia; MPE: mild preeclampsia; SPE: severe preeclampsia; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; UPRO: 24-hour urinary protein; OR: odds ratio; CI: confidence interval; TNF- α : tumor necrosis factor- α ; NF- κ B p65: nuclear factor-kappa B p65.

tolic blood pressure, diastolic blood pressure, mean arterial pressure, 24-hour urinary protein quantification, TNF- α mRNA, and NF- κ B p65 mRNA were included to calculate the odds ratio (OR) and 95% confidence interval (CI) for each variable. The results of the univariate analysis are presented in **Table 3**. Variables with $P < 0.1$ in the univariate analysis (including systolic blood pressure, diastolic blood pressure, mean arterial pressure, 24-hour urinary protein quantification, TNF- α mRNA, and NF- κ B p65 mRNA) were subsequently entered into a multivariate logistic regression model. The assignment of variables is shown in **Table 4**. The results (**Table 5**) showed that after adjusting for clinical indicators such as blood pressure and urinary protein, TNF- α mRNA (OR = 2.84, 95% CI: 1.67-4.82, $P < 0.001$) and NF- κ B p65 mRNA (OR = 2.13, 95% CI: 1.28-3.54, $P = 0.004$) remained statistically strong for the severity of PE. In addition, 24-hour urinary protein quantification (OR = 3.21, 95% CI: 1.89-5.45, $P < 0.001$) and mean arterial pressure (OR = 1.15, 95% CI: 1.06-1.25, $P = 0.001$) were also identified as independent risk factors for severe PE.

Predictive value of TNF- α and NF- κ B p65 mRNA expression for the severity of PE

Based on the analysis results of the above experiments, ROC curve analysis was used to

evaluate the predictive value of TNF- α and NF- κ B p65 mRNA, both individually and in combination, for distinguishing severe PE from mild PE (**Figure 8**). The results showed that the sensitivity and specificity of TNF- α mRNA at the optimal cutoff value (2.35) were 82.4% and 78.0%, respectively. The area under the curve of NF- κ B p65 mRNA was 0.79, the sensitivity was 75.6%, and the specificity was 74.5%. A binary logistic regression model was further used to calculate the combined predicted probability of TNF- α and NF- κ B p65 mRNA, and the ROC curve was plotted. The results showed that the AUC for the combined prediction was 0.91 (95% CI: 0.85-0.96), with a sensitivity of 86.7% and a specificity of 84.3%, all of which were higher than those of either single indicator.

Discussion

PE is a condition that affects multiple systems and organs in the body, resulting in damage. Patients typically present with elevated levels of UPRO and high blood pressure, which can have an impact on the maternal and infant health [12, 13]. As research progresses, it has been discovered that inflammation induced by the immune response exerts a crucial effect in the progression of PE [14]. Certain placental-derived circulating factors in the maternal body can serve as predictive indicators for PE.

The present study found that the positive rate, mRNA expression, and protein expression levels of TNF- α in placental tissues of patients with mild and severe PE were significantly higher than those in the normal control group, with the highest levels observed in the sPE group. This finding is consistent with multiple previous reports. For instance, Ali et al. (2020) reported that TNF- α expression was elevated 2.79-fold in patients with PE [15]. The increase in TNF- α in the PE placenta may impair the invasive capacity of extravillous trophoblasts, leading to placental ischemia and hypoxia, which in turn amplifies local inflammation [16]. Additionally, TNF- α can increase vascular endothelial permeability and induce systemic endothelial dysfunction [17]. The present study also observed higher TNF- α expression in the sPE group than in the mPE group, suggesting that TNF- α levels are associated with disease severity and supporting the view that excessive

Table 4. Assignment of variables

Variable	Variable type	Assignment description
Severity of PE (dependent variable)	Binary	SPE = 1, MPE = 0
SBP (mmHg)	Continuous variable	Original value entered
DBP (mmHg)	Continuous variable	Original value entered
MAP (mmHg)	Continuous variable	Original value entered
UPRO (mg/24 h)	Continuous variable	Original value entered
TNF- α mRNA	Continuous variable	Original value entered
NF- κ B p65 mRNA	Continuous variable	Original value entered

Note: PE: preeclampsia; MPE: mild preeclampsia; SPE: severe preeclampsia; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; UPRO: 24-hour urinary protein; TNF- α : tumor necrosis factor- α ; NF- κ B p65: nuclear factor-kappa B p65.

Table 5. Multivariate logistic regression analysis of the severity of PE

Variable	β	Standard error	Wald χ^2	OR (95% CI)	P
TNF- α mRNA	1.04	0.27	14.83	2.84 (1.67-4.82)	<0.001
NF- κ B p65 mRNA	0.76	0.26	8.55	2.13 (1.28-3.54)	0.004
24-hour urine protein quantification	1.17	0.27	18.78	3.21 (1.89-5.45)	<0.001
Mean arterial pressure	0.14	0.04	12.25	1.15 (1.06-1.25)	0.001

Note: OR: odds ratio; CI: confidence interval; TNF- α : tumor necrosis factor- α ; NF- κ B p65: nuclear factor-kappa B p65.

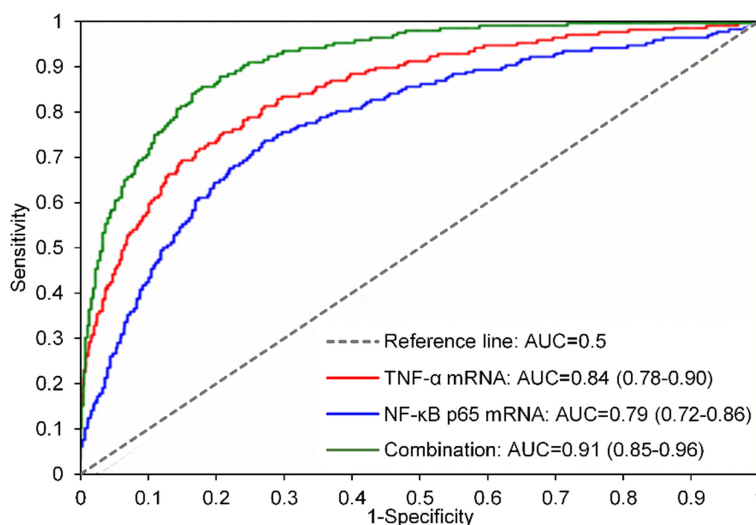


Figure 8. Receiver operating characteristic curve of TNF- α and NF- κ B p65 mRNA expression in predicting severe PE. Note: PE: preeclampsia; TNF- α : tumor necrosis factor- α ; NF- κ B p65: nuclear factor-kappa B p65.

inflammation exacerbates placental vascular defects and reduces blood flow [18-20].

Regarding NF- κ B p65, the present study found that the positive rate, mRNA expression, and protein expression levels of NF- κ B p65 in placental tissues of patients with mild and severe PE were significantly higher than those of normal pregnant women. A recent review summary

stated that, compared to pregnant women without a family history of PE, the expression level of NF- κ B in patients with PE was increased by 23.35% [21], and multiple studies had confirmed that NF- κ B was significantly elevated in the placenta and maternal circulation, which was closely associated with increased maternal inflammatory levels [22]. Activated NF- κ B serves as a transcriptional activation factor for important inflammatory factors such as TNF- α [23, 24]. Moreover, NF- κ B activation can promote the elevation of expression levels of growth factors, leading to smooth muscle cell proliferation, vascular reconstruction,

increased thickness of small arterial walls, and elevated mean arterial pressure, contributing to the occurrence of hypertensive disorders of pregnancy [25, 26]. In addition, the study showed that the expression levels of TNF- α and NF- κ B p65 in PE placenta were positively correlated, suggesting that there may be a synergistic amplification between them. TNF- α can increase the transcription of TNF- α by activat-

ing NF- κ B, which will aggravate placental inflammation and endothelial injury. This finding is consistent with previous research results. Zhu et al. [27] and Issotina Zibrila et al. [28] found that the expression of TNF- α in the placenta of PE rats was positively correlated, and blocking this signal pathway could alleviate the clinical symptoms of NF- κ B p65.

Regression analysis showed that TNF- α and NF- κ B p65 were independent risk factors, which had nothing to do with blood pressure and urine protein. The results show that inflammatory factors are central to the onset and progression of PE, and their role does not depend on common clinical detection indicators. A recent study also confirmed this. Li et al. [29] confirmed that the increase of circulating TNF- α is closely related to the increase of PE risk, which provided the basis for the results of this study. In addition, through receiver operating characteristic curve analysis, we found that the expression of TNF- α and NF- κ B p65 genes can effectively predict severe and mild PEa, and the AUC of their combination increased to 0.91, with both sensitivity and specificity also superior to those of either single indicator. This suggests that the above factors not only play an important role in the pathogenesis of PE but also may serve as supplementary markers for the timely detection and risk categorization of eclampsia. Pogula et al. [30] pointed out that the inflammatory factor represented by TNF- α can be used as an early warning index of PE, and its genetic variation is closely related to the risk of PEa. Combining the two methods is expected to improve the accuracy of early diagnosis of diseases and provide more accurate evidence for clinical treatment. It is worth noting that some studies have reported no significant correlation between TNF- α and blood pressure [29], whereas we observed a significant positive correlation ($r = 0.671$ with SBP). This discrepancy may be attributed to differences in sample collection time (antepartum vs. postpartum), patient ethnicity, or the distribution of disease severity. Our cohort included both mild and severe PE patients, whereas some studies enrolled only severe cases, which may have masked the dose-dependent relationship.

Despite these important findings, this study had several limitations. First, the retrospective, single-center design may have introduced

selection bias. Second, although the sample size is larger than that of many previous studies, it remains moderate and requires external validation in an independent cohort. Third, we only measured TNF- α and NF- κ B p65 at delivery (at the time of placental collection) and therefore do not know their dynamic changes during pregnancy. Fourth, we did not measure the circulating levels of these markers, limiting comparison with blood biomarker studies. Fifth, the optimal cutoff values for predicting severe PE (e.g., the TNF- α mRNA cutoff of 2.35) were derived from this dataset and require validation. Finally, the synergistic mechanism between TNF- α and NF- κ B p65 was inferred through correlation analysis and requires functional experiments (e.g., knock-down or inhibition studies) to confirm causality.

Conclusion

Expression levels of TNF- α , NF- κ B p65, and mRNA in the placenta of PE patients were increased (with statistical significance), and the patients in the sPE group had significantly higher levels than those of the mPE group. The levels of TNF- α and NF- κ B p65 in the serum of PE patients are closely related to the progress of PE. The analysis results show that TNF- α and NF- κ B p65 are key contributors to the development of PE, and both of them can well reflect the severity of PE, and can be used as an auxiliary diagnostic index. Therefore, TNF- α and NF- κ B-p65 are important regulatory factors of PE, and are also an important basis for the study of the pathogenesis of PE. In the future, this study will continue to use molecular biology and cell biology techniques to explore the molecular mechanism of the occurrence and development of PE from the molecular, cellular, and overall levels, thus offering a stronger theoretical foundation for both the early intervention of PE and the identification of therapeutic targets.

Disclosure of conflict of interest

None.

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