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ORIGINAL ARTICLE

Detection of global DNA methylation and paternally imprinted *H19* gene methylation in preeclamptic placentas

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Preeclampsia (PE) is a severe hypertensive disorder associated with pregnancy; despite substantial research effort in the past several years, the etiology of PE is still unclear. The role of epigenetic factors in the etiology of PE, including DNA methylation, has been poorly characterized. In the present study, we investigated global DNA methylation as well as DNA methylation of the paternally imprinted *H19* gene in preeclamptic placentas. Using 5-methylcytosine immunohistochemistry and *Alu* and *LINE-1* repeat pyrosequencing, we found that the global DNA methylation level and the *DNA (cytosine-5) methyltransferase 1* mRNA level were significantly higher in the early-onset preeclamptic placentas when compared with the normal controls. Data from methylation-sensitive high resolution melting demonstrated hypermethylation of the promoter region of the *H19* gene, and results of real-time PCR showed decreased mRNA expression of *H19* gene in the early-onset preeclamptic placentas as compared with the normal controls. Our results suggest that abnormal DNA methylation during placentation might be involved in the pathophysiology of PE, especially early-onset preeclampsia.
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Keywords: DNA methylation; *H19*; placenta; preeclampsia

INTRODUCTION

Preeclampsia (PE) is a pregnancy-specific syndrome, generally defined as the development of hypertension and proteinuria after 20 weeks of gestation in a previously normotensive woman. The disease affects 5–7% of pregnancies worldwide, and is one of the major causes of the maternal and neonatal mortality and morbidity.[1,2] PE has been termed the 'disease of theories', reflecting the confusion that surrounds the etiology of PE.[3] The pathogenesis of PE is complex, likely resulting from the interaction of numerous genetic, immunologic and environmental factors.[4] Although the prime cause remains unclear, it is generally accepted that the syndrome may be initiated by abnormal placental development during the first trimester, resulting in placental insufficiency and the release of impaired placental factors into the maternal circulation.[5–7]

It has been well documented that a successful pregnancy involves a complex and precisely coordinated modulation of gene expression in gestational tissues mainly including placenta and uterine decidua. Recent evidence suggests that epigenetic regulation of gene expression is likely to have a key role in this process. Serman *et al.* indicated that variations in the extent of DNA methylation could induce morphological and biochemical changes of trophoblast cells and endometrial cells, thus negatively impinging upon the delicate feto-maternal interaction during the early pregnancy. In addition, results from cloned animals also revealed that disruptions in DNA methylation profiles in placentas may be involved in the severe symptoms of placentomegaly.[8,9]

Recent mounting evidence strongly suggests the contribution of epigenetic factors to the etiology of PE. Results from two independent laboratories demonstrated that significant hypomethylation occurs in the promoter regions of the *serpinb5* and *serpina3* genes in preeclamptic placenta as compared with normal controls placentas.[10,11] Novakovic *et al.* reported decreased DNA methylation levels of the *cyp24a1* gene in preeclamptic placenta tissues.[12] However, most of these evidences are limited to the analysis of individual genes, and variations of global DNA methylation is still poorly understood in PE.

On the other hand, parent-of-origin specific imprinting of many genes in placenta is likely to have key roles in many aspects of placental function, including trophoblast cell proliferation, differentiation, angiogenesis and transportation of nutrients.[13,14] The variations of some imprinted genes were also reported to be associated with PE.

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These mainly included the single nucleotide polymorphorism of the *STOX1* gene (van Dijk *et al.*)[15] and the gene mutation of the parentally imprinted *p57* gene (Kanayama *et al.*)[16] The paternally imprinted *H19* gene is transcribed as an untranslated RNA that may serve as a riboregulator. The gene is located in close proximity to the maternally imprinted *IGF-2* gene on chromosome 11p15.5.[17] Notably, the *H19* null mice display hyperplasia of all layers of the placenta,[18] and recent studies demonstrated the regulatory role of *H19* in the invasive property of placental trophoblasts.[19,20] The evidences strongly indicate that the *H19* gene is critical to the early development of placenta. However, it remains unclear as to the DNA methylation status in the promoter region of *H19* gene in preeclamptic placenta.

In the present study, we investigated the extent of global DNA methylation in preeclamptic placentas obtained from a cohort of Chinese patients, and further compared the mRNA expression as well as the methylation change in the promoter region of the paternally imprinted *H19* gene between preeclamptic and normal placentas.

METHODS

Subjects

Placentas from normal and severe preeclamptic pregnant women were obtained from the Department of Obstetrics and Gynecology, Peking University First Hospital, China, from November 2005 to March 2007. The study was approved by the Research Ethic Committees of the Institute of Zoology, Chinese Academy of Sciences and Peking University First Hospital. A total of 24 preeclamptic cases and 24 controls were recruited for a nested case-control study. According to the onset time of the clinical symptoms, the 24 pre-eclamptic cases were categorized into 10 early-onset (E-PE, $\leqslant$34 weeks) and 14 late-onset PE (L-PE, $>$34 weeks). Severe PE was diagnosed according to the criterion of the International Society for the Study of Hypertension in Pregnancy. In brief, these study patients had no history of preexisting or chronic hypertension, but showed systolic blood pressure of $>$160 mm Hg or diastolic blood pressure of $>$110 mm Hg on at least two occasions, accompanied by significant proteinuria ($>$2 g per 24 h or 3+ by dipstick in two random samples collected at $>$4 h interval) after 20 weeks of gestation. Those who developed renal disease, transient hypertension in pregnancy, gestational diabetes, spontaneous abortion, intrauterine fetal death, fetal chromosomal or congenital abnormalities or pregnancies conceived by fertility treatment were excluded from this study. The clinical characteristics of the patients included in this study were summarized in Table 1. Placental Specimens were quickly dissected, snap frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ until prepared for further analyses.

Immunohistochemistry for 5-methylcytosine (5-MC)

Immunohistochemistry was performed as previously described.[21] Briefly, 4 μm thick tissue frozen sections were mounted onto microscope glass slides and fixed with the pre-cooled acetone for 10 min at room temperature. After the fixation, the slides were immersed in 3N HCL for 30 min at room temperature to expose the CpG sits. The slides were then treated with 1.0% hydrogen peroxide for 15 min, incubated with 1% bovine serum albumin for 1 h at room temperature, and subsequently incubated with a mouse monoclonal antibody against 5-MC (0.5 μg ml^{-1}) at 4 °C overnight. The negative control was prepared by replacing primary antibodies with non-immune mouse IgG at the same concentration. The slides were further incubated with horseradish peroxidase streptavidin -conjugated anti-mouse IgG (Zhongshan Goldenbrige Biotechnology, Beijing, China) for 30 min. Final visualization was performed by incubating the sections with a DAB Detection Kit (Zhongshan Goldenbrige Biotechnology), and the staining time was tightly fixed at 5 min for every slide. The slides were evaluated under a light microscope (Olympus BX51, Tokyo, Japan), and the staining intensity of the antibody was analyzed by a semi-quantitative analysis HSCORE.[22] The HSCORE was calculated using the following equation: HSCORE=ΣPi (i+1), where i was the staining intensity (1=weak, 2=moderate and 3=strong), and Pi was the percentage of the positively stained cells for each intensity varying from 0 to 100%. The assessment of staining intensity and HSCORE were evaluated in a double-blind condition by three independent observers who did not know the disease status of the samples.

RNA isolation and real-time PCR

Total RNAs from cells and placenta tissue were isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. A total of 2 μm of total RNA was used for reverse transcription with oligo-(dT)15 primer using moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). Real-time quantitative PCR was performed using the SYBR green detection system (TaKaRa Biotechnology, Dalian, China) in the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Reaction for each sample was performed in duplicate in a 20 μl reaction volume containing 1 μl cDNA, 10 μl SYBR Premix Ex Taq, 0.4 μl ROX Reference Dye II (50×) and 0.2 μM of each primer. The PCR conditions and primers sequence are shown in Table 2. To control for uniform amount of

Table 2 Primers and PCR conditions for real-time PCR

Gene	*PCR primers (5′–3′)*	*PCR conditions*	*PCR production*
DNMT1	F: TACCTGGACGACCCTGACCTC R: CGTTGGCATCAAAGATGGACA	Real-time PCR: 95 °C for 5 s,	103 bp
DNMT3a	F: GACAAGAATGCCACCAAAGC R: CGTCTCCGAACCACATGAC	60 °C for 30 s. (40 cycles)	190 bp
DNMT3b	F: GACTCGAAGACGCACAGCTG R: CTCGGTCTTTGCCGTTGTTATAG		98 bp
H19	F: CCGGACACAAAACCCTCTAGCT R:TGTTCCGATGGTGTCTTTGATG		142 bp
GAPDH	F: CCACATCGCTCAGACACCAT R: GGCAACAATATCCACTTTACCAGAGT		114 bp
β-actin	F: CCTGGCACCCAGCACAAT R: GCCGATCCACACGGAGTACT		70 bp

Abbreviations: F, forward; R, reverse.

Table 1 Clinical characteristics of the women enrolled in this study

Characteristics	*Normal pregnancy* (n=*24*)	*Early-onset preeclampsia* (n=*10*)	*Late-onset preeclampsia* (n=*14*)
Maternal age (years)	30.5652 ± 4.0768	31.2000 ± 5.1381	30.3571 ± 3.6712
BMI (kg m^{-2})	24.5114 ± 2.4750	24.1371 ± 3.2172	23.5179 ± 3.0814
SBP (mm Hg)	110.2174 ± 8.3228	161.8000 ± 29.0624*	158.2143 ± 24.8540*
DBP (mm Hg)	71.3043 ± 5.6844	104.2000 ± 15.9081*	106.0714 ± 22.9698*
24 h proteinuria (g per 24 h)	ND	3.9350 ± 3.6700*	3.1658 ± 3.1795*
50 g GCT (mmol l^{-1})	7.4633 ± 1.8116	7.2333 ± 1.3614	8.1900 ± 2.2650
Gestational age at delivery (day)	268.1739 ± 12.8511	226.3000 ± 8.7439*	257.9286 ± 14.5996*

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; ND, not determined; SBP, systolic blood pressure; 50 g GCT, 50 g glucose challenge test.
Data are expressed as means ± s.d. and compared by the one-way analysis of variance or independent sample t-test.
*P<0.05 *vs.* normal pregnancy.

Table 3 Primers and PCR conditions for pyrosequencing analyses

Gene/Repeat	*PCR primers (5′–3′)*	*Pyrosequencing primers (5′–3′)*	*Sequence analyzed (5′–3′)*	*PCR conditions*	*PCR products*
Alu	F: biotin-TTTTTATTAAAAATATAAAAATT R: CCCAAACTAAAATACAATAA	AATAACTAAAATTA CAAAC	G/AC/TG/AC/TG/ ACCACCA	95 °C for 90 s, 43 °C for 60 s, 72 °C for 120 s. (40 cycles)	144 bp
LINE-1	F: TTTTTTGAGTTAGGTGTGGG R: biotin-TCTCACTAAAAAATACCAAACAA	GGGTGGGAGTGAT	C/TGATTTTTTAGGTGC/ TGTTC/TG	95 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s. (35 cycles)	249 bp

Abbreviations: F, forward; R, reverse.

input RNA template, all results were normalized by using a geometric mean of the expression level of two internal control genes, *GAPDH* and *β-actin*.[23] To compare the expression levels among different samples, the relative mRNA level was calculated using the $2^{-\Delta CT}$ method recommended by Schmittgen *et al*.[24] In our experiments, $2^{-\Delta CT}$ means $2^{-(CTDNMT-CTcontrol)}$ or $2^{-(CTH19-CTcontrol)}$. In addition, product purity was confirmed by dissociation curve analysis and agarose gel electrophoresis.

Genomic DNA extraction and bisulfite treatment

Genomic DNA was extracted from cells or frozen tissues using a genomic DNA extraction kit (ZR Genomic DNA II Kit; Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. Bisulfite modification of genomic DNA was carried out by using the EZ DNA Methylation-Gold Kit (Zymo Research) following the manufacturer's instructions.

Bisulfite PCR and pyrosequencing

DNA methylation level was quantified using the methods of bisulfite PCR and pyrosequencing.[25,26] In brief, the bisulfite-modified DNA was PCR amplified using the primers shown in Table 3. The PCR products were purified and quantified using the PSQ HS 96 Pyrosequencing System (Pyrosequencing, Westborough, MA, USA). A biotin-labeled primer was used to purify the final PCR product using sepharose beads. The PCR product was bound to Streptavidin Sepharose High Performance (GE Healthcare, Milwaukee, WI, USA), and the Sepharose beads containing the immobilized PCR product were purified, washed, denatured using a 0.2 mol l^{-1} NaOH solution, and washed again using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing) according to the manufacturer's recommendations. Subsequently, 0.3 mM pyrosequencing primer was annealed to the purified single-stranded PCR product and pyrosequencing was done using the PSQ HS96 Pyrosequencing System. Quantification of cytosine methylation was performed using the PSQ HS96A 1.2 software (Pyrosequencing). The ratio of C to T nucleotides was evaluated for *LINE-1* methylation, and ratio of G to A nucleotides was evaluated for *Alu* methylation. The unmethylated human control DNA (EpiTect Control DNA; Qiagen, Hilden, Germany) was used to verify bisulfite conversion.

Methylation-sensitive high resolution melting analysis (MS-HRM)

MS-HRM was performed as previously described.[27,28] Universal methylated and unmethylated genomic DNAs were purchased from Qiagen (EpiTect Control DNA; Qiagen), and mixed to create a serial of reference DNA standards containing methylation levels of 100, 75, 50, 25 and 0%.

PCR amplification and MS-HRM were performed on the Applied Biosystems 7500 real-time PCR system (Applied Biosystems). Primers for amplifying 5′ promoter region of the *H19* gene were described previously.[29] The target region comprised the region from 9394 to 9529 within the sequence AF125183, spanning the upstream of the *H19* transcription start site and the downstream of the CTCF7 zinc-finger binding site. PCR was performed using Fast EvaGreen Master Mix for quantitative PCR and HRM (Biotium, Hayward, CA, USA) in a 20 μl reaction volume containing 10 μl 2× Fast EvaGreen Master Mix, 0.25 mM primers and 5 ng bisulfite-modified DNA. The primer sequences to bisulfite-modified DNA were as follows: *H19*F, 5′-GGGAGAGTTTGTGAGGT-3′ and *H19*R, 5′-AAATCCCCACAACCGCTAAAC-3′. The 137-bp fragment was amplified from bisulfite-modified DNA. Cycling was at 95 °C for 5 min, 40 cycles of 95 °C for 5 s, 60 °C for 35 s. HRM was carried out at 95 °C for 15 s, 50 °C for 1 min and with a ramping from 60 to 95 °C rising by 0.1 °C every second as recommended by the manufacturer. HRM data were analyzed using the HRM software v2.0 (Applied Biosystems). The melting curves were normalized by the calculation of two normalization regions before and after the major fluorescence decrease representing the melting of the PCR product. This algorithm allows the direct comparison of the samples that have different starting fluorescence levels. Normalization regions were in the range 74–76 and 85–88 °C. A differential profile was then evaluated for each sample by comparing fluorescence at the melting point against the value of fluorescence of the negative control (unmethylated DNA). Values of relative fluorescent density for reference DNA standards were plotted against the corresponding methylation level to generate a typical standard curve, against which the methylation level of each unknown sample was evaluated.

Statistical analysis

All data are presented as mean values ± s. d. calculated from three independent experiments. Statistical comparison between groups was estimated using two-sided Student's *t*-test or one-way analysis of variance, and $P<0.05$ was considered statistically significant. All statistical analyses were performed using the SPSS software for windows 13.0 (SPSS, Chicago, IL, USA) or GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

Clinical characteristics of the subjects

Of the 48 pregnant women enrolled in this study, 10 suffered from early-onset PE, 14 suffered from late-onset PE and 24 were normal pregnant subjects. The clinical characteristics of these study participants are described in Table 1. The systolic and diastolic blood pressures as well as the 24 h urine protein content were significantly higher in the preeclamptic groups than in the control group. There were no significant differences in age, body mass index and glucose tolerance (indicated by 50 g glucose challenge test) between the preeclamptic and the normal pregnant women.

Global DNA methylation status in preeclamptic and normal control placentas

5-MC immunohistochemistry. Global DNA methylation was first visually examined in frozen sections of placenta using an antibody directed against 5-MC. Figure 1 showed a representative pattern of immunohystochemical staining for 5-MC in placenta villus from preeclamptic and normal control women. HSCORE was used to semi-quantitatively evaluate the staining intensity. The HSCORE value of normal control, early-onset and late-onset preeclamptic placenta villus were 2.653 ± 0.03697, 2.953 ± 0.09952 and 2.763 ± 0.05038, respectively. Statistical analysis revealed that the intensity was significantly higher in the early-onset preeclamptic placenta villus (P=0.0036) as compared with the normal controls. The late-onset preeclamptic specimens exhibited relatively higher, but not statistically significant, HSCORE values than the normal controls (P=0.0826).

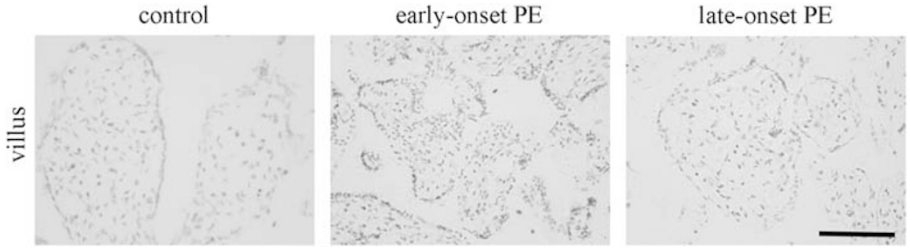

Figure 1 Immunohistochemical staining for 5-methylcytosine in preeclamptic and control placentas. Figures show a relatively higher staining intensity in the early-onset preeclamptic placenta villus than in the normal controls. Bar=100 μm.

Alu and LINE-1 pyrosequencing. To reliably compare the global methylation level in preeclamptic and normal placenta samples, we applied the pyrosequencing method to determine the methylation density in the *Alu* and *LINE-1* repeats, which are surrogate markers of global methylation. In normal control, early-onset and late-onset preeclamptic placentas, *Alu* methylation levels were 0.2553 ± 0.0091, 0.2952 ± 0.0065 and 0.2786 ± 0.0081, respectively, and *LINE-1* methylation levels were 0.3067 ± 0.0063, 0.3484 ± 0.0084 and 0.3217 ± 0.010, respectively. Statistical analysis showed that methylation of both *Alu* and *LINE-1* repeats were significantly higher in the early-onset preeclamptic placentas compared with the normal controls (P=0.0054 for *Alu*; P=0.0016 for *LINE-1*). *Alu* and *LINE-1* methylation in the late-onset preeclamptic placentas were also relatively higher than the normal controls, but the changes did not reach statistical significance (P=0.1006 for *Alu*; P=0.2136 for *LINE-1*) (Figure 2).

DNMTs mRNA expression. It is well established that DNA methylation at the CpG dinucleotides is catalyzed by various *DNMTs*, among which *DNA methyltransferase 1* (*DNMT1*), *3a* and *3b* are all highly expressed in human placenta.[30] We then examined expression of *DNMTs* mRNA in preeclamptic and control placentas using real-time PCR (Figures 3a–c). After normalization by the control genes, *GAPDH* and *β-actin*, the expression of *DNMT1* was significantly higher in the early onset (0.7523 ± 0.2135), but not in the late-onset preeclamptic placentas (0.3344 ± 0.1210) when compared with the normal controls (0.3020 ± 0.0711). Placental *DNMT3a* and *DNMT3b* expressions did not show any statistically significant difference among the three groups, though the level of *DNMT3a* in the early-onset preeclamptic placentas (0.0394 ± 0.0093) was relatively higher than in normal controls (0.0201 ± 0.0053).

Methylation status of the paternally imprinted H19 gene in preeclamptic and normal control placentas

The paternally imprinted *H19* gene has been indicated to be critical to the early development of placenta. We further examined *H19* mRNA expression and the methylation status in the promoter region of *H19* gene in preeclamptic placentas.

MS-HRM for H19. We applied a rapid and sensitive tool, MS-HRM, to selectively and reliably measure the degree of *H19* methylation in placentas. The melt curve and standard curve of the method was shown as Supplementary Figure 1. As shown in Figure 4a, methylation levels in the promoter region of the *H19* gene in normal control, early-onset and late-onset preeclamptic placentas were 47.87 ± 1.678, 57.19 ± 2.068 and 51.51 ± 2.182, respectively. Statistical analysis revealed a statistically significant difference of *H19* gene methylation in the early-onset preeclamptic placentas as compared with the normal controls (P=0.0023).

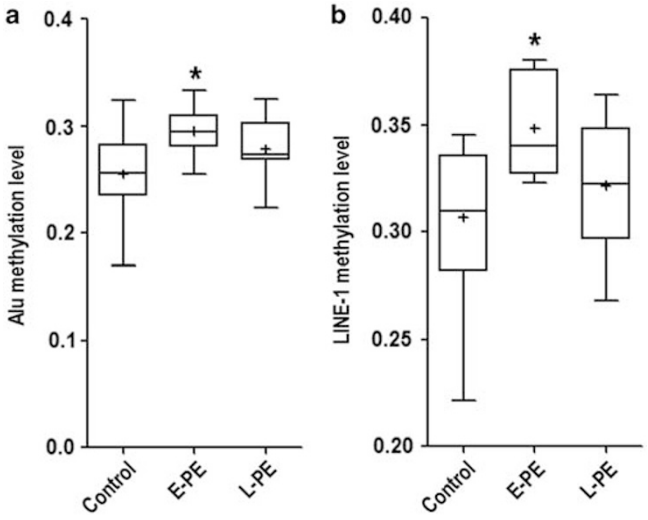

Figure 2 Quantitation of DNA methylation using *Alu* (**a**) and *LINE-1* (**b**) pyrosequencing. Statistical analysis showed that methylation of both *Alu* and *LINE-1* repeats was significantly higher in the early-onset preeclamptic placentas as compared with normal controls (P=0.0054 for *Alu*; P=0.0016 for *LINE-1*). *Alu* and *LINE-1* methylation in the late-onset preeclamptic placentas were relatively higher than the normal controls, but the changes were not statistically significant (P=0.1006 for *Alu*; P=0.2136 for *LINE-1*). Box plots were used to show the methylation level. The line within each box denotes the median. The crisscross denotes the mean. Limits of the box denote the 25th and 75th percentiles. Whiskers denote the 5th and 95th percentiles. *P<0.05 *vs.* Control.

H19 mRNA expression in the placentas. We further measured the expression of *H19* mRNA in preeclamptic and control placentas by using real-time PCR (Figure 4b). After normalization by the control genes, *GAPDH* and *β-actin*, the *H19* mRNA expression in control, early-onset and late-onset preeclamptic placentas were 14.22 ± 2.644, 2.720 ± 1.811 and 6.916 ± 4.266, respectively. As expected, *H19* mRNA level was appreciably lower in the early-onset preeclamptic placentas than in controls (P=0.0034). Though the level in the late-onset preeclamptic placentas was also lower than that in the normal controls, the change was not statistically significant (P=0.1408).

DISCUSSION

Epigenetic regulation in preeclamptic placenta has been scarcely investigated, and little evidence has been so far reported on the degree of global DNA methylation in this complex disease. In the present study, we measured the global DNA methylation level in preeclamptic placenta using immunohistochemistry for 5-MC and pyrosequencing of *Alu* and *LINE-1* repeats, which are two surrogate markers of global methylation. The data consistently revealed a marked hypermethyla-

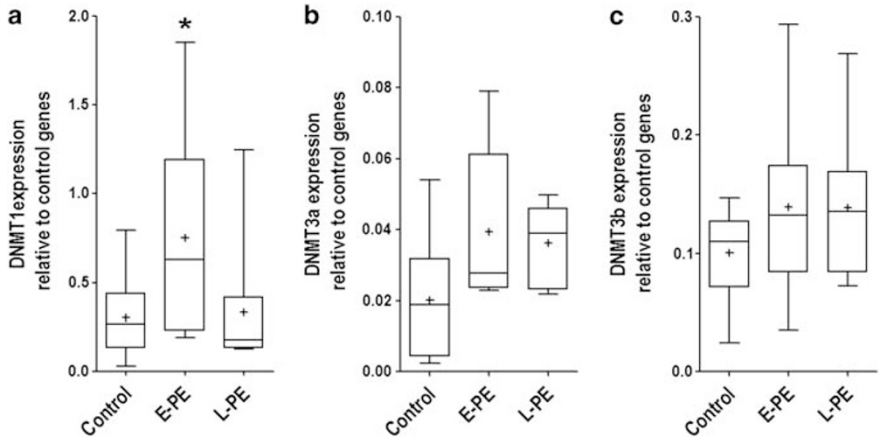

Figure 3 *DNMT1* (**a**), *DNMT3a* (**b**) and *DNMT3b* (**c**) mRNA levels in placentas were determined by real-time PCR. The expression of *DNMTs* was normalized by the control genes *GAPDH* and *β-actin*. The expression of *DNMT1* was significantly higher in the early onset, but not in the late-onset preeclamptic placentas when compared with the normal controls. Placental *DNMT3a* and *DNMT3b* expressions did not show any statistically significant difference among the three groups, though the level of *DNMT3a* in the early-onset preeclamptic placentas was relatively higher than in normal control. $*P<0.05$ *vs.* Control.

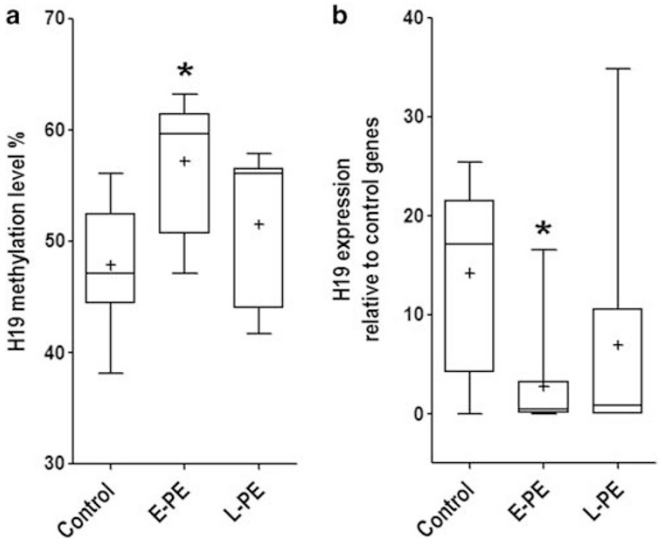

Figure 4 *H19* methylation and mRNA expression levels in control and preeclamptic placentas. (**a**) *H19* methylation levels were evaluated by methylation-sensitive high resolution melting (MS-HRM). The degree of methylation of clinical samples was evaluated by comparison with the standard curve. Statistical analysis revealed increased methylation of the *H19* gene in the early-onset preeclamptic placentas when compared with the normal controls ($P=0.0023$). The late-onset preeclamptic placentas also showed hypermethylation of the *H19* gene when compared with the normal controls, but the change was not statistically significant ($P=0.1944$). (**b**) The expression of *H19* was measured by real time PCR, and normalized by the control genes *GAPDH* and *β-actin*. *H19* mRNA level was appreciably lower in the early-onset preeclamptic placentas than in controls ($P=0.0034$). Though the level in the late-onset preeclamptic placentas was also lower than that in the normal controls, the change was not statistically significant ($P=0.1408$). $*P<0.05$ *vs.* Control.

tion in the genome of the early-onset preeclamptic placentas as compared with control placentas. It is well established that DNA methylation at the CpG dinucleotides is catalyzed by various *DNMTs*, among which *DNMT1*, *3a* and *3b* are all highly expressed in human placenta.[30] *DNMT1* is usually regarded as a maintenance methyltransferase being responsible for accurately replicating genomic DNA methylation patterns during cell division in mammalian cells. On the other hand, *DNMT3a* and *3b* are specific methyltransfersase involved in establishing DNA methylation patterns during embryonic development.[31] Our results thus indicated that the significantly higher expression of *DNMT1* may substantially contribute to the global hypermethylation in the early-onset preeclamptic placenta.

Recent studies may suggest cues as to the factors that may cause high *DNMT1* expression and global hypermethylation in PE patients. Folate, in the form of 5-methyltetrahydrofolate, is involved in remethylation of homocysteine to methionine, which is a precursor of SAM, the primary methyl group donor for DNA methylation. Adult rats subject to a short-term folate-deficient diet exhibited upregulation of *DNMT* as well as genomic DNA hypermethylation in the liver.[32] Choline, via betaine, provides methyl groups for the production of S-adenosylmethionine.[33] Feeding pregnant rats with a choline-deficient diet led to increased global DNA methylation in fetal liver and brain.[34] Deficiency of methyl group donors like folate or choline could result in hypomethylation of the regulatory CpGs within the *DNMT1* gene, leading to its overexpression and subsequent increased global DNA methylation.[35] Furthermore, Ray *et al.* reported that pregnant women with low levels of circulating folate had an increased risk of developing PE.[36] Lastly, results from two independent laboratories demonstrated that women with homozygous mutation of the T677 allele in the 5, 10-methylenetetrahydrofolate reductase (*MTHFR*) gene, an important enzyme in folate metabolism, had a significantly increased risk of PE.[37,38] Therefore, it may be worth investigating whether abnormality of folate or choline metabolisms is involved in the onset of PE via interfering with the status of DNA methylation during placentation.

Appropriate DNA methylation is indispensable for normal placenta development and function.[39] A number of studies have suggested critical links between alterations of appropriate epigenetic regulation in the placenta and diseases of gestation and early life. General disruption of DNA methylation consequent to certain clinical drug treatments has been shown to inhibit invasiveness of trophoblast cells and therefore to disrupt placental development.[40,41] Specific genetic approaches have shown that lack of *DNMT1* or *DNMT3L* genes results in impaired trophoblast differentiation and aberrant placenta development.[42,43] A recent report has revealed that *LINE-1* sequences are significantly hypermethylated in partial hydatidiform moles tissues

relative to normal placentas.[44] Partial hydatidiform moles is characterized by placenta overgrowth, trophoblastic hyperplasia and abnormal or absent fetus.[45] These placenta phenotypes are to some extent similar to the observations in preeclamptic placenta where immature intermediate trophoblasts are over proliferating.[46] Cases of PE in combination with partial hydatidiform mole have also been reported in literatures.[47,48] Taken together, these data demonstrate an epigenetic disorder in placentas of women who suffered from gestational diseases such as PE and partial hydatidiform mole. It remains to be investigated whether global DNA hypermethylation in placenta is a causal factor for gestational diseases.

The paternally imprinted *H19* gene is transcribed as an untranslated RNA that may serve as a riboregulator. *H19* is located in close proximity to the maternally imprinted *IGF-2* gene on chromosome 11p15.5[17] and the *H19* null mice display hyperplasia of all layers of the placenta.[18] These data strongly indicate that the *H19* gene is critical to the early development of placenta. The actual functions of the *H19* gene are still unclear. During pregnancy, the *H19* gene in the developing placenta can be expressed either biallelically or monoallelically. Its biallelic expression is confined to the placenta until 10 weeks of gestation, after which it is predominantly, but not exclusively, expressed from the maternal allele, and its paternal allele is proved to be methylated.[49] Some studies suggested that *H19* might act as an oncogene in human gestational trophoblastic tumors, based on the evidences that high level and biallelic expression of *H19* gene was observed in human choriocarcinoma cells, as well as that *H19* gene expression was much higher in the tumors formed by choriocarcinoma cells injected in nude mice.[50] In human placenta, *H19* expression was most pronounced in the invasive intermediate trophoblasts and villous cytotrophoblasts, whereas its expression was negligible in syncytiotrophoblasts. Such a differential expression pattern of *H19* may be consistent with its proposed role in regulating the invasive property of trophoblasts.[19,20] This possibility is supported by the data from Walsh *et al*., who showed an invasion-promoting effect of *H19* gene in trophoblastic cells.[51] The trophoblasts in preeclamptic placenta showed trophoblastic hyperplasia, over-proliferated immature intermediate trophoblasts and impaired trophoblast invasion.[46] The hypermethylation and reduced expression of the *H19* gene in early-onset preeclamptic placentas as reported in the present study, may indicate a role of *H19* in the abnormal proliferation and invasion phenotypes of the preeclamptic trophoblasts. Indeed, recent data suggest that *H19* might regulate expressions of specific target genes by acting as the precursor of microRNA-675.[52] To better address this issue, we are currently performing further studies on the molecular mechanisms underlying *H19* gene functions in human placental trophoblasts.

It has been suggested that early-onset and late-onset PE may have different etiologies and clinical expression, and should be regarded as different forms of the disease.[53] The different methylation status in placental genome as well as in the *H19* gene as observed in this study is likely supporting this idea. However, for the late-onset preeclamptic placentas, both the global methylation and *H19* gene methylation levels are in between the levels observed in the normal gestations and early-onset PE. Therefore, it is quite possible that early-onset and late-onset PE represent two degrees of severity of the disease, and maybe a pathological continuum. Patients with early-onset PE may have severe genetic or epigenetic disorders such as aberrant methylation in both genome and imprinted *H19* gene, which can disturb the proper early development of placenta during pregnancy.

One problem in our study is the difference in the duration of the pregnancy according to the disease status, which may cause gestation-dependent variation in DNA methylation levels. In our preliminary experiments, we examined the expression of DNMTs and *H19* as well as global methylation and *H19* methylation in placentas derived from six pre-term birth patients who delivered at gestational weeks 32–35. No evident differences in either *DNMTs* and *H19* expression or global methylation and *H19* methylation were found between these samples and term placentas (Supplementary Figure 2). Therefore, we used normal term placentas as universal controls in our study. Meanwhile, the extensive microarray data of Winn *et al.* did not show the expression change of *DNMTs* and *H19* genes during various developmental stages in human placentas.[54] What's more, Fuke *et al.* demonstrated that the global methylation (as measured by 5-MC content) in human placenta increased on average by 10% at the first half of pregnancy, but exhibited slight gestation-dependent difference at weeks 32 to term.[55] The early-onset, late-onset preeclamptic placentas and normal control ones used in this study were at average gestational weeks 32.2, 36.8 and 38.3, respectively. Taken these evidences together, we propose that the difference in the duration of the pregnancy according to the disease status may have little influence on both global methylation and *H19* methylation and expression.

In general, the present study is the first report on the change of global DNA methylation and *H19* gene methylation in human preeclamptic placenta. The influence of such epigenetic change on placenta development as well as the occurrence of gestational diseases needs further investigations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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