Hypomethylation of tissue factor pathway inhibitor 2 in human placenta of preeclampsia

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Abstract

Objectives: To investigate the expression, DNA methylation status and its regulatory mechanism of tissue factor pathway inhibitor 2 (TFPI-2) in human placenta tissues of preeclampsia (PE).

Material and methods: We studied the mRNA and protein expression and the promoter methylation levels of TFPI-2 in the PE placentas compared with those in the normal pregnant (NP) women. Quantitative real-time polymerase chain reaction, immunohistochemistry, western blot, and Sequenom MassARRAY were used for placenta tissue detection.

Results: The expressions of TFPI-2 mRNA and protein were significantly elevated in the PE placentas when compared with those in the NP ones ($P<0.05$).

Hypomethylation of the TFPI-2 promoter was detected both in PE patients and NP women, with a significant decrease in PE placentas ($P=0.005$). The methylation level was significantly decreased at CpG_6 (-168 to -167), CpG_15 (-98 to -97) and CpG_18.19 (-68 to -65) in PE patients than that in normal placentas ($P<0.05$).

However, the expression of DNMT-1 didn't show significant difference between the two groups ($P>0.05$).

Conclusion: Over-expression of TFPI-2 and aberrant promoter mythylation status presented in the PE placentas, suggesting that epigenetic mechanism might contribute to the pathogenesis of PE.

Keywords: DNMT-1, methylation, placenta, preeclampsia, TFPI-2.

Abbreviations list: TFPI-2, tissue factor pathway inhibitor 2; PE, preeclampsia; DNMT, DNA methyltransferase enzymes; NP, normal pregnant; BMI, body mass index; MAP, mean arterial pressure; LSD, lysine-specific demethylase

Introduction

Preeclampsia (PE), affecting pregnancy by 3-5%, is a pregnancy-specific syndrome routinely diagnosed with the combined presentation of high blood pressure and proteinuria [1]. PE is one of the main causes of maternal, fetal and neonatal mortality, especially in developing countries [2]. Although a great deal of research has been conducted to explore the precise pathogenesis, the complicated molecular mechanism of PE development and progression remains unclear. Accumulating evidence supports the hypothesis that thrombosis, accompanied with many changing coagulation factors such as tissue factor (TF) and tissue factor pathway inhibitor (TFPI), is an important contributing factor of PE development [3-5].

The tissue factor pathway inhibitor 2 (TFPI-2), located on chromosome 7q22, is a 32-kDa Kunitz-type serine proteinase inhibitor [6, 7]. The human TFPI-2 gene, expressed abundantly in full-term placenta and widely in a variety of adult human tissues such as liver, skeletal, muscle, heart, kidney and pancreas, has also been shown to reduce tumor invasion and metastasis [8]. TFPI-2 may play an important role in pregnancy maintenance, because the serum TFPI-2 can extremely increase during pregnancy and return to prepregnancy level several days after delivery, as evidenced by our previous report that demonstrated a significantly increased expression of TFPI-2 in PE placenta based on immunohistochemical analysis [9]. Expression of TFPI-2 inhibited migration, proliferation and invasion in Bewo and in JEG-3 cell lines, which may contribute to the pathogenesis of PE [10]. However, the molecular mechanisms regulating the differential placental expression of TFPI-2 have not been

identified.

DNA methylation of the imprinted genes is an intriguing mechanism linking environmental cues to placental pathology [11]. Aberrant DNA methylation has been demonstrated to control multiple physiological and pathological processes in trophoblast and placenta [12, 13]. As an imprinted gene cluster, the promoter of TFPI-2 has been reported to contain a CpG island region of 220 bp that spans exon1 and three transcription initiation sites, associated with transcriptional silencing of the gene [14-16]. Studies have also shown that TFPI-2 is frequently inhibited by promoter hypermethylation in non-small-cell lung cancer, gastric stromal tumor, cervical cancer and nasopharyngeal carcinoma [8, 17-19]. The development of placenta involves proliferation, invasion and migration of placental trophoblasts into the myometrium of the uterus, in a way that is similar to that in most malignant tumors [20]. In light of the close resemblance between the process of tumorigenesis and placental development, we hypothesized that promoter methylation could be the possible regulatory mechanism for the expression of TFPI-2.

To explore the role of methylation in regulating the differential expression of TFPI-2, we investigated the expressions of TFPI-2 mRNA and protein, and evaluated the methylation status of TFPI-2 in the human placenta tissues from PE patients and normal pregnancy. To date, there have been no such reports, as indicated by our literature review, on the methylatic mechanism regulating the TFPI-2 expression involved in the development of PE.

Material and Methods

Study participants

A total of 29 placenta samples, 10 diagnosed with normal pregnancy and 19 with PE, were obtained from the pregnant women attending the Obstetrics and Gynecology Hospital of Fudan University from July 2014 to June 2015. The diagnosis was made of PE according to the criteria of ACOG 2013: onset of hypertension in a previously normotensive woman and proteinuria (at least 0.3g of protein in a 24-hour urine sample) [21]. The cases diagnosed as chronic hypertension, diabetes mellitus, chronic nephritis and systemic lupus erythematosus were excluded. All of the participants, who were of Han ethnicity, received cesarean section. The women in the group of normal pregnancy chose cesarean delivery according to their personal request or had a history of uterine scar. The current study adhered to the principles of the Declaration of Helsinki and was approved by the institutional ethics committee. Written informed consent was obtained from all participants.

Sample collection and preparation

After placentas birth, three blocks (1×1×1cm) of chorionic villi were dissected from the central cotyledon on the maternal surface of each subject. When the samples were washed with phosphate buffered saline (PBS), one sample block was fixed in 4% formalin, and the other two blocks were frozen in liquid nitrogen to be stored at -80°C.

Immunohistochemistry

Formalin-fixed and paraffin-embedded, the samples of the placenta tissues were

studied. The 4- μ m-thick tissue sections were deparaffinized and rehydrated before immersed in EDTA buffer and subjected to antigen microwave retrieval. The sections were incubated with rabbit monoclonal anti-human TFPI-2 (Abcam, USA; dilution 1:100), DNMT-1 (Abcam, USA; dilution 1:100), DNMT-3a (Abcam, USA; dilution 1:100) or DNMT-3b (Abcam, USA; dilution 1:100) individually at 4°C overnight in a humidified chamber. Immunostaining for TFPI-2 and DNMTs was then revealed using a standard streptavidin-biotin peroxidase complex kit with diaminobenzidine as a chromogen (Tiangen Biotech, China). The negative controls were established by omitting the primary antibody of the same species.

When the images were acquired with an Olympus BX50 microscope digital camera (Olympus, Japan), staining intensity of TFPI-2 was analyzed using the computerized image analysis software (Image-Pro® Plus v6.0 Cybernetics) [22-24]. As DNMTs were nuclei expressed, all the slides for DNMTs analysis were independently reviewed by two investigators (XR Xiao and X Tao). The nuclei were regarded as being stained specifically only when there was an agreement between the two observers. The stained nuclei were scored by estimating the percentage of the syncytiotrophoblast cells [25, 26].

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the placenta samples using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. The first-strand complementary DNA (cDNA) was synthesized with M-MLV reverse transcriptase (Promega, USA). The primer sequences for TFPI-2 cDNA were (5'→3'):

TTCTGCGCACCAAAGAAA (F), TCTTGGATTAAAATAATAGCGAGTCA (R).

qRT-PCR were conducted to detect the relative mRNA levels using Power SYBR Green PCR Master Mix (Applied Biosystems, USA) on the Bio-Rad IQ5 Real Time PCR System (Bio-Rad, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The primer sequences for GAPDH cDNA were (5'→3'): TTCGACAGTCAGCCGCATCTT (F), CCCAATACGACCAAATCCGTT (R). The results were calculated using $2^{-\Delta\Delta Ct}$.

Western blot

From the placenta tissues the total protein was extracted using radioimmunoprecipitation assay (RIPA) buffer containing 1 mmol/L protease inhibitor cocktail. Upon 5-min heating at 95°C, 20µg of denatured protein for each reaction was used to load a 12% polyacrylamide SDS for electrophoresis. Polyvinylidene difluoride (PVDF) membranes were treated as the transfer by a wet transferring system. With 2-h blocking at room temperature, the membrane was probed with primary antibody (Abcam, USA; dilution 1:1000) at 4 °C overnight, and with secondary HRP-conjugated antibody for 1h at room temperature. The immunoreactive bands were detected by chemiluminescence and photographed in ChemiDoc™ Imaging Systems. GAPDH was used as an endogenous protein for normalization. The blot was analyzed using Image J software, and the ratio of the absorbance volume was normalized to GAPDH.

DNA preparation and bisulfite conversion

To avoid the potential effects on DNA methylation of glucocorticoid, we studied the

methylation status in 10 PE patients and all 10 normal pregnancy. Genomic DNA was isolated from the placentas using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. An RNase step was included during DNA isolation to avoid the contamination by RNA. The concentration and purity of genomic DNA were measured with absorbance at 260 and 280 nm using a NanoDropTM 1000 spectrophotometer (Thermo Scientific, Wilmington, USA). The extracted DNA was bisulfite modified using an EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) according to the protocols recommended. When the sequencing results were confirmed, cytosine residues were converted by more than 99.0%.

Quantitative MassARRAY analysis of gene methylation status

An analysis was made of quantitative DNA methylation with MassARRAY EpiTYPER (Sequenom, San Diego, CA, USA), combining the base-specific enzymatic cleavage with MALDI-TOF mass spectrometry, which is considered to be a highly accurate and sensitive approach to the quantitative analysis of DNA methylation at CpG islands [27]. The detailed primers were designed using the online software Epidesigner (at <http://www.epidesigner.com/>, Supplementary files: Table S1). The quantitative methylation data for each CpG site or unites of multiple CpG islands were analyzed with the EpiTYPER software (Sequenom). Strict quality control was ensured before analysis. The samples were discarded missing more than 30% of data points as the unreliable and the CpG units failing to produce data from more than 30% of the samples as the unreliable [28].

Statistical analysis

All statistical analysis was performed on SPSS (Version 19.0; IBM, New York, USA) and GraphPad Prism (Version 5.0; GraphPad Software Inc., San Diego, CA, USA). The quantitative data were presented as mean $\pm$ standard deviations, and the methylation levels and DNMT-1 expression levels were classified as quartiles based on the distributions. All the quantitative data in the two independent groups were analyzed through Mann-Whitney *U* test. The analysis of Spearman Rank correlation was performed to evaluate the correlations between the levels of TFPI-2 protein expression and mean arterial pressure (MAP). All statistical analysis was two-sided and $P < 0.05$ was considered to be statistically significant.

Results

Patient characteristics evaluation

As indicated by the demographic and clinical characteristics of the study groups (Table 1), the maternal age and antepartum body mass index (BMI) were comparable between of the PE patients and the NP women, while the former had lower mean gestational age at delivery and mean birthweight than the latter. As expected, the levels of systolic BP (mmHg), diastolic BP (mmHg) and proteinuria (g/24h) were higher in the PE than in the NP group, respectively.

TFPI-2 expression and localization in placentas

As evidenced by the immunohistochemistry (IHC) assay to detect the expression and localization of TFPI-2 in the placenta samples with PE, the brown signal was observed mainly in the cytoplasm of the syncytiotrophoblast layers, but not in the villi interstitium (Fig.1A). The semi-quantitation of immunostaining showed the mean density was significantly higher in the PE than in the NP group (0.0978 vs. 0.0537; $P=0.0002$; Fig.1B). According to the analysis on the correlation between MAP before cesarean section and the mean density of TFPI-2 expression in the 29 samples, a significant association was observed between the TFPI-2 expression and MAP ($r=0.677$; $P<0.0001$; Fig.1C).

The evaluation of the differential expression of TFPI-2 mRNA through the performance of qRT-PCR in the preeclamptic and normal placentas, upon GAPDH standardization, indicated that significantly higher levels of TFPI-2 expression were observed in the PE placentas than in the controls. Approximately, the level of TFPI-2

mRNA was 4-fold higher in the former than in the latter (3.52 [0.80-10.44] vs. 0.86 [0.28-2.07]; median; $P<0.01$; Fig.1D).

The results from the western blot performed on the PE and NP placentas to further confirm the expression of TFPI-2 protein were found to be consistent with those obtained using IHC and qRT-PCR. The level of TFPI-2 protein, measured by densitometric analysis, was nearly 1.5-fold higher in the PE than in NP placentas (0.92 [0.50-1.55] vs. 0.6 [0.5-1.29]; median; $P<0.05$; Fig.1F).

Methylation profiles of TFPI-2 in preeclamptic placentas

As indicated by the analysis of the methylation level of TFPI-2 promoter region sequences on the Sequenom MassARRAY platform, where one region with the highest percentage of CpG sites in the promoter of TFPI-2 (-272 to 96, nucleotide positions relative to the translation start sites) was analyzed, it was found that the amplicon contained 31 CpG sites which could be divided into 15 CpG unites, and hypomethylation of the TFPI-2 promoter was detected both in the PE and NP placentas (Fig.2A). The methylation level was significantly lower in the PE placentas, with a median value of 5.5% (interquartile range: IQR 4.44%-7.03%), than in the controls, with a median value of 8.75% (IQR 7.05%-10.18%; $P=0.005$; Fig.2B).

In the evaluation of the methylation status of every CpG unit to investigate the methylation level at different CpG sites, an analysis was made of 12 available CpG unites containing 22 CpG sites, and the results showed that the methylation level was different at each CpG unit, ranging from 0 to 47% (Fig.2C), and that the methylation status at CpG_6 (-168 to -167), CpG_15 (-98 to -97) and CpG_18.19 (-68 to -65) was

significantly decreased in the PE patients than in the normal women ($P < 0.05$; Fig.2D-F). The difference at the other CpG units was not significant between the groups ($P > 0.05$).

Expression of DNMTs in preeclamptic placentas

In the evaluation of the expression of DNA methyltransferase enzymes (DNMTs) to investigate the potential mechanism regulating the methylation level in the placenta, IHC staining showed that DNMT-1 was mainly expressed in the nuclei of syncytiotrophoblasts. However, the semi-quantitation of immunostaining for DNMT-1 showed no significant difference between the groups, with a median value of 46.67% in the PE group relative to 28.33% in normal one ($P > 0.05$; Fig.3A-B). As DNMT3a and DNMT3b were faintly or negatively expressed in the nuclei of syncytiotrophoblasts either in the PE or in the normal placentas, their roles on TFPI-2 expression were not evaluated in the current study (Fig.3C-D).

Discussion

In the current study, we showed that the expressions of TFPI-2 mRNA and protein were significantly elevated in the PE placentas when compared with those in the NP ones, and that hypomethylation presented in the promoter of TFPI-2, with a significant decrease in PE placentas, suggesting that TFPI-2 regulated by epigenetic mechanism might contribute to PE pathogenesis.

TFPI-2 regulates negatively the enzymatic activity of plasmin, trypsin and VIIa-tissue factor complex and consequently plays an important physiological role in blocking endothelial dysfunction initiated coagulation in normal pregnancy [6, 9]. TFPI-2 may also inhibit trophoblast cell proliferation and tumor-related angiogenesis [29-31]. Over-expression of TFPI-2 could inhibit intervillous remodeling, normal decidualization and appropriate placentation when it played a role in the development of PE [32]. These observations provided a new evidence for the potential pathogenesis of PE.

The current study showed an elevation of TFPI-2 mRNA and protein expression in the PE relative to the NP placentas, which confirmed our previously reported finding [9]. Moreover, it revealed a significant association between TFPI-2 protein expression and MAP, which is an important prognosis predictor in PE [33]. These findings were consistent with those reported by Erez *et al* and Dusse *et al* [34, 35] but inconsistent with those revealed by Ogawa *et al* and Chinni *et al* [36, 37]. Several possible factors might account for these differences: TFPI-2 elevation could be related to the severity of PE; coordinated with the higher blood pressure, TFPI-2 could be

strongly induced from the vascular smooth muscle cells exposed to the higher fluid shear stress in the placenta [38]; and the expression of TFPI-2 could be heterogeneous and unstable in different individuals due to varied coagulation status, diet and local environment.

There is an increasing body of evidence showing that epigenetic features are involved in the pathogenesis of PE; however, the role of DNA methylation of TFPI-2 in placenta dysfunction has not been explored. The current study showed that hypomethylation of the TFPI-2 promoter region was detected both in the PE and in the NP group, and that the general level of methylation decreased more significantly in the former than in the latter, which is consistent with many other studies on other diseases [14, 39-41].

However, not all hypomethylation status was inversely correlated with transcription of the gene. It was demonstrated that methylation of CpG sites downstream of transcription initiation site couldn't inhibit elongation in mammalian cells [42]. TFPI-2 promoter was reported to have 3 transcription initiation sites, and in the current study the hypomethylated CpG sites were found to be all at the upstream or nearby the major transcription initiation sites at position -75 upstream from the translation start site [39]. Hube *et al* revealed that unmethylated CpG sites between -112 and -63, where CpG_15 (-98~- to -97) and CpG_18.19 (-68 to -65) were located in the current study, allowed TFPI-2 transcription in human choriocarcinoma cells [39]. Dong *et al* demonstrated that CpG_15 and CpG_18.19 of TFPI-2 were more significantly hypermethylated in invasive cervical carcinomas than in cervical

intraepithelial neoplasm tissues and healthy controls, which was consistent with the finding in the current study [19]. As indicated by our literature review, this is the first study showing the aberrant methylation status at CpG₆ (-168 to -167) of the TFPI-2 promoter. In view of this, we hypothesized the methylation status at the three hypomethylated CpG sites could regulate the expression of TFPI-2.

Normally, DNA methylation is orchestrated by DNMTs. DNMT-1 was reported to be the predominant DNA methylating enzyme and maintain the DNA methylation patterns during DNA replication [43]. Unexpectedly, DNMT-1 was highly expressed both in the PE and the NP group and no significant difference was observed between the two groups, which was consistent with the finding previously reported [44]. This might be explained by the evidence that the methylation status of TFPI-2 in the placenta could be regulated by some other factors. Actually, it was revealed that genomic hypomethylation didn't require DNMT-1 down-regulation at all [45]. Recent studies have shown that histone methylation plays an important role in the establishment of DNA methylation. Ribarska *et al.* showed that enrichment of histone modification presented at TFPI-2 promoter [14]. More effort is merited to get a deeper insight into the mechanism in the regulation of TFPI-2 expression and promoter methylation in placenta.

As one of the limitations in the current study, the gestation age was younger in the PE than in the control group. In our previous study, we had revealed that TFPI-2 increased steadily with gestational age and reached a peak at the 39th gestational week with similar changes in placenta [9]. The current study showed that the expression of

TFPI-2 protein in the PE group was nearly two times of that in the NP group at a smaller gestation, which indicated that the elevation of TFPI-2 could play an important role in PE pathogenesis. Additionally, the sample size was limited, which may affect the precision for large populations.

In conclusion, the current study confirmed the potential role of TFPI-2 in the pathogenesis of PE. Aberrant DNA methylation of TFPI-2 promoter, especially the methylation status at CpG₆ (-168 to -167), CpG₁₅ (-98 to -97) and CpG_{18.19} (-68 to -65), could regulate the expression of TFPI-2. However, the expression of DNMT-1 didn't show any significant difference between the two groups, which suggests other potential mechanisms involved in regulating DNA methylation of TFPI-2 promoter. Such findings should provide a heuristic basis for further studies on the role of histone methylation in placenta tissues and cell lines.

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Conflict of interest

None declared.

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Table 1. Clinical characteristics of the study population

Characteristics	PE (n=19)	NP (n=10)	<i>P</i> -value [*]
Maternal age (years)	31.9±2.6	29.6±4.60	0.08
BMI (kg/m ²)	27.9±2.3	26.2±2.0	0.07
Gestational age (weeks)	35.71±1.95	38.98±0.78	0.003
Systolic BP (mmHg)	155.16±11.00	120.56±12.78	<0.001
Diastolic BP (mmHg)	96.74±10.54	76.78±8.24	<0.001
Proteinuria (g/24h)	3.23±2.39	0	<0.001
Infant birthweight (g)	2347.11±579.59	3446.67±275.04	<0.001
5' appgar score	9	9	—

All results presented as mean ± SD.

* Obtained using the Mann-Whitney *U*-test on SPSS software.

Figure legends

Figure 1 Expression and localization of TFPI-2 in PE Vs NP patients. A, Representative images of immunostaining of TFPI-2 in placenta lesions from 19 PE patients and 10 NP placentas. TFPI-2 was mainly expressed in the cytoplasm of the syncytiotrophoblast layers and stronger staining could be detected in PE group. Original magnification is 400 fold . Scale bars (100 μ m) are shown in each image. B, Quantification of TFPI-2 expression were analyzed using the software of Image-Pro® Plus. The expression of TFPI-2 was significantly increased in PE placentas than that in NP group. C, Spearman Rank correlation analysis showed a significant association between MAP and TFPI-2 expression levels. Further confirmation of TFPI-2 mRNA and protein expression is shown. D, The relative mRNA expression of TFPI-2 gene was evaluated by qRT-PCR with GAPDH as endogenous control. E and F, TFPI-2 were determined using western blot with GAPDH as a loading control. The representative images of western blot of TFPI-2 are shown. The relative quantity of the TFPI-2 expression were both consistent with IHC, suggesting over-expression of TFPI-2 in PE patients than that in NP group. All experiments were repeated three times at least. The mean values are shown and the data is presented as the mean $\pm$ SD. *** P <0.0001, ** P <0.01, * P <0.05.

Fig 2 MassARRAY results for TFPI-2 methylation analysis in 10 PE placentas and 10 NP placentas. A, The blue column represents the distribution of the CpG sites examined in the TFPI-2 gene in the schematic diagram. The studied sequence represents a 368 base pair fragment (-272 to 96 bp, relative to the TSS) of TFPI-2 gene. The different methylation levels at each site are shown. Red signal presented in both PE and NP placentas, suggesting hypomethylation of TFPI-2

promoter in the placenta. B, The mean methylation level was analyzed between groups. The methylation level of TFPI-2 was significantly decreased in PE placentas. C, Mean methylation levels of 12 CpG units in TFPI-2 gene between the PE patients and the NP placentas are shown. D-F, The methylation status at CpG_6, CpG_15 and CpG_18.19 were significantly decreased in PE patients than that in NP group (The methylation status at CpG_6 in one case and CpG_15 in another case was not analyzed for unreliable data). TSS, translation start site. $**P<0.01$, $*P<0.05$.

Fig 3 Expression of DNMTs in PE and NP patients using IHC. A, Representative images of immunostaining of DNMT-1 staining in placentas lesions from 19 PE patients and 10 NP placentas. DNMT-1 was mainly expressed in the nuclei of the syncytiotrophoblast layers. Original magnification is 400 fold. Scale bars (100 μ m) are shown in each image. B, Quantification of DNMT-1 expression was analyzed using the percentage of staining as described aforementioned. The expression of DNMT-1 didn't show significant difference between groups ($P>0.05$). C, DNMT3a was faintly expressed in either PE placentas or normal placentas. D, DNMT3b was faintly or negatively expressed in either PE placentas or normal placentas.

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