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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.

1. 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

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

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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.

2. Material and methods

2.1. 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 0.3 g 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.

2.2. Sample collection and preparation

After placentas birth, three blocks ($1 \times 1 \times 1$ cm) 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.

2.3. Immunohistochemistry

Formalin-fixed and paraffin-embedded, the samples of the placenta tissues were studied. The 4-μm-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 syncytiocytotrophoblast cells [25,26].

2.4. 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.

2.5. Western blot

From the placenta tissues the total protein was extracted using radioimmuneprecipitation assay (RIPA) buffer containing 1 mmol/L protease inhibitor cocktail. Upon 5-min heating at 95 °C, 20 μg of denatured protein for each reaction was used to load a 12% polyacrylamide SDS for electrophoresis. Polyvinylidenedifluoride (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 1 h 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.

2.6. 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 NanoDrop™ 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 >99.0%.

2.7. 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 >30% of data points as the unreliable