Correlation between *MTHFR* gene methylation and pre-eclampsia, and its clinical significance

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ABSTRACT. We investigated the correlation between 5,10-methylenetetrahydrofolate reductase (MTHFR) gene methylation and pre-eclampsia, and its clinical significance, by comparing methylation in the *MTHFR* gene promoter of the placenta and peripheral venous blood in pre-eclampsia and normal gravidas. We enrolled 259 gravidas from the People's Liberation Army 202nd Hospital, China, between January 2011 and September 2011, including 127 pre-eclampsia and 132 normal gravidas. Methylation levels of the *MTHFR* gene in placentas in two sets of gravidas were detected by methylation-specific polymerase chain reaction, plasma homocysteine levels were detected by enzyme-linked immunosorbent assay, and folic acid and vitamin B12 levels were detected by electrochemiluminescence. The chi-square test results were analyzed using the SPSS19.0 statistical software. In placentas, the methylation indices were 26.8% (34/127) and 15.2% (20/132) in the pre-eclampsia and normal groups, respectively (χ^2 = 5.30, P < 0.05, odds ratio (OR) = 2.04, 95% confidence interval (95%CI) = 1.10-3.73). In peripheral venous blood, the methylation indices were 22.8% (29/127) and 12.1% (16/132) in pre-eclampsia and normal groups, re-

spectively ($\chi^2 = 5.17$, $P < 0.05$, OR = 2.15, 95%CI = 1.11-4.15). The plasma methylation level of the pre-eclampsia group was consistent with the normal group. The plasma homocysteine level in the pre-eclampsia group was higher than in the normal group ($P < 0.05$). Levels of folic acid and vitamin B12 in the pre-eclampsia and normal groups were not statistically significant ($P > 0.05$). Patients with pre-eclampsia have hypermethylation in the *MTHFR* gene promoter, which may be one of its causes.

Key words: MTHFR; Methylation; Hcy; Pregnancy hypertension

INTRODUCTION

Pre-eclampsia is a serious complication of pregnancy and a threat to the health of both mother and child. It is the main cause of mortality in gravidas and perinatal infants, and has an incidence rate as high as 7-10%. Nearly three years' statistical data show that the incidence rate in Liaoning Province is 13.4%, making it the most prone area in the northeast of China. Currently, both Chinese and international scholars agree that an increased homocysteine (Hcy) concentration is closely related to pre-eclampsia (Yi et al., 2008; Mao et al., 2010). The enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) plays a key role in Hcy metabolism, and its effects on pre-eclampsia are of great interest to researchers in the field (Klai et al., 2011; Xia et al., 2012). International research on MTHFR and pre-eclampsia has focused on gene polymorphisms. As research has progressed, the correlation between MTHFR and pre-eclampsia has become more apparent. In this research, the levels of *MTHFR* gene methylation in the placental tissue of gravidas were detected using methylation-specific polymerase chain reaction (MSP). We explored the possible mechanism of *MTHFR* gene methylation and its correlation with pre-eclampsia to provide a genetic basis for the prevention, early diagnosis, and treatment of the disorder.

MATERIAL AND METHODS

Research subjects

Pre-eclampsia groups

We chose 127 patients from the People's Liberation Army 202nd Hospital, China, between January 2011 and September 2011. The gravidas were aged 20-40 years, with an average age of 29.1 ± 4.3 years, and the gestation period was 33-37 weeks, with an average of 35.1 ± 1.5 weeks. We measured blood pressure and urine protein according to the diagnostic criteria for pre-eclampsia in Obstetrics and Gynecology (Le, 2008).

Control group

The control group comprised 132 normal gravidas from the People's Liberation Army 202nd Hospital, China, chosen between January 2011 and September 2011. The gravidas were aged 20-35 years, with an average age of 28.5 ± 3.2 years, and the gestation period was 34-41

weeks, with an average of 38.4 ± 1.2 weeks.

All gravidas were Han women without a history of primary hypertension, diabetes, chronic nephritis, or heart disease. There was no statistically significant difference in age and progestation body mass index between the two groups (P > 0.05). The difference in the periods of gestation was statistically significant between the two groups (P < 0.01), with an abnormally short gestation in the pre-eclampsia group. We acquired the informed consent from all gravidas (see Table 1).

Table 1. Comparison of the clinical data from the pre-eclampsia and normal groups.

Variable	Pre-eclampsia group (N = 127)	Normal group (N = 132)	U	P
Age (years)	29.1 ± 4.3	28.8 ± 3.2	0.6350	>0.05
BMI during pregnancy	23.6 ± 4.5	23.2 ± 4.1	0.7469	>0.05
Gestation time (weeks)	35.1 ± 1.5	38.4 ± 1.2	19.5046	<0.01

BMI = body mass index.

Methods

Specimen collection and processing

On admission, 15 mL peripheral blood was taken from each patient and placed in three test tubes with ethylenediaminetetraacetic acid anticoagulant. Within half an hour of sampling, the tubes were shaken gently and centrifuged for 10 min at 3000 revolutions per minute to obtain the plasma as supernatant, which was stored at -80°C for testing in batches. Three placental tissue samples, one from the central area and one from either side of it, were taken from each gravida immediately following birth. The samples measured 1.0 x 1.0 x 1.0 cm, and areas of infarction and calcification were avoided. They were stored at -80°C until required for molecular genetic testing.

Genomic DNA extraction

The samples were washed with normal saline until the rinse solution became clear, and tissue genomic DNA and blood genomic DNA were extracted using a kit supplied by the Beijing Ding National Biotechnology Co., Ltd., according to manufacturer instructions for tissue DNA extraction.

Genomic DNA bisulfite modification

For genomic DNA methylation, we used a methylation modification kit (Invitrogen). DNA was denatured using 3 M NaOH, modified by sodium bisulfite, and stored in a 50°C water bath in the dark overnight. Unmethylated cytosines were translated into uracil while methylated cytosines remained unchanged, and the modified DNA was recovered by ethanol precipitation and resuspended in ionized water for the MSP.

MTHFR promoter methylation detection

We used two pairs of allelic MSP primers: MTHFR-M, corresponding to the sequence containing 5-methylcytosines that had not been affected by sodium bisulfite treatment; and

unmethylated MTHFR-U, corresponding to a C-T-transformed sequence that had been affected by sodium bisulfite. The primers were researched by reviewing the literature (Wang et al., 2007), and synthesized by the Beijing Huada Sheng Biotechnology Co. The polymerase chain reaction (PCR) regimen was: pre-denaturation at 95°C for 5 min; denaturation at 94°C for 30 s; annealing for 30 s; extension at 72°C for 30 s, 35 cycles; and connection at 72°C for 5 min. The PCR products were examined by agarose gel electrophoresis. We used two pairs of primers for amplification in each sample (2U and 2M); if there was methylation in the gene promoter, the methylation-specific primers amplified according to the size of the bands, as represented by m in Figures 1 and 2; otherwise, the methylation-specific primers amplified according to the size of the bands represented by u; if there was mixed methylation, u and m existed simultaneously. The primer sequences are given in Table 2.
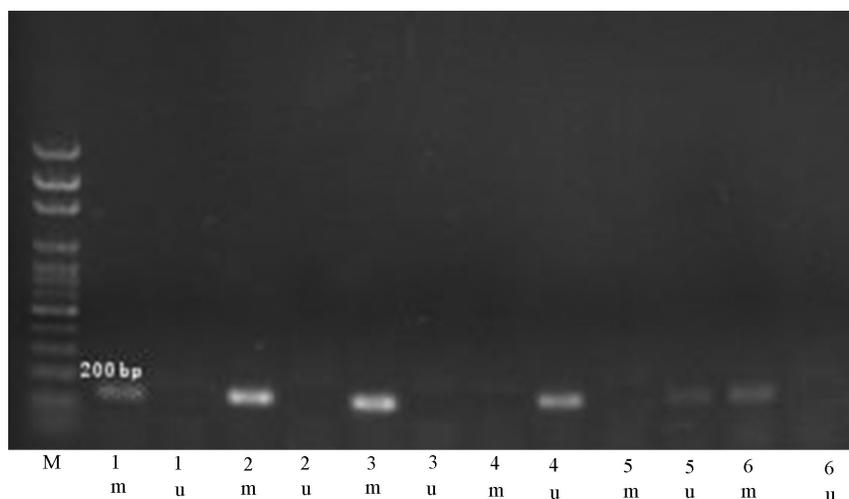

Figure 1. Agarose gel electrophoresis of MTHFR gene methylation-specific polymerase chain reaction (MSP) products from placental tissue. *Lane M* = marker; m represents methylation-specific primers; u represents unmethylated-specific primers. *lanes 1*, *2*, and *3* represent pre-eclampsia groups. *lanes 4*, *5*, and *6* = normal groups.
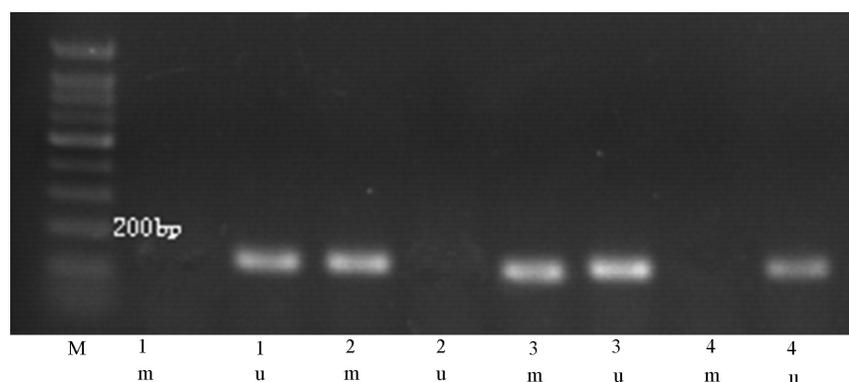

Figure 2. Agarose gel electrophoresis of MTHFR gene methylation-specific polymerase chain reaction (MSP) products from peripheral blood. *Lane M* = marker; m represents methylation-specific primers; u represents unmethylated-specific primers. *Lanes 1* and *2* = pre-eclampsia groups, *lanes 3* and *4* = normal groups.

Table 2. Methylation-specific polymerase chain reaction (MSP) primer sequences.

Primer	Forward primer	Reverse primer
MTHFR-M	TTTTTAGTTAGGATTTGCGTTTTAC	GTTAAA AACCGTACCTTTATCGTC
MTHFR-U	TTTAGTTAGGATTTGTGTTTTATGT	CATTAAAAACCATACCTTTATCATC

Detection of the levels of Hcy and vitamin B12 in plasma

Hcy levels were detected by enzyme-linked immunosorbent assay using AxSym Chemiluminescence Apparatus (Mabtech AB, Swedish). Reagents were purchased from Abbott Laboratories. Folic acid and vitamin B12 levels were detected by electrochemiluminescence. We used original reagents.

Statistical analysis

Data were recorded in a Microsoft Excel 2000 database and were analyzed by the SPSS 13.0 software. Data are reported as means ± SD. Comparisons were performed using the U- and *t*-tests. We calculated odds ratios (ORs) to estimate the correlation between *MTHFR* gene methylation and pre-eclampsia. Differences of $P < 0.05$ were considered to be statistically significant.

RESULTS

Agarose gel electrophoresis of the MSP products

In each group, the methylation primer amplified specific PCR bands, and unmethylated primers also amplified specific PCR bands. However, there was a large difference in the methylation index. In the placental tissue, the methylation indices were 26.8% (34/127) and 15.2% (20/132) in the pre-eclampsia and normal groups, respectively, and the difference was statistically significant ($\chi^2 = 5.30$, $P < 0.05$, OR = 2.04, 95% confidence interval (95%CI): 1.10-3.73). In the peripheral blood, the methylation indices were 22.8% (29/127) and 12.1% (16/132) in the pre-eclampsia and normal groups, respectively, and the differences were statistically significant ($\chi^2 = 5.17$, $P < 0.05$, OR = 2.15, 95%CI: 1.11-4.15). When the methylation indices of the placental tissue and the peripheral blood in the two groups were compared, the differences were not statistically significant ($P > 0.05$) (see Table 3). Comparison of the levels of Hcy, folic acid, and vitamin B12 is shown in Table 4.

Table 3. Comparison of *MTHFR* gene methylation in the pre-eclampsia and normal groups.

Groups	Placental tissue		Peripheral blood	
	Methylated	Unmethylated	Methylated	Unmethylated
Pre-eclampsia group (%)	34 (56.7)	93 (43.3)	29 (37.4)	98 (62.6)
Normal group (%)	20 (36.4)	112 (63.6)	16 (23.9)	116 (76.1)
χ^2	5.30		5.17	
P	<0.05		<0.05	
OR (95%CI)	2.04 (1.10-3.73)		2.15 (1.11-4.15)	

OR = odds ratio; 95%CI = 95% confidence interval.

Table 4. Comparison of the levels of homocysteine (Hcy), folic acid, and vitamin B12 (VitB12).

Variable	Pre-eclampsia group (N = 127)	Normal group (N = 132)	U	P
Hcy (mg/L)	10.37 ± 1.29	28.8 ± 3.27	60.0769	<0.05
Folic acid (nM)	607.21 ± 200.37	605.43 ± 206.32	0.070437	>0.05
VitB$_{12}$ (pM)	341.72 ± 2.9	341.97 ± 4.1	0.56823	>0.05

DISCUSSION

Pre-eclampsia is exclusive to pregnancy and remains one of the main causes of maternal and perinatal mortality. Its etiology and pathogenesis have not been clearly elucidated. The current study suggests that the pathogenesis of pre-eclampsia is closely related to placental ischemia, immune factors, vascular endothelial injury, and genetic factors (Kanasaki and Kalluri, 2009; Buurma et al., 2012).

Hcy is an important metabolite of the human body and changes in its plasma concentration have an impact on pre-eclampsia, a fact that has been fully affirmed both in China and internationally. Its mechanism of action may be one of the following.

First, elevated Hcy concentrations may affect the invasion of extravillous trophoblasts, causing inadequate recasting of spiral arteries. Owing to the lack of high-flow and low-resistance spiral arteries, placental perfusion is reduced, resulting in placental stunting. In such cases, placental function cannot meet the needs of normal pregnancy and the uterine placenta experiences long-term ischemia and hypoxia. Maternal endothelial cells are activated and inflammatory cytokines released, ultimately resulting in the various clinical manifestations of pre-eclampsia (James et al., 2010).

Second, hyperhomocysteinemia causes a change in nitric oxide bioavailability, increases oxidative stress and matrix metalloproteinase activity, and injures the structure and function of vascular endothelial cells (Steed et al., 2007). Vascular endothelial cell injury can increase the secretion of endothelin-1, reduce vascular endothelium-derived relaxing factor and prostacyclin secretion, and lead to vasomotor factor balance disturbances, causing pre-eclampsia (Zhou et al., 2006).

MTHFR is one of the key enzymes of Hcy metabolism. In humans, about 50% of cells under the action of Hcy in N5-MTHFR use methylenetetrahydrofolate as a methyl donor, are subject to re-methylation, and re-synthesize methionine (Ingrosso and Perna, 2009), which is involved in the body's normal metabolism. The fact that high Hcy may lead to the occurrence of pre-eclampsia has been widely recognized. We speculate that Hcy may lead to increased MTHFR expression, which is possibly one of the mechanisms of pre-eclampsia. At present, Chinese and international research focuses on the correlation between polymorphisms and pre-eclampsia, but research on the correlation between the *MTHFR* promoter methylation level and pre-eclampsia from the perspective of epigenetic DNA methylation is rare. During DNA methylation, the DNA sequence does not change but the bases are modified, resulting in altered gene activity. DNA methylation is closely related to cancer, genetic diseases, autoimmune diseases, and senility (McCabe et al., 2009). Kulkarni (2011) researched the correlation between *MTHFR* gene methylation and pre-eclampsia in the placental tissue of 57 pre-eclampsia gravidas and 30 normal gravidas in India. The results suggest that placental *MTHFR* gene methylation and plasma Hcy levels increased simultaneously in pre-eclampsia. Because gene promoter methylation levels are affected by race and geographical origin, we selected 127 pre-eclampsia gravidas and 132 normal gravidas from the Shenyang Han population to

study the correlation between *MTHFR* gene methylation and pre-eclampsia in placental tissue and peripheral blood.

The results showed that in the placental tissue, the methylation indices were 26.8% (34/127) and 15.2% (20/132) in the pre-eclampsia and normal groups, respectively, and the difference was statistically significant ($\chi^2 = 5.30$, $P < 0.05$, OR = 2.04, 95%CI: 1.10-3.73). In peripheral venous blood, the methylation indices were 22.8% (29/127) and 12.1% (16/132) in pre-eclampsia and normal groups, respectively, and again the differences were statistically significant ($\chi^2 = 5.17$, $P < 0.05$, OR = 2.15, 95%CI: 1.11-4.15). The methylation level in the plasma of the pre-eclampsia group was consistent with that of the normal group. The Hcy level in the plasma of the pre-eclampsia group was higher than in the normal group, and the differences were statistically significant ($P < 0.05$). However, when the levels of folic acid and vitamin B12 in the pre-eclampsia and normal groups were compared, the differences were not statistically significant ($P > 0.05$).

To date, the specific mechanism of the *MTHFR* genomic hypermethylation cause of pre-eclampsia has not been fully elucidated. Previous studies have shown that when *MTHFR* gene promoter methylation occurs, there may be a reduction in *MTHFR* gene expression resulting in a decrease in MTHFR production, which in turn reduces Hcy metabolism. This leads to an accumulation of Hcy in the body, leading to a series of pathological changes. Folic acid and vitamin B12 have a major impact on Hcy levels. In this study, there were no significant differences in folic acid and vitamin B12 levels between the pre-eclampsia and normal groups. This confirms that *MTHFR* gene promoter methylation may be an independent risk factor for pre-eclampsia. In summary, we conclude: 1) in pre-eclampsia, placental tissue and peripheral venous blood both exhibit *MTHFR* genomic hypermethylation; and 2) *MTHFR* genomic promoter methylation may lead to pre-eclampsia by affecting Hcy metabolism.

This study provides a genetic basis for the clinical prediction and treatment of pre-eclampsia. If *MTHFR* gene methylation can be detected before pregnancy, early intervention and intensive care of gravidas with gene promoter methylation may reduce the risk of pre-eclampsia, help guide treatment, and improve the prognosis.

Conflicts of interest

The authors declare no conflict of interest.

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