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Altered DNA methylation and transcription of *WNT2* and *DKK1* genes in placentas associated with early-onset preeclampsia

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ABSTRACT

Purpose: The purpose of this study was to characterize the changes in DNA methylation and transcription of *WNT2 and DKK1* genes in placentas associated with early-onset preeclampsia.
Methods: The study includes three groups: patients with early-onset preeclampsia, normotensive preterm and term births. Placental tissues were collected and pyrosequencing was performed on *DKK1 and WNT2* proximal promoters. Transcriptional levels of *DKK1 and WNT2* genes were determined with real-time PCR.
Results: DKK1 gene methylation levels were lower in placentas associated with early-onset preeclampsia compared to those associated with preterm birth ($P < 0.05$). *DKK1* mRNA expression was higher in early-onset preeclampsia placentas than those in preterm placentas ($P < 0.05$). *WNT2* mRNA expression in early-onset preeclamptic placentas was lower than that in other two groups ($P < 0.05$). In the preterm and early-onset preeclampsia groups, the mRNA levels for *WNT2 and DKK1* were negatively correlated ($P < 0.05$). In all the subjects, the levels of *DKK1* mRNA and methyaltion were negatively correlated ($P < 0.05$).
Conclusion: Decreased methylation of *DKK1* promoter in early-onset preeclamptic placenta tissues may up-regulate the expression of *DKK1*. The increased expression of *DKK1* and decreased expression of *WNT2* may be involved in the pathogenesis of early-onset preeclampsia.

1. Introduction

Preeclampsia is a pregnancy-specific hypertensive disorder characterized by the onset of hypertension and proteinuria after 20 weeks of gestation, affecting 2–8% of all pregnancies [1]. Preeclampsia accounts for 15% of preterm births worldwide [2]. Preeclampsia increases the risks of cardiovascular as well as metabolic diseases in the mother and fetus [3], and tends to increase the risk of preeclampsia in the pregnancy of female offspring [4]. Usually preeclamptic manifestations do not appear until the third trimester of pregnancy, and its early diagnosis remains a research subject. Currently, symptomatic management is widely applied in clinical practice, and the only definitive treatment of preeclampsia is termination of pregnancy [5]. While many pathological changes, such as systemic inflammation, oxidative stress, inadequate nutrition supply and genetic factors, the exact molecular mechanisms of preeclampsia are unclear. Epigenetic factors play a crucial role in the pathological mechanisms of preeclampsia. Changes of DNA methylation and DNA methyltrasferases (DNMT), the enzyme family catalyzing the addition of methyl group to cytosines in human genome are well recognized in preeclamptic placentas [6–8].

The canonical Wnt/β-catenin signaling pathway is the most widely studied Wnt pathway, known to be involved in cell proliferation, differentiation, invasion and migration. Wnt ligands interact with a heterodimeric receptor consisting of lipoprotein receptor-related pretein-5 and -6 (LRP-5/6) and a seven transmembrane Frizzled (Fzd) protein [9]. This interaction inhibits the degradation of β-catenin through a series of events, resulting in the accumulation of β-catenin in the cell plasma and its translocation to nucleus. In the nucleus, β-catenin replaces the transcriptional inhibitors and binds to T-cell-specific factor (TCF)/lymphoid enhancer-binding factor 1 (LEF1) transcription factor

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family. TCF/β-catenin complex activates the expression of target genes involved in the process of cell proliferation and migration [10]. To date, 19 different Wnt ligands and 10 different Fzd receptors have been described [11], and 14 of the 19 Wnt ligands, 8 of the 10 Fzd receptors have been found in the first trimester placenta [12]. As one of the Wnt ligands, WNT2 is a secretory glycoprotein that is reported to the overexpressed in various human malignancies, such as gastric, esophageal, breast, lung and colorectal cancers [12–16]. Knockdown of *WNT2* in WNT2 over expressing cancer cells can induce cell apoptosis and inhibit cell proliferation, angiogenesis and invasion, leading to suppression of tumor growth *in vitro* and *in vivo* [17]. Exogenous WNT2 could induce endothelial cell proliferation [18], supporting the involvement of WNT2 in angiogenesis, an important function of placenta implicated in the development of preeclampsia [19].

DKK1 represents a best studied member of the DKK family (DKK1, DKK2, DKK3 and DKK4) [20]. DKK1 have been found in many tissues and cell types including skin, prostate, endothelium, osteoblasts and osteocytes [21]. DKK1 functions as an antagonist of the canonical Wnt signaling pathway through binding to LRP5/6 to inhibit the formation of the Wnt-induced Fz-LRP5/6 complex [22]. Indeed, DKK1 was found to be related to the development of various malignancies such as colorectal cancer, medulloblastoma, and mesothelioma [23,24]. Dysfunction of Wnt signaling may inhibit the proliferation and invasion of extravillous trophoblast cells in placenta, leading to shallow trophoblast invasion and failure of uterine spiral artery remodeling that are frequently observed in preeclamptic placenta [25]. The purpose of this study was to investigate the DNA methylation and the transcription levels of *WNT2* and *DKK1* genes in early-onset preeclamptic placenta. Knowledge along this line may help us to better understand the pathogenic mechanisms of this life-threatening disease.

2. Materials and methods

2.1. Study subjects

Preeclampsia was diagnosed according to the criteria of ACOG (American Congress of Obstetricians and Gynecologists) [26]: 1) Systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg with proteinuria ≥ 300 mg/24 h after 20 weeks of gestation; 2) Systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg without proteinuria, but with pregnancy complications such as early-onset thrombocytopenia, impaired liver function, impaired kidney function, pulmonary edema and damaged vision or brain. Patients with other pregnancy complications including gestational diabetes mellitus, chronic hypertention, thyroid dysfunctions and kidney disease were excluded from the study. Other factors that may influence DNA methylation levels including smoking, alcohol abuse, assisted reproduction and multiple pregnancies were also excluded from the study. The preterm birth patients displayed varied pathologies such as premature rupture of membranes, cervical incompetence and placenta preposition. All the patients were accrued from the Obstetric Department of the Third Affiliated Hospital of Zhengzhou University between August 2017 and March 2018. The study included three groups: early-onset preeclampsia pregnancy (n = 25), normotensive term pregnancy (n = 25) and normotensicve preterm pregnancy (n = 25). Early-onset preeclampsia was defined as preeclampsia diagnosed before 34 weeks of gestation. Preterm births were defined as deliveries before 37 weeks of gestation. This investigation was approved by the Ethics Committee of the Third Affiliated Hospital of Zhengzhou University. All study subjects were properly informed and written consents were obtained.

2.2. Sample collection

The placental tissues were collected within 15 min following caesarean section. After the removal of the membranes, placental villous samples in the size of 5 × 5 × 5 mm were collected from the center part of maternal side 5 mm beneath the upper layer of the placenta. The placental villous samples were washed with cold PBS to remove the maternal and fetal blood. One portion of the samples was kept for DNA extraction, another portion of the samples was kept in RNAstore (Cwbio, China) for RNA extraction. Samples were snap frozen in liquid nitrogen for 10 min before storage at −80 °C until for use.

2.3. Pyrosequencing

Genomic DNA was exacted from 60 mg of placental villous tissue using TIANamp Genomic DNA Kit (TIANGEN BIOTECH, Beijing, China) according to the manufacturers' instructions. DNA samples were qualified with $A_{260/280}$ readings fell between 1.8 and 2.0, and $A_{260/230}$ between 2.0 and 2.5. The DNA was bisulfide-converted with Qiagen EpiTect Bisulfite Kit (Qiagen, 59104, Germany) as instructed by the manufacturers. Sequences for pyrosequencing were evaluated according to the following criteria: 1. Proximal to the transcriptional start site; 2. Contain mass CpG sites; 3. Suitable for designing more appropriate primers and resulting in a higher successful rate for pyrosequencing. *DKK1* gene was located on Chromosome 10, the locations of the start and the end CpG sites were 52314262 and 52314281 (Genome assembly: GRCh38.p12) respectively, and the CpG sites from the start to the end were named form CpG1 to CpG5. *WNT2* gene was located on Chromosome 7, the location of the start and end CpG sites were 117323490 and 117323534 (Genome assembly: GRCh38.p12) respectively, and the CpG sites from the start to the end were named from CpG1 to CpG7. The primers were designed with the use of PyroMark Assay Design 2.0 (Shanghai Geneland Biotech, Shanghai, China). The primers used for *WNT2* gene pyrosequencing were: Forward primer, AGTAATG AGTTGAGAATTATTTTTGGATT; Reverse primer, ACCTTTAAAAAAACT CCAAACCA; Sequencing primer, TTTAGGGATTTGTTTGTTAG. The primers used for *DKK1* pyrosequencing were: Forward primer, GAAGAGT GTTAAAGGTTTTTTTTTATGTAT; Reverse primer, CCAAAATCCTAACT ACAAAAAACACA; Sequencing primer, GGGTTAGAGGTATAAAGGTAG. Reverse primers were labeled with biotin at the 5′ end.

2.4. RNA extraction and quantitative real-time PCR

RNA was extracted from 80 mg of placental villous tissue with the use of TRIzol® Reagent (Ambion, Life Technologies, USA) following the manufacturers' instructions. RNA that satisfied $A_{260/280}$ ratio no < 1.8 and $A_{260/230}$ ratio no < 1.5 was considered acceptable. cDNA was produced with 800 ng RNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Japan) following the manufacturers' instructions. Real-time PCR was performed with the use of TB Green™ Premix Ex Taq™II(TaKaRa, Japan) in 20 μL reactions under the following conditions: Initial denaturation at 95 °C for 30 s; 40 cycles of denaturation at 95 °C for 5 s, annealing at appropriate temperatures for 30 s, extension at 72 °C for 15 s. Melting curves were analyzed under the following conditions: 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The Ct (cycle of threshold) value was normalized to that of the *GAPDH* mRNA and the difference in relative expression levels between groups under comparison was analyzed with the $2^{\triangle\triangle Ct}$ method. The mean value of the levels of *WNT2* mRNA in the preeclampsia placentas and the mean value of the levels of *DKK1* mRNA in the preterm placentas were set as 1.The sequences of *WNT2* PCR primers were: Forward primer, GTTCTTGAAACAAGAGTGCAAGTG; Reverse primer, CCCATTGTACTTCCTCCAGAGATA. The sequences of *DKK1* PCR primers were: Forward primer, AGTGTGTACCAAGCATAGGAGAAAA; Reverse primer, TTAGTGTCTCTGACAAGTGTGAAGC. The sequences of *GAPDH* PCR primers were: Forward primer, AGAACGGGAAGCTTGTC ATC; Reverse primer, CATCGCCCCACTTGATTTTG.

2.5. Statistical method

SPSS (Version 19.0, IBM, New York, USA) and GraphPad Prism (Version 5.0, GraphPad Software Inc., San Diego, CA, USA) were used

Table 1
Characteristics of the early-onset preeclampsia, preterm and term pregnancies.

Characteristics	Preeclampsia (n = 25)	Preterm (n = 25)	Term (n = 25)
Maternal age (years)	29.92 ± 6.24	31.06 ± 5.25	29.52 ± 4.30
Gestational age at delivery (week)	33.91 ± 1.39	34.14 ± 1.26	38.48 ± 0.88
Proteinuria (g/24 h)	3.866 ± 0.823	0.235 ± 0.156	0.235 ± 0.120
SBP (mmHg)	167.20 ± 18.22	116.88 ± 7.67	111.88 ± 9.24
DBP(mmHg)	104.76 ± 7.58	72.13 ± 8.72	69.80 ± 9.03
Fetal gender (male/female)	15/10	12/13	11/14
Neonatal birth weight (g)	1740.00 ± 297.55	2146.88 ± 277.77	3043.20 ± 921.04

for statistical analysis. The quantitative date was expressed as mean ± standard deviation. For the quantitative date, One-Way ANOVA or Kruskal-Wallis test was performed to compare among the three groups, student *t*-test was performed to compare between two groups. Pearson correlation analysis was performed to analyze the relationship of two continuous variables. $P < 0.05$ was considered statistically significant. R^2 represents the contribution of the independent variable to the regression relation, and r is defined as correlation coefficient.

3. Results

3.1. Demographic and clinical characteristics of study subjects

The demographic and clinical characteristics of the early-onset preeclampsia, normotensive preterm and normotensive term pregnancies are shown in Table 1. As expected, the gestational ages were significantly higher in the normotensive term group than that in the early-onset preeclampsia group ($P < 0.05$) and the normotensive preterm group ($P < 0.05$), and there was no significant difference between the early-onset preeclampsia group and the normotensive preterm group ($P > 0.05$). The proteinuria in the early-onset preeclampsia group was significantly more severe than that in the normotensive preterm group ($P < 0.05$) and the normotensive term group ($P < 0.05$), and there was no significant difference between the term group and the preterm group ($P > 0.05$). The systolic and diastolic blood pressure in the early-onset preeclampsia group was significantly higher when compared with the normotensive preterm group ($P < 0.05$) and the normotensive term group ($P < 0.05$), and there was no significant difference between the preterm group and the term group ($P > 0.05$). The normotensive term group had a significantly higher neonatal birth weight than the normotensive preterm group ($P < 0.05$) and the early-onset preeclampsia group ($P < 0.05$), and the neonatal birth weight in normotensive preterm group was significantly higher than that in the early-onset preeclampsia group ($P < 0.05$). There was no significant difference for maternal age and fetal gender among the three groups.

3.2. Methylation status of WNT2 and DKK1

The methylation levels of the 5 CpG sites and the average of *DKK1* are shown in Table 2. The methylation levels of CpG1, CpG2, CpG3, CpG4 and the average were all significantly higher in the preterm group than that in the preeclampsia and term groups ($P < 0.05$), and there were no significant differences between the early-onset preeclampsia group and the term group ($P > 0.05$). CpG5 was nearly unmethylated in all the subjects except 4 in the preterm group and 4 in the term group, but no significantly different clinical or demographic characteristics were observed within these 8 subjects.

The methylation levels with means of the 7 CpG sites and the average of *WNT2* gene are shown in Fig. 1. We did not observe any significant difference of the methylation levels of *WNT2* gene among the three groups ($P > 0.05$). The majority of the subjects demonstrated methylation levels below 10% in all the CpG sites of *WNT2* gene. There was no significant difference in the percentage of subjects that showed methylation levels of *WNT2* lager than 10% among these three groups. The methylation levels larger than or smaller than 10% were not significantly different among these three groups. We did not find significant correlation of the methylation levels of *WNT2* with the blood pressure, proteinuria or neonatal birth weights.

Table 2
Methylation levels of *DKK1*.

CpG sites	Preeclampsia (n = 25)	Preterm (n = 25)	Term (n = 25)
CpG1 (%)	4.22 ± 1.15	5.47 ± 1.37	3.97 ± 1.08
CpG2 (%)	4.22 ± 0.85	5.08 ± 1.11	4.08 ± 0.65
CpG3 (%)	3.81 ± 1.47	5.57 ± 1.79	4.15 ± 0.81
CpG4 (%)	3.88 ± 1.95	5.66 ± 1.32	3.63 ± 2.16
CpG5 (%)	0.00	0.99 ± 1.79	0.66 ± 1.27
Average (%)	3.23 ± 0.80	4.52 ± 1.13	3.31 ± 0.94

3.3. Relative levels of WNT2 and DKK1 mRNA in placentas

The relative levels of *WNT2* and DKK1 mRNA are shown in Table 3. The levels of *WNT2* mRNA in the early-onset preeclampsia group was significantly lower when compared with the normotensive preterm group ($P < 0.05$) or the normotensive term group ($P < 0.05$), and there was no significant difference between the preterm and term groups ($P > 0.05$). The levels of *DKK1* mRNA were significantly lower in the preterm placentas than that in the normotensive term placentas ($P < 0.05$) and the early-onset preeclampsia placentas ($P < 0.05$), and there was no significant difference between the early-onset preeclampsia placentas and the term placentas ($P > 0.05$). In the preterm and the early-onset preeclampsia groups, the Pearson correlation analysis showed that the expression levels of *WNT2* were negatively correlated with the expression levels of *DKK1* (Fig. 2) ($r = -0.539$, $P < 0.05$), and R^2 was 0.291.

3.4. Analysis of the correlation between the methylation levels and the levels of DKK1and WNT2 mRNA

Significant correlations were not found between the methylation levels of any CpG sites and the levels of *WNT2* mRNA. In the preterm, term and early-onset preeclampsia groups, the Pearson correlation analysis showed that the levels of *DKK1* mRNA were significantly negatively correlated with the methylation levels of CpG2 ($r = -0.660$, $P < 0.05$) and CpG4 ($r = -0.357$, $P < 0.05$)(Fig. 3), with R^2 as 0.436 and 0.128, respectively.

4. Discussion

Preeclampsia is considered a pregnancy complication of placental origin, because the symptoms of preeclampsia including hypertension and proteinuria disappear in short time after the delivery of placenta, and preeclampsia has been observed as a complication of molar pregnancy where a fetus is absent, which suggest that placenta is sufficient to cause such disease. Trophoblast cells and cancer cells share the character of invasion, and they are both immune tolerant to human

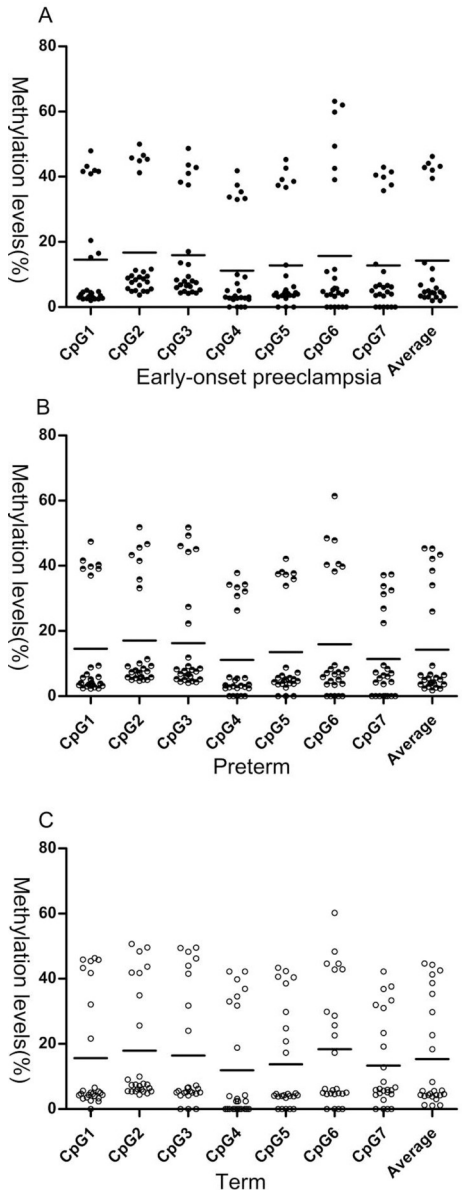

Fig. 1. Methylation levels of the seven CpG sites and the average of *WNT2* gene. The Y-axis of column scatter graph indicated methylation rates and the X-axis represented CpG sites or the average, and the bars within the column scatters represent the mean methylation levels. A. Methylation levels of *WNT2* gene in early-onset preeclampsia placentas. B. Methylation levels of *WNT2* gene in preterm placentas. C. Methylation levels of *WNT2* gene in term placentas. There was no significant difference of the methylation levels of *WNT2* gene among the three groups ($P > 0.05$), and no significant difference in the percentage of subjects that showed methylation levels of *WNT2* lager than 10% among these three groups.

Table 3
Levels of *WNT2 and DKK1* mRNA.

Genes	Preeclampsia	Preterm	Term
WNT2	1.25 ± 0.77	4.64 ± 1.68	4.61 ± 1.87
DKK1	3.46 ± 1.34	1.14 ± 0.60	4.01 ± 1.85

body. Preeclampsia occurs as a result of the reduced invasion of trophoblasts into the uterus and failed remodeling of the uterine spiral arteries. Because of the inadequate maternal blood perfusion into the placenta, the fetuses are exposed to insufficient nutrient and oxygen

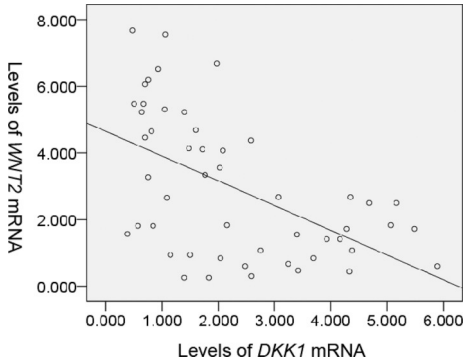

Fig. 2. Correlation between the mRNA levels for *WNT2* and *DKK1* in early-onset preeclampsia and preterm placentas. Pearson correlation analysis showed a significant negative correlation between the mRNA levels for *WNT2* and *DKK1* ($r = -0.539$, $P < 0.05$), and the R^2 was 0.291.

supply in the uterus [27], and intrauterine growth restriction in some cases accompanies preeclampsia.

Recently there are various studies focused on the normal and pathogenic placental genomic methylation profiles or the methylation changes of specific pathogenesis-associated genes. Methylation plays an important role in the placenta development. Alterations in methylation patterns may cause gene expression changes, and abnormal methylation patterns may affect the morphologies and functions of the placenta.

In the previous studies, gestational age is an important factor affecting DNA methylation. In a study by Shi-Wen Jiang et al. [28], LINE-1 DNA methylation status representing global DNA methylation was observed to be significantly decreased in the third trimester placentas when compared to first trimester placentas. The subjects in this study were classified into three groups: early-onset preeclampsia, normotensive preterm birth and normotensive term birth. The classification of the subjects in our study was different from most of the previous studies, in which the subjects were classified into preeclampsia and preterm groups or preeclampsia and normotensive term groups. In the classification of preeclampsia and term pregnancies, the older gestational ages in the term groups might be an additional factor affecting the genomic methylation status. On the other hand, when the subjects were classified into preeclampsia and preterm groups, the underlying factors causing preterm might affect the genomic methylation status. In our study, we set the normotensive preterm and term pregnancies as the control groups to take into account the effects of both the gestational ages and underlying factors causing preterm on the methylation and transcription of *DKK1* and *WNT2*. In the study by C.B.van den Berg et al. [29], the researchers preformed Illumina Humanmethylation 450 K BeadChip tests to investigate the genomic methylation profiles in the placenta, and more methylation alterations were identified in the early-onset preeclamptic placentas in comparison with the late-onset preeclamptic placentas, probably due to the longer time of early-onset preeclamptic placentas exposed to oxidative stress [29]. Early-onset preeclampsia but not late-onset preeclampsia was selected in our study for the greater methylation alterations that early-onset preeclampsia might demonstrate. It should be noted that there are gray areas in the distinction of early-onset and late-onset preeclampsia [30]. Some early-onset preeclampsia may be diagnosed after 34 weeks of pregnancy, or the gestational ages may be calculated incorrectly. Fetal gender is another important factor affecting gene methylation, and methylation alterations were more frequently observed in female placentas [31]. In our study, there was no significant difference of fetal gender among these three groups, thus excluding the effects of fetal gender on the methylation and transcription levels of *DKK1* and *WNT2* genes.

Methylation alterations of *DKK1* gene have been implicated in various diseases, such as colon cancer, osteoporosis, and systemic sclerosis [32–34]. But to the best of our knowledge, there have been no

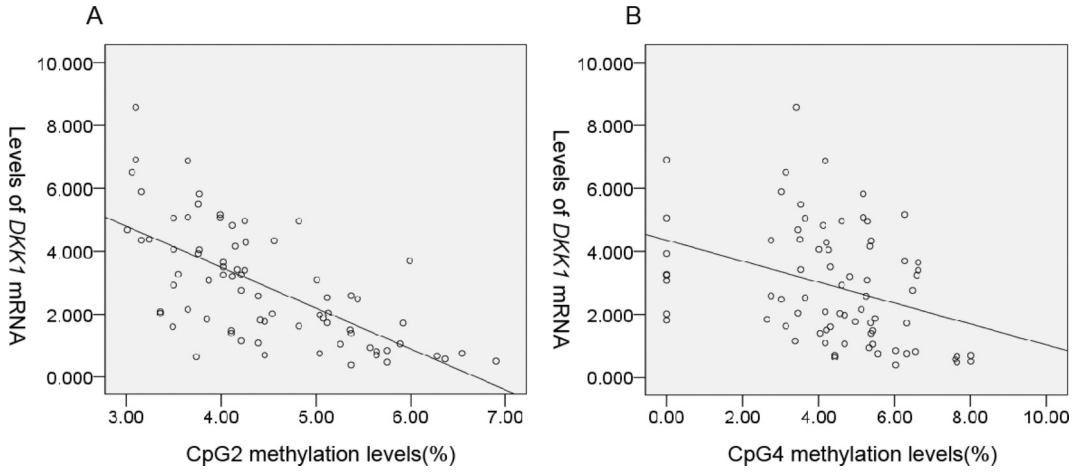

Fig. 3. A. Correlation between the levels of *DKK1* mRNA and the methylation levels of CpG2 in early-onset preeclampsia, preterm and term placentas. Pearson correlation analysis showed a significant negative correlation between the mRNA levels for *DKK1* and methylation rates of CpG2 ($r = -0.660$, $P < 0.05$), and the R^2 was 0.436. B. Correlation between the levels of *DKK1* mRNA and the methylation levels of CpG4 in early-onset preeclampsia, preterm and term placentas. The Y-axis indicated levels of *DKK1* mRNA and the X-axis represented CpG4 methylation levels. Pearson correlation analysis showed a significant negative correlation between the mRNA levels for *DKK1* and methylation rates of CpG4 ($r = -0.357$, $P < 0.05$), and the R^2 was 0.128.

studies focusing on the methylation alterations in preeclamptic placentas. The methylation levels of CpG1, CpG2, CpG3, CpG4 and the average were all higher in normotensive preterm pregnancies than those in early-onset preeclampsia and term pregnancies, and there was no significant difference between the early-onset preeclampsia and term groups. Because there was no significant difference of gestational ages between the early-onset preeclampsia and preterm groups, the decreased methylation of *DKK1* gene might be used as a biomarker of early-onset preeclampsia in preterm cases. CpG5 was almost unmethylated in the placentas, and the minority subjects that demonstrated methylation of CpG5 demonstrated no significant different clinical or demographic characteristics. The rare occurrence of CpG5 methylation should be regarded as a scientific observation although it couldn't be explained at present. Studies have shown that DKK1 may inhibit the implantation of the placentas to the uterus. DKK1 was demonstrated to inhibit the proliferation of cytotrophoblasts in human villous explants [35]. DKK1 counteracted the Wnt-induced cell motility, reduced basal migration and invasion of primary trophoblasts and SGHPL-5 cells [36]. The levels of *DKK1* mRNA were higher in the early-onset preeclampsia placentas than those in the preterm placentas, a result consistent with a study by Zhan Zhang et al. [37]. There was no significant difference in the levels of *DKK1* mRNA between early-onset preeclampsia and term groups, the increased levels of DKK1 mRNA might serve as a biomarker of early-onset preeclampsia in preterm cases. In Zhan Zhang's study, the DKK1 levels were investigated by western blot and immunohistochemistry, and levels of *DKK1* mRNA were performed by quantitative real-time PCR, the results confirmed the consistency of the DKK1 levels and levels of *DKK1* mRNA in both preeclampsia and control placentas [37]. Based on this previous study, it was speculated higher DKK1 levels in early-onset preeclampsia placentas in comparison with preterm placentas. The higher leves of DKK1 might be involved in the pathogenic mechanisms of preeclampsia through inhibiting Wnt signaling pathway, subsequently suppressing the proliferation and invasion of trophoblastic cells. The promoter methylation status is usually negatively correlated with the expression levels, and the increased levels of *DKK1* mRNA might be regulated by the decreased methylation status in early-onset preeclamptic placentas. Based on the significant negative correlation between levels of *DKK1* mRNA and the methylation levels of CpG2 and CpG4, the levels of *DKK1* mRNA were more likely to be regulated by these two CpG sites. The lower methylation status and higher trancription levels of *DKK1* in the term placentas than that in the preterm placentas also suggested that DKK1 might be involved in the termination of pregnancy, because the only difference between these two groups was the gestational age. The similar methylation and transcription patterns of *DKK1* in early-onset preeclampsia and term placentas suggested that early-onset preeclampsia placentas might be considered premature. The methylation status of *DKK1* gene even within the same group were usually different, for the methylation alterations might be correlated with disease severity [38], which may vary among the individuals in the same group, and multiple environmental factors affected the methylation status, such as the gestational ages within the same group were approximately but not all the same.

Methylation alterations of *WNT2* gene have been observed in placental tissues of preeclampsia [39], placental tissues of fetal growth restriction [40], and colorectal cancer tissues [41]. In the study by Kristen R Yeung et al. [39], the researchers employed Illumina Infinium Methylation 450 BeadChip array, and identified the increased methylation of *WNT2* gene in preeclamptic placental tissues, and the hypermethylation of *WNT2* in preeclamptic placental tissues was confirmed by bisulfite pyrosequencing. But in our study, there was no significant difference of the methylation status of *WNT2* gene among these three groups, and we did not find significant difference in the proportion of subjects that showed methylation levels larger than 10% among these three groups, no significant difference was observed in the methylation levels larger than or smaller than 10% among these three groups. In addition, we did not find a significant correlation in the methylation levels of *WNT2* with any clinical characteristics. The methylation levels of *WNT2* gene couldn't serve as a potential biomarker of early-onset preeclampsia. The difference between this study and that of Kristen R Yeung might be due to the different subjects selected. Because the methylation status is affected by numerous factors, any underlying difference of the study subjects could result in the different methyaltion status between the two studies. In the study by Kristen R Yeung, the gestational ages in the preeclampsia group was significantly lower than that in the control group, but the classification of early-onset and late-onset preeclampsia was unclear; whereas in our study, the study subjects were restricted to early-onset preeclampsia, which was different from those in Kristen R Yeung's study. The different CpG sites selected for investigation could also cause the difference of the methylation status of *WNT2* between this and the previous studies. In another study by Zhan Zhang et al. [42], the researchers performed immunohistochemistry and Western blot on the levels of WNT2 in placentas, and quantitative real-time PCR was employed to investigate the levels of *WNT2* mRNA, and they found decreased levels of WNT2 and the mRNA for *WNT2* in severe preeclampsia placental tissues,

suggesting a correlation between the decreased levels of WNT2 in placentas and preeclampsia. In our study, the levels of *WNT2* mRNA were decreased in the early-onset preeclampsia placentas relative to the preterm and the term placentas, and there was no significant difference between the term and the preterm placentas. The decreased levels of *WNT2* mRNA might be used as a biomarker of early-onset preeclampsia. Referring to the previous study, the levels of WNT2 could be decreased in early-onset preeclampsia placentas, suggesting that the lower levels of WNT2 might be involved in the pathogenesis of early-onset preeclampsia *via* inhibiting the activation of Wnt signaling pathway and eventually decreasing trophoblastic cell proliferation, invasion and placental angiogenesis. Because no significant difference of *WNT2* methylation among these three groups was detected, the lower levels of *WNT2* mRNA in early-onset preeclampsia placentas might not be regulated by DNA methylation. In the preterm and the early-onset preeclampsia groups, the levels of *WNT2* mRNA were negatively correlated with the levels of *DKK1* mRNA. Because WNT2 activates and DKK1 inhibits the Wnt signaling, it was possible that the lower levels of WNT2 collaborated with the higher levels of DKK1 to contribute to the pathogenesis of early-onset preeclampsia.

Pyrosequencing is the golden standard for methylation studies, and it can accurately determine the methylation levels of the exact CpG sites. In our methylation studies of *DKK1* and *WNT2*, pyrosequencing accurately determined the methylation status of *DKK1* and *WNT2* genes in the placentas. The limitations of pyrosequencing is that it could only determine the methylation levels of a few CpG sites, which might not represent the overall promoter methylation levels. Future studies are expected to investigate the methylation levels of *WNT2* and *DKK1* genes in early-onset preeclampsia placentas in other CpG sites or with other methods. Further studies *in vitro* as well as in animal models are required to determine the exact effects of methylation alterations of *WNT2* and *DKK1* on gene expression and placentation.

5. Conclusion

In summary, decreased methylation of *DKK1* promoter in early-onset preeclamptic placenta tissues may up-regulate the expression of *DKK1*. The increased expression of *DKK1* and decreased expression of *WNT2* may be involved in the pathogenesis of early-onset preeclampsia.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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