Hypermethylation of the HLA-G promoter is associated with preeclampsia

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Abstract: Preeclampsia (PE) is a severe pregnancy-induced disorder characterized by hypertension and proteinuria and a leading cause of perinatal maternal–fetal mortality and morbidity in developing countries. Dysregulated human leukocyte antigen (HLA)-G was found in placentas as well as in maternal sera from PE patients; however, the reason for this difference is unknown. As accumulating evidence has confirmed that DNA methylation is an important mechanism for regulating gene expression, we sought to test the hypothesis that alteration in the DNA methylation of the HLA-G promoter region is responsible for decreased expression of HLA-G in PE. Bisulfite pyrosequencing showed that a series of CpG sites in the HLA-G promoter region were significantly more highly methylated in PE than in normal pregnancy (NP). Interestingly, the hypermethylated CpG sites were mostly reported to be binding sites of active transcription factors. To further investigate the regulation of HLA-G methylation, we also defined the expression patterns of DNA methyltransferases (DNMTs) in placental tissue using immunohistochemistry and quantitative polymerase chain reaction analyses. Here, we demonstrate that DNMT-1 is overexpressed and HLA-G expression is reduced in PE women when compared with NP. Furthermore, both treatment with the DNMT inhibitor 5-aza-2′-deoxycytidine and specific knockdown of DNMT-1 using siRNAs can significantly increase the expression level of HLA-G in a trophoblastic cell line, indicating the potential mechanism of DNMT-1-mediated DNA methylation in HLA-G regulation. Taken together, our research confirms that DNMT-1-mediated promoter hypermethylation of HLA-G is associated with PE. These findings provide new insights into the diagnosis and treatment of PE.

Key words: HLA-G / preeclampsia / DNA methylation / DNMT-1

Introduction

Preeclampsia (PE) is a pregnancy-induced disorder characterized by hypertension, proteinuria and, sometimes, mild-to-severe edema. This multisystemic disorder can lead to severe clinical complications, such as HELLP (hemolysis, elevated liver enzymes and low platelets) when a hemolytic process is observed, and, more rarely, to eclampsia, when generalized seizures appear (Steegers et al., 2010). PE is a leading cause of perinatal maternal–fetal mortality and morbidity, especially in developing countries (Duley, 2009).

While the etiology and pathogenesis of PE remain largely unclear, it is generally accepted that the syndrome consists of two successive processes that include poor placenta in early pregnancy followed by placental oxidative stress (Steegers et al., 2010). Genetic and epigenetic factors, as well as immunological and nutritional factors, are believed to contribute to the mechanism of PE (Serrano, 2006; Chelbi and Vaiman, 2008). In a successful pregnancy, human leukocyte antigen (HLA)-G is a fundamental molecule that induces maternal immune tolerance and protects the fetal-derived placenta and fetal antigens from immune rejection by uterine natural killer cells and antigen-processing cells (Chumbley et al., 1994). Dysregulated HLA-G has been found in most PE placentas, and HLA-G expression is reduced or absent in PE placentas compared with normotensive placentas (Hara et al., 1996; Goldman-Wohl et al., 2000; Zhu et al., 2012). Consistent with placental HLA-G expression, the serum HLA-G concentration is also decreased during the third trimester of PE pregnancies, as well as during the early gestational weeks in females who eventually develop PE, which strongly argues that soluble HLA-G may be an early predictor of PE (Yie et al., 2004, 2005). A low level of HLA-G expression in both the placenta and maternal serum might account for the disorder by mediating immune maladaptation at the maternal–fetal interface (Yie et al., 2004). In addition, our previous work demonstrated that alterations in HLA-G might directly interfere with the invasiveness function of trophoblast cell lines through different cell signaling pathways, confirming its significant role in the mechanism of PE (Li et al., 2011; Liu et al., 2013a, b).
However, the regulation of HLA-G in PE remains unknown. Unlike other HLA class I molecules, the sequences involved in the transcriptional regulation of HLA class I genes are disrupted in the HLA-G gene (Moreau et al., 2009). The demethylation reagent 5-aza-2′-deoxycytidine (5-Aza-dC) has been reported to reverse HLA-G gene repression in a number of tumor cell lines (Moreau et al., 2003). Spontaneous demethylation in the HLA-G promoter region has also been found in ovarian tumor cells compared with normal epithelial cells, and this demethylation is accompanied by an increased level of HLA-G protein in tumor cells (Menendez et al., 2008). Because promoter methylation is assumed to be a crucial mechanism that regulates gene expression (Deaton and Bird, 2011), the methylation status of the HLA-G promoter might account for its expression level, at least in part. However, whether the methylation status of the HLA-G promoter region is altered in PE patients, resulting in changes in its expression, has not been investigated.

Our study aims to investigate whether elevated methylation of the HLA-G promoter is related to PE. In addition, we hypothesized that the key enzymes that regulate DNA methylation [DNA methyltransferases (DNMTs)] contribute to the altered methylation pattern of the HLA-G promoter region in trophoblastic cells. So far, we are the first group to use PE placentas to study HLA-G. As the traditional methods used by previous studies, such as methylation-specific polymerase chain reaction (MSP) and bisulfite-sequencing PCR (BSP), cannot accurately reflect low levels of CpG methylation and most likely have a high level of false-negative results, we chose to perform bisulfite pyrosequencing, which is considered a gold standard for methylation quantification (Dupont et al., 2004), to determine the promoter methylation status of HLA-G in PE. Our findings increase our understanding of the role of epigenetics in HLA-G regulation in PE.

Materials and Methods

Patients and samples collection

Placentas and sera samples from 20 normal and 19 PE pregnancies were collected for this case–control study. The inclusion of PE patients followed the criteria of the American College of Obstetricians and Gynecologists (hypertension (blood pressure ≥140/90 mmHg after 20 weeks’ gestation) and proteinuria (≥300 mg/24 h or ≥1+ dipstick)). The normal pregnancies (NPs) consisted of healthy women undergoing selective cesarean. The clinical characteristics of the recruited pregnancies are shown in Table I. Several tissue samples (0.5 cm × 0.5 cm) were obtained from the maternal side of the placenta soon after each selective cesarean section. After removal of the maternal blood cells by washing the tissue in sterile phosphate-buffered saline, a block of tissue was fixed in 4% formalin for immunohistochemistry (IHC), and the remaining tissue was aliquoted and snap frozen in liquid nitrogen and transferred to storage at −80°C for later use. Five milliliters of blood was taken from PE and NP by venipuncture in sterile conditions and collected in sterile tubes. Blood samples were centrifuged for 15 min at 600g at 4°C. Serum was separated within 15 min after collection of blood and frozen at −80°C until assayed. Duration of the storage before measurement was up to 6 months.

Ethical approval

This study was approved by the local ethics committees of Obstetrics and Gynecology Hospital of Fudan University (Shanghai, China), and written consent was obtained from all of the patients before the collection of the placenta and blood.

Genomic DNA extraction and quantification of DNA methylation

Genomic DNA from placental tissue and cell lines was extracted using the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China) according to the manufacturer’s instructions. The DNA concentration was evaluated using an Eon Microplate Spectrophotometer. Purified DNA with an optical density (OD) value between 1.8 and 2.0 was assumed to be of good quality. One thousand nanograms of DNA was modified using the EZ DNA Methylation-Gold Kit (ZYMO, USA). The modified DNA was stored at −80°C.

To assess the HLA-G methylation status of the tissue and quantify the percentage of methylation of each individual CpG, bisulfite-modified DNA was amplified using bisulfite PCR primers with a biotin label on the 5′ end of the reverse primer, according to Menendez et al. (2008). The pyrosequencing primers were designed by Pyromark Assay Design software (QIAGEN, Germany) according to the HLA-G reference sequence GenBank J03027.1. All primers are shown in Table II. The PCR reactions were performed using HotStart PCR enzymes (TAKARA, Japan) following the manufacturer’s instructions. The optimized PCR conditions were 35 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 1 min and a final step of 72°C for 5 min. The PCR products were then processed and sequenced to measure the percentage of DNA methylation using the Pyromark Q96 MD Pyrosequencer system (QIAGEN).

Table I Characteristics of the study population.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>NP (N = 20)</th>
<th>PE (N = 19)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>30.70 ± 5.10</td>
<td>30.53 ± 4.67</td>
<td>0.8104</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>38.21 ± 0.85</td>
<td>37.77 ± 0.93</td>
<td>0.1178</td>
</tr>
<tr>
<td>Pregnancy body mass index (kg/m²)</td>
<td>28.36 ± 3.38</td>
<td>29.88 ± 4.23</td>
<td>0.3394</td>
</tr>
<tr>
<td>BP systolic (mmHg)</td>
<td>115.05 ± 10.02</td>
<td>153.79 ± 20.39</td>
<td>0.0001</td>
</tr>
<tr>
<td>BP diastolic (mmHg)</td>
<td>73.40 ± 6.72</td>
<td>97.79 ± 10.26</td>
<td>0.0001</td>
</tr>
<tr>
<td>Proteinuria (g/24 h)</td>
<td>—</td>
<td>1.70 ± 1.80</td>
<td>—</td>
</tr>
<tr>
<td>Neonatal weight (g)</td>
<td>3286.75 ± 488.58</td>
<td>3023.68 ± 592.10</td>
<td>0.1031</td>
</tr>
<tr>
<td>Placenta weight (g)</td>
<td>510.00 ± 82.10</td>
<td>470.26 ± 74.81</td>
<td>0.0408</td>
</tr>
<tr>
<td>Gender ratio of newborns (male/female)</td>
<td>10/10</td>
<td>10/9</td>
<td>0.8695</td>
</tr>
</tbody>
</table>

All results are presented as mean ± SD.

*Obtained using the Mann–Whitney U test on Graph Pad version 5.0.
**Immunohistochemistry (IHC)**

For IHC staining, the placentas were harvested and fixed in 4% paraformaldehyde, embedded in paraffin, and processed into 4-μm-thick sections. After incubation at 60°C for 2 h, the slides were deparaffinized in xylene, rehydrated in a graded alcohol series, and washed in running water. Endogenous peroxidase activity was blocked with 3% H2O2 in methanol for 15 min. For antigen retrieval, the slides were incubated in 0.01 M citric acid buffer at 100°C for 20 min. The sections were incubated with rabbit monoclonal antibodies directed against DNMT-1 (Epitomics, USA; dilution 1:100) and HLA-G (Abcam, USA; dilution 1:200) at 4°C overnight in a humidified chamber. A horseradish peroxidase (HRP)-labeled (Gene Company, Hong Kong) secondary antibody was incubated with the slides for 30 min at room temperature followed by diaminobenzidine staining and hematoxylin counterstaining. Negative controls were created by omitting the primary antibody. All images were captured by an Olympus BX51 microscope. The original magnification was 200x for all the panels.

Estimation of HLA-G and DNMT-1 expression was performed according to the German ImmunoReactive Score (Soslow et al., 2000). The staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong) and staining quantity (0, no staining; 1–1 to 10%; 2–11 to 50%; 3–51 to 80%; and 4–81 to 100%) were evaluated successively by two researchers without any knowledge of the patients’ clinical data. The intensity and quantity of the staining were documented initially by Y.T. and verified by Hayan Liu. The overall score was expressed as the summation of the intensity and quantity scores and defined as the quantity-intensity product with a range of 0–12.

**Enzyme-linked immunosorbent assay (ELISA)**

The concentration of sHLA-G protein in sera samples from PE and NP patients was determined by a specific double monoclonal sandwich enzyme immunoassay ELISA technique (BioVendor Laboratory Medicine, Heidelberg, Germany) according to the manufacturer’s instructions. Briefly, standard and analyzed samples were incubated in microplate wells pre-coated with monoclonal anti-sHLA-G antibody. After 20 h incubation and washing, monoclonal anti-human β2-microglobulin antibody labeled with HRP was added to the wells and incubated for 60 min with captured sHLA-G. Following another washing step, the remaining HRP conjugate was allowed to react with the substrate solution. The reaction was stopped by addition of acidic solution, and absorbance of the resulting products was measured at wavelengths 450 and 630 nm. Standard curve was used to establish the concentrations of sHLA-G protein in analyzed samples.

**RNA isolation and quantitative real-time PCR**

Total RNA was extracted from cells and placenta samples using the TRIZOL reagent (Invitrogen, USA) according to the manufacturer’s instructions. Two micrograms of total RNA was then reverse transcribed, and first-strand complementary DNA was synthesized using the RevertAid™ First Strand cDNA Synthesis Kit (THERMO, USA). Quantitative real-time PCR (qRT-PCR) was conducted to detect the relative mRNA levels of genes using SYBR® Premix Ex Taq™ II (TAKARA) in a 7900HT Fast Real-time PCR System. All qRT-PCR reactions were performed in triplicate in a final volume of 15 μl according to the manufacturer’s protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers for qRT-PCR are shown in Table II. The relative expression of target genes in the samples was expressed as the averaged, normalized Ct value of each sample compared with the GAPDH Ct value of the corresponding sample based on the 2−ΔΔCt method.

**Cell culture and treatment**

The trophoblast-like cell line HTR-8/SVneo was a gift from Professor Graham at the University of Toronto, Canada. The cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO2 humidified incubator. The culture medium was replaced every 24 h.

Lipofectamine 2000 transfection reagent (Life Technologies, Invitrogen, USA) was used to transfect the HTR-8/SVneo cell line with sRNAs that targeted DNMT-1 mRNA according to the manufacturer’s protocol. The medium was replaced with fresh growth medium 6 h after transfection. The efficiency of cell transfection was evaluated by performing real-time PCR analysis. The cells were collected for the appropriate experiments 48–72 h after transfection.

Demethylating treatment was carried out for 72 h with 5-aza-dC at different concentrations of 2, 4 and 8 μM. The trophoblastic-like cell line HTR-8/SVneo was cultured for 12 h before the treatment at a confluence of 70%. The cells were collected for the following experiments.

**Statistical analysis**

Statistical calculations were performed using GraphPad Prism 5.1 (GraphPad Software, Inc., USA). Quantitative data are presented as the mean ± SEM. The statistical significance of the variances was determined by the non-parametric Mann–Whitney U test, chi-square test or Student’s t-test for comparisons. A P-value of <0.05 was considered statistically significant.

**Results**

Promoter methylation patterns of the HLA-G promoter in placentas from PE patients

To obtain the methylation pattern of the HLA-G promoter region, we performed bisulfite pyrosequencing in placentas from PE and NPs and used three sequencing primers to detect the average % methylation...
for CpG sites located from \(-8\) to \(-394\) bp 5’ to the HLA-G transcriptional start site, which were reported to be regulated by DNA methylation in trophoblastic cell lines (Fig. 1A). The HLA-G promoter displays a different methylation profile in PE placentas compared with NP placentas. The average methylation levels across the promoter region are shown in order according to the location (Fig. 1B–D). Among all of the CpG sites examined, the \(-188\), \(-68\) and \(-65\) sites of the HLA-G promoter region have significantly higher methylation levels in PE than in NP tissues (\(P = 0.0165, 0.0222\) and 0.0037, respectively, Fig. 1C and D). The methylation percentage of the \(-188\) CpG site was \(15.6 \pm 1.2\%\) in PE placentas and \(11.8 \pm 0.9\%\) in NP placentas (Fig. 1C). The maximum difference was found at the \(-65\) CpG site, with a methylation percentage of \(8.6 \pm 1.3\%\) in PE placentas versus \(3.8 \pm 0.9\%\) in NP placentas, while the minimum difference was found at the \(-68\) site, with a methylation percentage of \(9.5 \pm 1.1\%\) in PE placentas versus \(6.3 \pm 0.8\%\) in NP placentas (Fig. 1D). As the region from \(-290\) to \(-211\) is reported to be a hypoxia-response element (HRE) that functions by binding the transcription activation factor HIF1-alpha (Menendez et al., 2008), we assayed the overall methylation level of this region except for \(-211\) CpG sites, which was overlapped by sequencing primer. Interestingly, although we did not find any significant difference in the percentage of methylation in any of the four CpG sites in the HRE (Fig. 1B), we found overall hypermethylation in the HRE in PE versus NP placentas (Fig. 1E, \(46.0 \pm 0.6\%\) in NP versus \(48.5 \pm 1.0\%\) in PE; \(P = 0.0399\)).

**HLA-G is down-regulated in PE and is accompanied by altered expression of DNMT-1**

Because a high methylation level is believed to be closely associated with gene silencing and DNMTs are considered key enzymes that regulate

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**Figure 1** Bisulfite pyrosequencing to reveal the methylation patterns in the promoter region of HLA-G in placentas from PE and NP patients. (A) Schematic diagram of the promoter of the HLA-G gene. Each CpG site is indicated by vertical lines. The locations of the primers used for bisulfite PCR and pyrosequencing are shown by black and red arrows, respectively. The transcription start sites are indicated by boldface arrows. (B–D) The percentages of methylation at each CpG site in the promoter region of HLA-G in placentas from PE (\(n = 19\)) and NP (\(n = 20\)) patients using three sequencing primers. E shows the overall methylation level of the HRE element (from \(-290\) to \(-211\)). Several CpG sites in the promoter region and HRE element are relatively hypermethylated in PE compared with NP placentas. Data are shown as the mean \(\pm\) SEM (\(^*P < 0.05\); \(^**P < 0.001\)). PE refers to preeclampsia. NP refers to normal pregnancy.
DNA methylation, we attempted to examine the expression levels of DNMTs and HLA-G. Immunostaining experiments on slides of placental tissue indicated that HLA-G was specifically expressed on the membranes of extravillous cytotrophoblasts (EVTs) (Fig. 2A), while DNMT-1 was highly expressed in the nuclei of both EVTs (Fig. 2A) and syncytiotrophoblasts (data not shown). The semi-quantitation of immunostaining for DNMT-1 and HLA-G was examined according to the German ImmunoReactive Score (Soslow et al., 2000). The immunostaining of DNMT-1 was significantly overexpressed in PE placentas than that in NP placentas (6.2 ± 0.9 in PE versus 2.6 ± 0.4 in NP, P = 0.0069, shown in Fig. 2B), while the immunostaining of HLA-G showed us an opposite trends between the two population (3.8 ± 0.4 in PE versus 9.5 ± 0.5 in NP, P = 0.0001, shown in Fig. 2C). However, we failed to detect and locate the DNMT3a/3b proteins in slides of placental tissue from both PE and NP in our IHC experiments (data not shown). We supposed that the DNMT3a/3b expression levels may be too low to detect, or they may be completely silenced in human placentas. As it was reported that HLA-G could be secreted or shed from trophoblastic cells of the placenta into maternal blood and soluble HLA-G was believed to be an early predictor of PE, we also determined the serum concentrations of sHLA-G in our study by performing ELISA. The sera concentration level of sHLA-G in PE was found to be significantly depressed when compared with that in NP (10.9 ± 2.3 in PE versus 39.0 ± 17.9 in NP, P = 0.0348, shown in Fig. 2D). Furthermore, we also performed a qRT-PCR analysis to determine the mRNA expression levels of DNMT-1 and HLA-G in our study population of placentas. DNMT-1 was expressed at approximately 3- to 4-fold higher levels in placentas from PE placentas compared with NP placentas (3.5 ± 0.7 in PE

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**Figure 2**. DNMT-1 and HLA-G localization and expression in PE versus NP patients. (A) IHC and localization of HLA-G and DNMT-1 in placentas. Tissue sections were deparaffinized, rehydrated and stained with DNMT-1 and HLA-G antibodies. Biopsies from all PE and NPs were analyzed. HLA-G and DNMT-1 are co-expressed in extravillous trophoblastic cells. HLA-G is a specific marker for extravillous trophoblastic cells. ▲ indicates extravillous trophoblastic cells. The original magnification in the representative results is 200 fold. Scale bars (10 μm) are shown on right side of each image. (B and C) The IHC quantifications of DNMT-1 and HLA-G in placenta tissues were estimated according to the German ImmunoReactive Score. DNMT-1 was significantly overexpressed in PE placentas, while HLA-G expression was decreased in PE compared with NP placentas. (D) The concentrations of sHLA-G (units/ml) in sera samples from PE and NP patients were measured by ELISA. sHLA-G were significantly decreased in PE patients. (E and F) The fold change of the transcription levels of DNMT-1 and HLA-G in placentas from PE versus NP patients was evaluated using qRT-PCR analysis. The results indicate that HLA-G expression is significantly decreased, whereas DNMT-1 is significantly increased in PE placentas compared with NP placentas. The data are the mean ± SEM that were acquired from 19 PE and 20 NPs (**p < 0.001).
versus $1.0 \pm 0.2$ in NP, $P = 0.0022$, shown in Fig. 2E), while HLA-G was decreased by nearly half in PE placentas compared with placentas from NP patients ($0.5 \pm 0.1$ in PE versus $1.0 \pm 0.1$ in NP, $P = 0.0033$, shown in Fig. 2F). Interestingly, the results of qRT-PCR analysis were consistent with the quantifications of the IHC staining. We did not perform a correlation analysis because the statistical relationship of the two molecules did not indicate a biological relationship, and the placental tissues might be composed of multiple cell types.

**HLA-G is regulated by DNA methylation and DNMT-1**

To further investigate the role of promoter DNA methylation in the deregulation of HLA-G in trophoblastic cells, we evaluated the effect of the methylation inhibitor 5-Aza-dC on HLA-G expression in HTR-8/SVneo cells, which are human immortal trophoblastic cells that express a low level of the HLA-G molecule. The data indicated that the HLA-G transcripts were robustly increased after treatment with 2–8 μM 5-Aza-dC for 72 h (Fig. 3A). Bisulfite pyrosequence analysis of HLA-G promoter region in HTR-8/SVneo cell line before and after being incubated with 2 μM 5-Aza-dC indicated that there was an overall promoter demethylation in most of its CpG sites including −188 site (Fig. 3C, $28.7 \pm 0.9$ before versus $10.9 \pm 0.9$ after demethylation treatment, $P = 0.0001$) and −68 site (Fig. 3D, $30.5 \pm 1.8$ before versus $1.4 \pm 1.4$ after demethylation treatment, $P = 0.0002$) as well as HRE region (Fig. 3E, $76.6 \pm 2.7$ before versus $44.7 \pm 0.7$ after demethylation treatment, $P = 0.0003$), which were hypermethylated in PE placentas. However, the 265 CpG site did not show a significant demethylation after 5-Aza-dC treatment as it was initially hypomethylated in HTR-8/SVneo cell line (Fig. 3D, $1.3 \pm 1.3$ before versus $0.9 \pm 0.9$ after demethylation treatment, $P = 0.8$). To better understand the methylation-dependent mechanism of the change in HLA-G, we further manipulated the expression of the primary DNMT, DNMT-1, by RNA interference. After the cells were incubated with two different siRNAs targeting DNMT-1 for 72 h, an increase in HLA-G expression was observed, although the increase was less than that observed following the 5-Aza-dC treatment (Fig. 3F). We hypothesize that this effect may be due to a different effect of the siRNA on DNMT activity. We did not evaluate the influence of DNMT3a/3b on HLA-G expression as these proteins were hardly detected in our placenta samples.

**Discussion**

Recent evidence has indicated that multiple epigenetic mechanisms contribute to PE (Chelbi and Vaiman, 2008). Meanwhile, a wide range of PE-specific genes with aberrant DNA methylation and gene expression patterns have been identified, including apoptosis-related genes, cytokine receptor genes and obesity-related genes (Reimer et al., 2002; Tsoi et al., 2003; Yuen et al., 2010). In the current study, we have shown that a series of CpG sites in the HLA-G promoter region are hypermethylated in placenta from PE pregnancies compared with...
DNMT-1 expression of HLA-G as well as the overexpression of DNMT-1. Furthermore, both DNA demethylation reagent and the specific inhibition of DNMT-1 in vitro can lead to increased HLA-G expression, confirming the epigenetic regulation of the HLA-G gene in PE.

In the present study, the percentage of HLA-G methylation was elevated in PE. Moreover, the expression level of HLA-G in both placenta tissues and maternal blood is decreased in PE patients when compared with those from NPs, which is consistent with previous studies by other research groups (Darmochwal-Kolarz et al., 2012). A hypermethylated promoter correlates with decreased gene expression because the methylated cytosine hinders the binding of transcription factors to the chromosome. Chelbi et al. (2007, 2012) have demonstrated that specific CpG demethylation in the SERPINA3 promoter region might help render the chromatin accessible to transcription factors, thus promoting its expression and participating in the onset of PE. Hence, we analyzed the particular CpG sites that exhibit different methylation patterns in our study population. Interestingly, the HRE (−211 to −290) in the HLA-G promoter had a significantly higher overall methylation level in PE than in NP. HRE is a binding site for the transcription factor HIF-1, which plays a critical role in the cellular response to hypoxia and is reported to induce HLA-G expression in the HLA-G-negative MB melanoma cell line (Moullot et al., 2007). Thus, we supposed that the hypermethylated HRE might become less sensitive to HIF-1, which is induced by placental physiology and/or pathology caused by hypoxia during pregnancy, and thus fail to induce HLA-G expression and eventually disrupt the fetal–maternal immune tolerance or influence the trophoblastic cell functions that are mediated by HLA-G. Similarly, other CpG sites displaying a significantly higher level of methylation are located close to intact binding sites of regulatory proteins known to facilitate gene expression (CCAAT box at −76 and enhancer kb1 at −188) (Menendez et al., 2008). The hypermethylation of these sites is likely to inhibit the activation of HLA-G expression through a cis-acting mechanism in PE.

DNMTs are key enzymes that regulate DNA methylation. DNMT-1 acts as a ‘maintenance’ methyltransferase and is the primary DNMT in mammals, while DNMT3a/3b act as ‘de novo’ methyltransferases (Bestor, 2000). We demonstrate an up-regulation of DNMT-1 in PE placentas in our research. High levels of DNMT-1 expression were usually believed to be responsible for global hypermethylation. Accordingly, a global hypermethylation was shown PE placentas in Gao’s and Kulkarni’s studies (Gao et al., 2011; Kulkarni et al., 2011). However, this up-regulation of DNMT-1 contrasts with recent findings from several microarray analysis in which no significant alteration of DNMT-1 was observed (Herse et al., 2007; Nishizawa et al., 2007). The discrepancy between our work and earlier work probably arises from the differences in methodologies used to detect DNMT-1 levels. The fact that some microarray designs might not distinguish all the isoforms of DNMTs separately as we did by performing real-time PCR would probably account for differences in the results. Moreover, the gestational week and the patient ethnicity may also have effects on it.

DNMT-1 is closely correlated with the methylated CpG island phenotype (Teodoridis et al., 2008). Robert had shown us that DNMT-1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells, although the mechanisms of which DNMT1 involves with locus-specific methylation are uncertain (Robert et al., 2003). Consistently, our research indicated that DNMT-1 participated in HLA-G gene silencing through DNA methylation in HTR-8/SVneo. It was reported that HLA-G gene transcription is inhibited by DNA methylation in a series of tumor cell lines and reversal of methylation-mediated repression by DNA demethylation reagent 5-Aza-dC could directly induce HLA-G expression (Moreau et al., 2003). We find a similar phenomenon in HTR-8/SVneo. Furthermore, although to a lesser extent than 5-Aza-dC treatment, a reversion in HLA-G expression was also observed following specific DNMT-1 knockdown, which supports the critical role of DNMT1 in the epigenetic regulation of HLA-G expression.

The aberrant DNMT-1 expression observed in PE in our study was consistent with Gao’s study (Gao et al., 2011). As a putative HRE was discovered in the 5’UTR of DNMT-1 and HIF-1α-mediated hypoxia regulation of DNMT enzymes eventually alters the DNA methylation pattern (Watson et al., 2014), we guess that the pathophysiology due to hypoxia in the placenta from PE patients may have induced abnormal DNMT-1 expression. Combined with other transcription factors, increased levels of DNMT-1 might cause the hypermethylation of HLA-G promoter region and suppress HLA-G transcription. However, further research should be done to testify this hypothesis in the future.

As we did not find clear expression of DNMT3a/3b in placentas at either the mRNA or protein levels, DNMT3a/3b knockdowns were not performed, and their impact on HLA-G expression was not evaluated in our in vitro experiment. However, these proteins may still contribute to DNA methylation. A possible reason for the low expression of these proteins is that de novo methylation mainly occurs during epigenetic reprogramming in the gametes and preimplantation embryo (Yin et al., 2012). After embryo implantation, methylation is maintained by DNMT-1, and no noticeable changes in de novo methylation occur in the post-differentiated human villous (Yin et al., 2012).

Consistent with previous studies, our results indicate that the epigenetic regulation by promoter methylation is central for HLA-G expression as well (Moreau et al., 2003; Menendez et al., 2008; Holling et al., 2009). Our results represent a slightly different but more accurate assessment of the methylation percentage of each CpG site than previous studies due to the advanced methylation detection system that we employed.

There are several limitations to our study. First, the purity and cell proportion of placental tissues may have an influence on the results. Repeating the experiment with primary cells might be a more preferable and less contrived approach to experiments in an immortalized trophoblast cell line that expresses a low level of HLA-G as primary trophoblast culture is reported to retain the PE phenotype for a few days (Zhou et al., 2013). Second, the study population was mostly in the late stages of pregnancy, while the onset of PE begins during early pregnancy; thus, we cannot clearly conclude whether the dysfunctional regulation of HLA-G is a cause or a consequence of PE. Thirdly, it is controversial to use GAPDH as a reference gene as some studies indicate that it was unstably expressed in the placenta across pregnancy. However, Kaituu-Lino proved that GAPDH did not vary in PE compared with normotensive pre-term and term placentas (Kaituu-Lino et al., 2014).

In summary, we have characterized the expression of DNMTs in PE placentas and have demonstrated the involvement of DNMT-1-mediated promoter hypermethylation in the regulation of HLA-G in PE. Further research is needed to illustrate the underlying mechanisms that regulate gene-specific methylation dysfunction in PE, which will help us identify specific epigenetic markers for PE, find new strategies to treat PE and thus improve the outcomes of affected women and their children.
Authors’ roles

Y.T. formed the study design, collected clinical samples, obtained results, performed data analysis and wrote this manuscript. Haiyan Liu and Han Li also participated in clinical samples collection and performed data analysis. W.G. edited the manuscript, and she is the corresponding author; he formed the study idea and study design and also participated in critical discussion. X.L. participated in the formation of study design, researched the data, reviewed and edited the manuscript. T.P. participated in the formation of study design and also in critical discussion.

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Conflict of interest

None declared.

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