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Original Article

Significant hypomethylation of *TNFAIP8* and increased expression in the placenta and peripheral blood cells from early-onset preeclamptic patients

Liang Lin[1,2], Yanhong Yu[1], Zhenhai Zhang[1], Yin Yang[2]

[1]*Department of Obstetrics, Affiliated Southern Hospital of Southern Medical University, Guangzhou 510515, Guangdong, China;* [2]*Provincial Clinical College of Fujian Medical University, Fuzhou 350001, Fujian, China*

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Abstract: Objective: To investigate the methylation and expression of tumor necrosis factor α-induced protein 8 (TNFAIP8) in the placenta and peripheral blood from preeclamptic patients. Study design: Placental and peripheral blood samples were obtained from women with early-onset and late-onset preeclampsia (EOPE, LOPE) and healthy pregnant women. TNFAIP8 methylation, mRNA and protein expression and its localization in the placenta were examined by pyrosequencing, quantitative RT-PCR, Western blotting, immunohistochemistry, respectively. Results: We observed significant hypomethylation of TNFAIP8 gene in placentas of EOPE patients compared with those of LOPE and control patients. TNFAIP8 was mainly localized in syncytiotrophoblasts and vascular endothelial cells of placenta. Moreover, TNFAIP8 mRNA and protein expression in placenta and peripheral blood cells significantly decreased following the order of EOPE→LOPE→control patients. Conclusions: Significant hypomethylation and high expression of TNFAIP8 in the placenta and peripheral blood cells were observed in EOPE, suggesting TNFAIP8 may be associated with the pathogenesis of preeclampsia.

Keywords: Preeclampsia, methylation, TNFAIP8, placenta, peripheral blood

Introduction

Preeclampsia (PE) is a critical pregnancy-related disease that is clinically characterized by hypertension and proteinuria [1]. PE affects 3-5% of all pregnancies and is the leading cause for maternal mortality, morbidity, perinatal death, preterm birth, and intrauterine growth restriction [2, 3]. Pathological changes in the placenta with PE mainly include vascular lesions in the decidua, infarction, placental abruption, villus dysplasia, and incremental syncytial knots [1, 4]. Increasing evidence has suggested that PE results from placental dysfunction during the first trimester, which is primarily caused by the shallow invasion of extravillous trophoblast cells and impaired vascular development in the placental bed [5]. All PE-associated symptoms resolve after the delivery of the placenta. PE has been classified into two types: early-onset (<34 weeks) and late-onset (>34 weeks). Early-onset PE (EOPE) is a distinct and more severe clinical entity compared with late-onset PE (LOPE). High proteinuria is the most frequent symptom in EOPE [6]. It has been reported that placental lesions associated with maternal underperfusion occur more frequently in EOPE than in LOPE [7]. Moreover, these two types of PEs have been proposed to have different pathophysiological mechanisms and significant differences in placental findings, but the differences at the molecular level are still not well clarified.

DNA methylation is the most commonly studied epigenetic process, which is primarily observed in CpG dinucleotides that are underrepresented in the genome at low density, but are enriched in the promoter regions of genes [8, 9]. Elevated DNA methylation in one particular gene is generally associated with gene inactivity, whereas a lower level of DNA methylation indicates the potential for gene expression [9]. Aberrant gene expression in the placenta has been linked to PE [10]. Tumor necrosis factor α-induced protein 8 (TNFAIP8, also known as

Table 1. Sequences of primers for pyrosequencing

Name	Sequences (5'→3')
TNFAIP8-F	GGGGAGGTTTTGATTTTAGTGGTT
TNFAIP8-R-B	ATCCTACCTTCCTTAAATTCTTCTACTT
*TNFAIP*8-S	GGTTAGTTAGAGTATATGTGAG

SCC-S2, GG2-1, and MDC-3.13) is a 21-kDa cytosolic protein that is induced by TNF-αand transcription factor nuclear factor-κB activation [11]. TNFAIP8 primarily functions as an oncogenic and antiapoptotic molecule by enhancing cell survival and inhibiting the activity of apoptotic proteins caspase 3 and caspase 8 [12, 13]. Previous studies have demonstrated that TNFAIP8 is overexpressed in various tumors and is correlated with chemotherapy resistance in tumors and its clinical outcomes [14, 15]. Moreover, Liu *et al*. reported that TNFAIP8 overexpression is positively correlated to higher histological grade, deep myometrial invasion, lymphovascular space invasion, lymph node metastasis, and recurrence in endometrial cancer [16]. Because the occurrence of PE is closely associated with the shallow invasion of extravillous trophoblast cells into the endometrium, we assume that TNFAIP8 may play a specific role in the pathogenesis of PE. However, the potential roles of TNFAIP8 and its methylation in the placenta and placenta-related diseases like PE have never been investigated. Therefore, in the present study, we aimed to investigate the methylation of TNFAIP8 in the placenta and examine TNFAIP8 expression in the placenta and peripheral blood of patients with EOPE and LOPE.

Materials and methods

Patient enrollment and sample collection

A total of 64 pregnant women who underwent labor delivery at Fujian Provincial Hospital between March 2013 and August 2014 were recruited for this study. They included 23 pregnant women with EOPE (<34 weeks, mean age 29.39±5.69 years), 16 pregnant women with LOPE (≥34 weeks, mean age 28.00±6.55 years), and 25 healthy pregnant women who delivered at full term (control, mean age 28.32±4.55 years). The diagnosis of PE was made according to previously described criteria [17]. The exclusion criteria for pregnant women included: (1) stillbirth and fetal malformation; (2) kidney disease, diabetes, immune disease, cancer, epilepsy, and other obstetrical complications; (3) multiple pregnancies (≥3 fetuses); and (4) alcohol and drug addiction. This study was approved by the local ethics committee of Fujian Provincial Hospital, and written consent was obtained from all recruited participants.

Peripheral blood samples (3 ml × 2 tubes) was collected into tubes with EDTA (ethylenediaminetetraacetic acid) from all PE patients and control participants before labor delivery and stored at -80°C. The blood cells were collected by centrifugation and used for RNA and protein extraction. Four placental tissue samples of 1.0 × 1.0 × 1.0 cm^3 were collected from different locations of every placenta, washed with phosphate-buffered saline (PBS), and frozen at -80°C.

DNA and total RNA isolation

DNA isolation from placental issues was carried out using a DNA Extraction Kit (Qiagen, Cat No. 59124, Valencia, CA). The DNA concentration was measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Tewksbury, MA), and its quality was examined on an agarose gel. Total RNA from placental tissues and blood cells was isolated using the RNeasy Mini Kit (CWbio Co. Ltd., Cat No. CW0581, Beijing, China). The RNA quality was assessed by electrophoresis on a 1% denaturing agarose *gel*.

Evaluation of TNFAIP8 methylation by bisulfite pyrosequencing

DNA methylation of *TNFAIP8* was confirmed by bisulfite pyrosequencing as previously described [18]. Methylation-unbiased pyrosequencing primers (**Table 1**) were designed by PyroMark Assay Design 2.0 to include the same CpG sites targeted by the Illumina probes. A total of 1-2 μg DNA was transformed using the EpiTect Plus DNA Bisulfite Kit (Qiagen) and used as a template for PCR. The assay was run on a Qiagen PyroMark Q24 system (Qiagen) following the manufacturer's instructions. The quantitative levels of *TNFAIP8* methylation for the CpG dinucleotide were evaluated using the Pyro Q-CpG software (Biotage, Uppsala, Sweden).

Immunohistochemistry

Placental samples were fixed in formalin and embedded in paraffin. Sections (4 m) were

Table 2. Primer sequences for qPCR

Name	Sequences (5'→3')
TNFAIP8-F	GGGGAGGTTTTGATTTTAGTGGTT
TNFAIP8-R	ATCCTACCTTCCTTAAATTCTTCTACTT
β-actin-F	ACTTAGTTGCGTTACACCCTT
β-actin-R	GTCACCTTCACCGTTCCA

deparaffinized, rehydrated, and subjected to antigen retrieval at 95°C for 10 min in 0.05 mol/L Tris-EDTA solution (pH 6.0). Then sections were treated with 4% hydrogen peroxide for 10 min in the dark at room temperature, blocked in normal goat serum, and then incubated with rabbit antihuman TNFAIP8 polyclonal antibody (1:100 dilution, Bioss, China) at 37°C for 2 h. The sections were incubated with secondary antibody (goat anti-rabbit 1:1,000; Beijing Zhongshan Biotechnology) for 20 min at 37°C after three washes in PBS. The sections were counterstained with hematoxylin and mounted with a cover glass. The positive and negative images were captured using a BX51T-PHD-J11 microscope (Olympus Corp., Tokyo, Japan).

Quantitative reverse transcription polymerase chain reaction (RT-PCR)

The cDNA synthesis was conducted using HiFi-MMLV cDNA kit (CWbio. Co. Ltd., Cat. No. CW-0744). The primer sequences for quantitative PCR (qPCR) are listed in **Table 2**. qPCR was carried out with an ABI prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). After heating at 95°C for 5 min, the samples were run for 45-48 cycles at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec, and then incubated at 72°C for 10 min. The relative quantification of gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and using β-actin gene expression as an endogenous control.

Western blotting

The placenta tissues were homogenized, and blood cells were lysed in lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCL, 1% NP-40, 10 mM EDTA, 1 mM phenylmethane sulfonyl fluoride, 50 mM NaF, 40 mM β-glycerophosphate, 200 μM sodium orthovanadate, 40 mM benzamidine, and protease inhibitor cocktail (EMD, Gibbstown, NJ) on ice for 20 min. The lysates were collected and centrifuged at 12,000×*g* for 10 min at 4°C. The concentration of protein in lysates was detected using the Pierce BCA Protein Assay Kit (Life Technologies, Grand Island, NY). Aliquots of 30 μg protein were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blocked with Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST) and 5% skim milk powder. The membranes were then incubated with antibodies to TNFAIP8 (1:100 dilution, Bioss) and rabbit polyclonal β-actin (loading control, 1:100 dilution, Beijing Zhongshan Biotechnology) with gentle shaking at room temperature for 1 h. After a few washes with TBST, the membranes were incubated with secondary antibody for 1 h, washed, and then developed using an ECL Western Blotting Detection System (Amersham Biosciences, Buckinghamshire, England). The intensities of the bands were scanned using ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical analysis

The data are presented as mean ± standard deviation (SD) and were analyzed using SPSS 19.0 software (SPSS, Inc., Chicago, IL). The data among three groups with normal distribution were analyzed by single factor analysis of variance (ANOVA) and using the least significant difference (LSD) method. Linear regression was used to evaluate the correlation between *TNFAIP8* methylation in the placenta and mean arterial pressure (MAP) and fetal birth weight. A P value <0.05 was considered statistically significant.

Results

General characteristics of participants

The general characteristics of the participants are presented in Supplementary Table 1. No significant differences were noted in maternal age or body mass index at early pregnancy among the three groups. The urine protein concentration at 24 h did not differ significantly between EOPE and LOPE patients (P>0.05). Gestational age at delivery and fetal birth weight in the EOPE group were significantly lower than those in the LOPE and control groups (P<0.05). The MAP at delivery in PE patients was significantly higher than that in control participants (P<0.05). Twenty-one cases (91%) in the EOPE group and four cases (25%) in LOPE group delivered preterm.

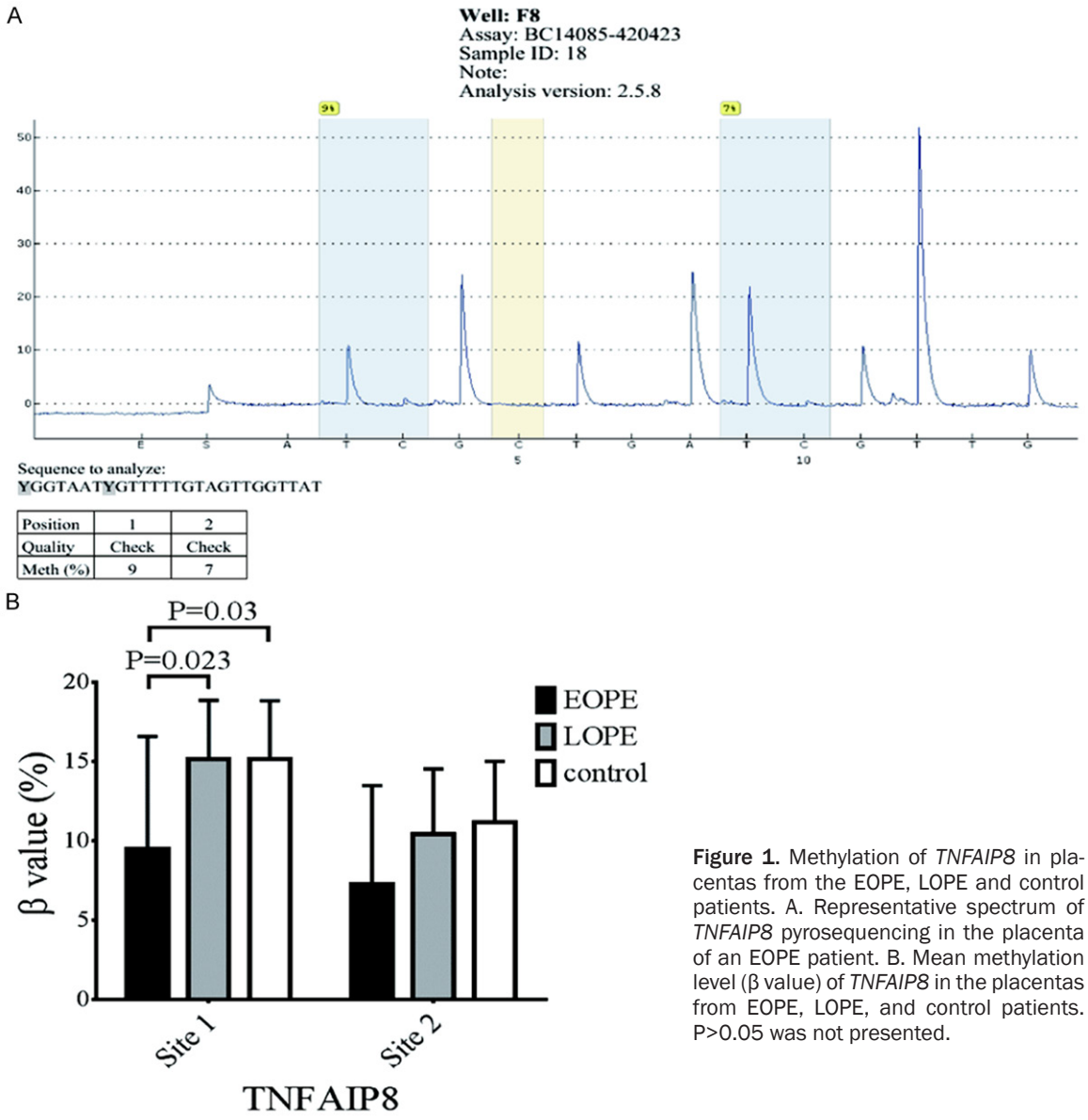

Figure 1. Methylation of *TNFAIP8* in placentas from the EOPE, LOPE and control patients. A. Representative spectrum of *TNFAIP8* pyrosequencing in the placenta of an EOPE patient. B. Mean methylation level (β value) of *TNFAIP8* in the placentas from EOPE, LOPE, and control patients. P>0.05 was not presented.

TNFAIP8 methylation in the placentas of patients with PE

TNFAIP8 methylation was examined by bisulfite pyrosequencing, because Δβ was approximately 14% when the EOPE group was compared with the LOPE and control groups. Two Illumina CpG sites in the 5' untranslated region (UTR) and 1st exon of *TNFAIP8* were selected to ensure a comprehensive assessment of the gene region. The sequence analyzed was YGGTAATYGTTTTTGTAGTTGGTTAT. Moreover, two significant methylation sites in *TNFAIP8* were observed in the placenta (**Figure 1A**). The methylation of site 1 was not detected (value =0) in four specimens in the EOPE group (17.4%), but was detected in all specimens from the LOPE and control groups. Likewise, no methylation of site 2 was found in five patients in EOPE group (21.7%), but only in one patient in each of the LOPE and control groups. We further compared the methylation percentage of the two sites in *TNFAIP8* among the three groups. We found the methylation percentage of site 1 in the EOPE group was significantly lower than that in the LOPE and control groups (P<0.05), but there was no significant difference in the methylation of site 1 between the LOPE and control groups (P>0.05, **Figure 1B**). Surprisingly, we did not observe a significant

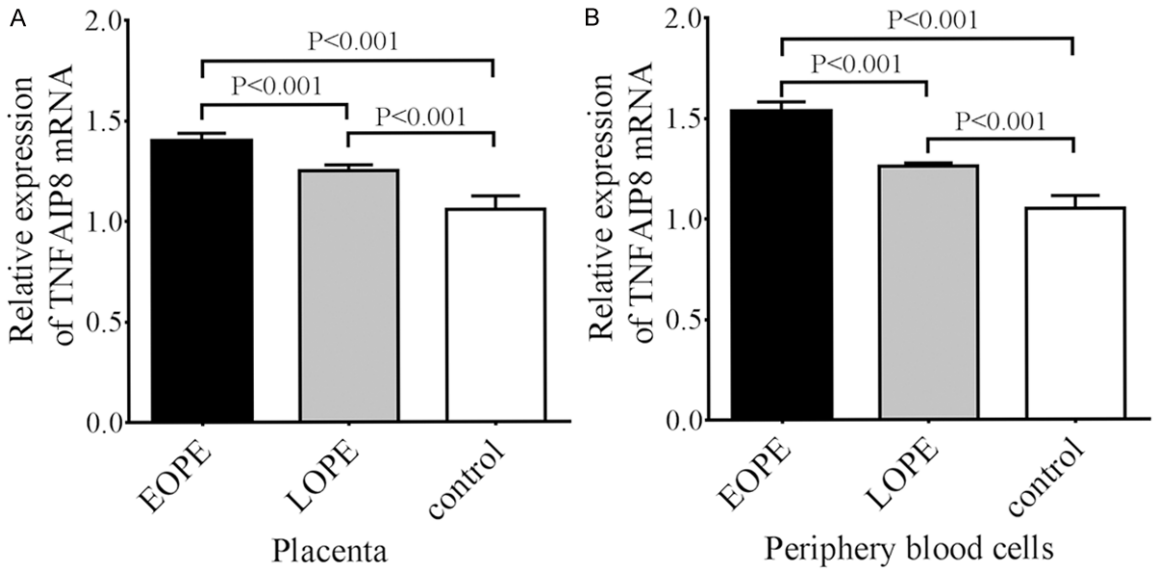

Figure 2. TNFAIP8 mRNA expression in the placentas (A) and peripheral blood cells (B) from EOPE, LOPE, and control patients. The data are presented as mean ± SD (n=23 for EOPE, n=16 for LOPE, and n=25 for controls). The relative expression of TNFAIP8 mRNA was determined according to the ratio of TNFAIP8 mRNA expression to endogenous control β-actin mRNA expression.

difference in the methylation percentage of site 2 among the three groups (P>0.05, **Figure 1B**). These data suggest that the degree of *TNFAIP8* methylation in the placentas of EOPE patients is significantly less than that in placentas of LOPE and control patients.

TNFAIP8 mRNA expression in placenta and peripheral blood cells of patients with PE

To assess the effect of *TNFAIP* methylation on its mRNA expression, the TNFAIP8 mRNA expression in the placenta and peripheral blood samples from the three groups was examined by qRT-PCR. We observed that TNFAIP8 mRNA expression in the placenta of EOPE patients was significantly higher than that in the LOPE and control groups (P<0.01, **Figure 2A**). Also, TNFAIP8 mRNA expression in the placenta of LOPE patients was significantly higher than that in control participants (P<0.01, **Figure 2A**). Interestingly, we also examined the TNFAIP8 mRNA expression in the peripheral blood samples in the three groups and found that its expression pattern in the peripheral blood cells was completely consistent with that in the placenta for the three groups. That is, TNFAIP8 mRNA expression in the peripheral blood cells from EOPE patients was significantly higher than that in LOPE and control patients (P<0.01, **Figure 2B**).

TNFAIP8 protein expression in placenta and peripheral blood cells of patients with PE

To further confirm the results of TNFAIP8 mRNA expression, we examined the protein expression of TNFAIP8 in the placenta and peripheral blood cells by Western blotting. Similar to the trend in mRNA expression, TNFAIP8 protein expression in the placenta of EOPE patients was significantly higher than that in the LOPE and control groups (P<0.01, **Figure 3A**). Moreover, TNFAIP8 protein expression in the placenta of LOPE patients was significantly higher than that in control participants (P<0.01, **Figure 3A**). In the meantime, we also observed that TNFAIP8 protein expression in the peripheral blood cells decreased significantly according to the following order EOPE→LOPE→control patients (P<0.01, **Figure 3B**). Immunohistochemical staining showed that positive TNFAIP8 expression was mainly localized in the syncytiotrophoblasts and vascular endothelial cells of the placenta in all three groups, and the staining patterns also indicated that TNFAIP8 expression gradually decreased following the trend of EOPE→LOPE→control patients (**Figure 4A-F**).

Associations between TNFAIP8 methylation in the placenta and clinical parameters

Because we observed significant differences in MAP and fetal birth weight and *TNFAIP8* methylation (site 1) was significant lower in EOPE

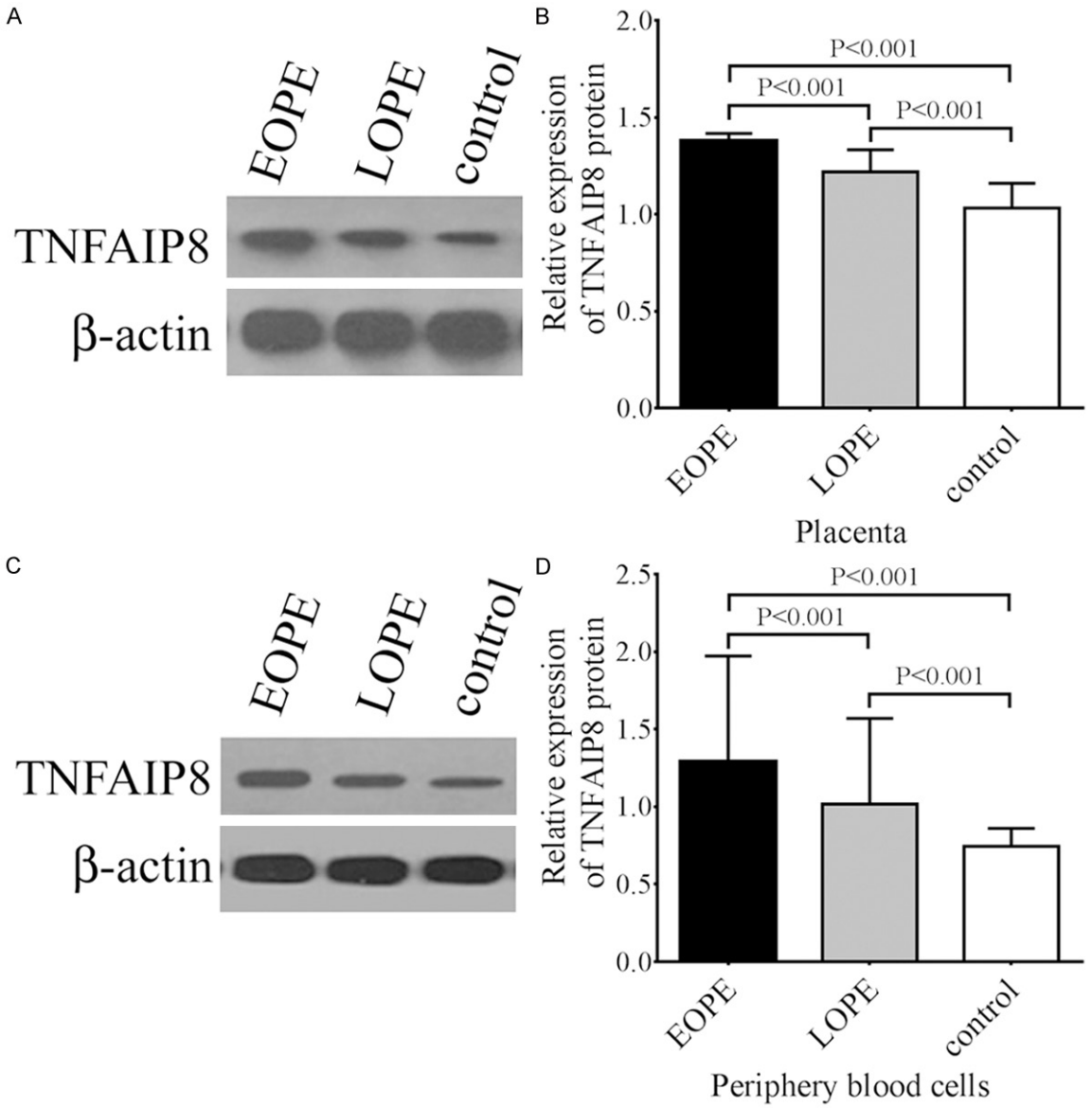

Figure 3. TNFAIP8 protein expression in the placentas and peripheral blood cells from EOPE, LOPE, and control patients. A. Representative image for TNFAIP8 protein expression in the placentas from EOPE, LOPE, and control patients. B. Statistical analysis of TNFAIP8 protein expression in the placentas. C. Representative image for TNFAIP8 protein expression in the peripheral blood cells from EOPE, LOPE, and control patients. D. Statistical analysis of TNFAIP8 protein expression in the peripheral blood cells. The data are presented as mean ± SD (n=23 for EOPE, n=16 for LOPE, and n=25 for controls). The relative expression of TNFAIP8 protein was determined according to the ratio of TNFAIP8 protein expression to endogenous control β-actin protein expression.

patients, we sought to examine whether there is an association between *TNFAIP8* methylation in the placenta and fetal birth weight and MAP. Although the MAP in PE patients (including EOPE and LOPE) was significantly higher than that in control patients, we did not find any correlation between *TNFAIP8* methylation in the placenta and MAP in both PE patients and control patients (**Figure 5A** and **5B**). However, we observed a significant positive correlation between *TNFAIP8* methylation in the placenta and fetal birth weight in PE patients (including EOPE and LOPE; P<0.01, **Figure 5C**), and this correlation was not significant in control patients (P>0.05, **Figure 5D**) and the individual EOPE and LOPE groups (data not shown).

Discussion

Placental function is highly associated with fetal development and health, and placental

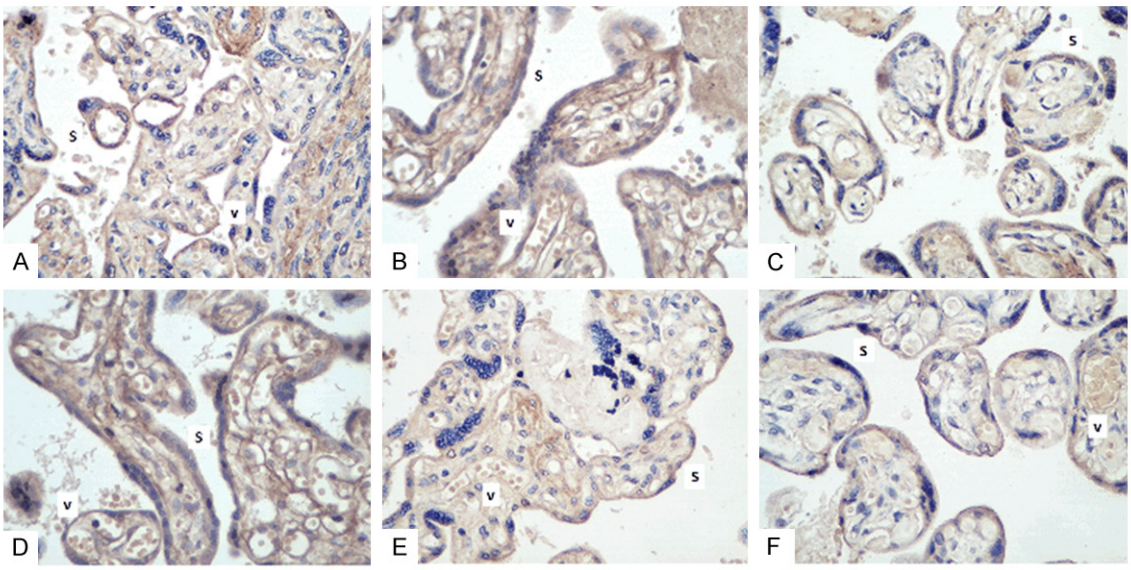

Figure 4. TNFAIP8 expression in placentas of the three groups (immunohistochemistry, 400 ×). (S: syncytiotrophoblast; V: vascular endothelial cell). TNFAIP8 expression in the placenta in the EOPE group (A and B), LOPE group (C and D), and control group (E and F).

dysfunction, particularly aberrant placental gene expression, has been linked to many pregnancy-associated diseases including PE, intrauterine growth restriction, gestational diabetes mellitus, and gestational trophoblastic disease [10, 19]. Placenta-specific methylation patterns have been demonstrated in the placenta and maternal plasma of PE patients. Previous studies have demonstrated that gene expression is altered in pregnancies with PE and PE is associated with altered DNA methylation [18, 20]. Given our limited understanding of the epigenetic alterations in PE, it is necessary to verify the reported associations between DNA methylation and PE in further studies. Therefore, in this study, we examined *TNFAIP8* methylation in the placenta and its expression in the placenta and peripheral blood from EOPE, LOPE, and control patients. We found a lower level of *TNFAIP8* methylation in the placenta from LOPE patients but higher expression of TNFAIP8 mRNA and protein in the placenta and peripheral blood from EOPE patients than in those from LOPE and control patients.

Because DNA methylation profiling in preeclamptic placentas has been reported, some researchers initiated studies of the methylation of specific genes or a group of genes in preeclamptic placentas and their functions in the pathogenesis of PE, especially EOPE. Xiang *et al*. reported that promoter hypomethylation of *TIMP3* is associated with PE [21]. Moreover, hypomethylation of multiple genes such as GATA zinc finger domain containing 1 (*GATAD1*), [22] *LEP*, [23] and *matrix metalloproteinase 9* [24] have been identified in preeclamptic placentas. In addition, hypermethylation of some genes such as *SH3PXD2A* [23] and human endogenous retrovirus (*HERV*) [25] is also observed in preeclamptic placentas. Here we report significantly greater hypomethylation of *TNFAIP8* in the placentas of EOPE patients compared with that in LOPE and control patients, although the significant difference was only observed in one of two methylated sites in *TNFAIP8*. These data implicate that hypomethylation of *TNFAIP8* in the placentas of EOPE patients may influence its expression and regulate placental function during the pathogenesis of EOPE.

The hypomethylation of genes is associated with the activation of gene expression, and thus, it is reasonable to speculate that hypomethylation of *TNFAIP8* in the placentas of EOPE patients may lead to its high expression. Indeed, we observed that both mRNA and protein expression of *TNFAIP8* was significantly upregulated in the placentas of EOPE patients compared with LOPE and control patients. Interestingly, we also found that the expression

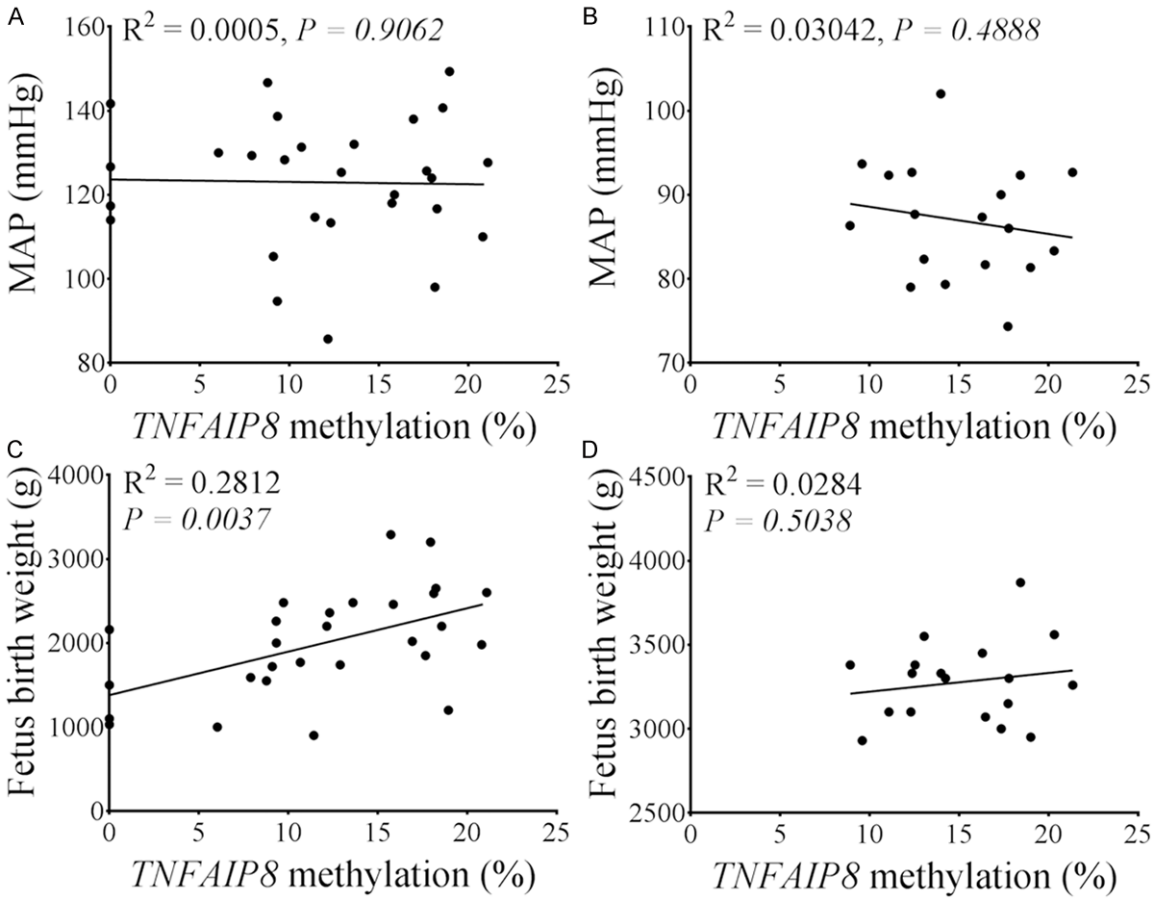

Figure 5. Correlation analyses between *TNFAIP8* methylation in the placenta and clinical parameters. The correlation between *TNFAIP8* methylation in the placenta and MAP in PE patients (including EOPE and LOPE) (A) and control participants (B) was analyzed by linear regression. Similarly, the correlation between *TNFAIP8* methylation in the placentas and fetal birth weight in PE patients (including EOPE and LOPE) (C) and control participants (D) was assessed. R squared and *P* values are presented in the graphs.

of TNFAIP8 mRNA and protein in the placentas of LOPE patients was significantly higher than that in control participants, although no significant difference in *TNFAIP8* methylation was observed between the two groups. These data suggest that TNFAIP8 may be associated with the severity of PE. It has been found that TNFAIP8 is highly related to the growth, invasion, metastasis, and clinical prognosis of some cancers, [12-16] but to the best of our knowledge, this is the first report to investigate the function of TNFAIP8 in the placenta and a placenta-associated disease like PE. Recent work has demonstrated that the production of TNF-α and other inflammatory cytokines is elevated in women with pE, [26] and TNF-α blockade can partially attenuate the associated hypertension in the reduced uterine perfusion pressure (RUPP) animal model of PE [27]. These findings can help to explain the high expression of TNFAIP8 in the placentas of EOPE patients and its potential role in the pathogenesis of PE. Moreover, PE is closely associated with defective trophoblast cell invasion, endothelial cell dysfunction, and dysregulated uteroplacental vascularization [28]. It has been suggested that aberrant placental expression of matrix metalloproteinases (MMPs) may cause shallow cytotrophoblastic invasion and incomplete remodeling of the spiral arteries, which is thought to link placental ischemia to the cardiovascular alterations of PE [29]. Antisense inhibition of endogenous TNFAIP8 expression results in decreased expression of vascular endothelial growth factor (VEGF) receptor-2, MMP-1, and MMP-9 in tumor cells [30]. Here we observed that TNFAIP8 was predominantly present in the syncytiotrophoblasts and vascu-

lar endothelial cells of the placenta. This evidence suggests that TNFAIP8 may cause aberrant invasion of placenta villi via mediating the expression of MMPs. Additionally, consistent with the TNFAIP8 expression pattern in the placenta, we also observed higher mRNA and protein expression of TNFAIP8 in the peripheral blood of EOPE patients compared with LOPE and control patients, which is consistent with the significantly elevated serum TNF-α concentration in women with severe PE [31]. High expression of TNFAIP8 in the peripheral blood cells of EOPE patients indicates that TNFAIP8 may be used as a potential biomarker for the diagnosis of EOPE. More importantly, we observed that the level of *TNFAIP8* methylation in the placentas of PE patients was significantly correlated with fetal birth weight, a correlation that was not obvious in control patients. This evidence indicates that TNFAIP8 may be an important factor that affects placental function and fetal growth during the progression of PE, although this finding requires further investigation.

In conclusion, we observed significant hypomethylation of *TNFAIP8* in the placenta and increased expression of TNFAIP8 in the placenta and peripheral blood cells in EOPE patients. The level of *TNFAIP8* methylation in the placenta was significantly correlated with fetal birth weight in PE patients. These findings demonstrate that TNFAIP8 may be a critical factor in the pathogenesis of PE.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yanhong Yu, Department of Obstetrics, Affiliated Southern Hospital of Southern Medical University, 1838 North Guangzhou Avenue, Guangzhou 510515, Guangdong, China. Tel: +86 020-61648687; Fax: +86 020-62787562; E-mail: yuyh1010@hotmail.com

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Supplementary Table 1. Clinical characteristics of study participants

	EOPE group (n=23)	LOPE group (n=16)	Control group (n=25)
Maternal age (years)	29.826±5.523	28.000±6.552	28.320±4.553
Gravidity, median (range)	2.522 (1-4)	1.938 (1-4)	1.840 (1-4)
Primiparity, n (%)	12 (52.174)	12 (75)	15 (60)
Early pregnancy BMI (kg/m^2)	21.550±3.585	21.472±2.483	21.604±3.450
Gestational age at delivery (weeks)	33.161±2.727^{A,B}	38.063±1.534	39.360±0.732
Mean arterial pressure (mmHg)	124.014±12.938^{A,B}	119.125±14.246^{C}	87.733±9.272
24 h urine protein (g/L)	5.310±4.430	2.913±2.259	-
Birth weight (g)	1606.520±611.694^{A,B}	2740.000±565.108^{C}	3324.000±287.141

Data are presented as mean ± standard error of the mean (SEM). ^{A}EOPE group *vs*. LOPE group, ^{B}EOPE group *vs*. control group and ^{C}LOPE group *vs*. control group, $P<0.01$. BMI, body mass index.