

Methylenetetrahydrofolate reductase polymorphisms at 3'-untranslated region are associated with susceptibility to preterm birth

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Background: Etiology and mechanism of preterm birth (PTB) is complicated. Genetic susceptibility is one of the key factors involved in the pathogenic mechanism underlying PTB.

Methods: A subset of single nucleotide polymorphisms (SNPs) selected by bioinformatics approach from 3'-untranslated region (3'-UTR) of *methylenetetrahydrofolate reductase (MTHFR)* gene were subjected to SNaPshot analysis in a case-control study. Three SNPs (rs45451599, rs1537515, rs1537516) were simultaneously tested in one tube, among 1,135 DNA samples including 480 PTBs and 655 term controls.

Results: Two perfectly correlated ($r^2=1$) SNPs, rs1537515 and rs1537516, were found significantly associated with PTB susceptibility [P=0.012; OR: 0.65; 95% confidence interval (CI), 0.47-0.91]. The frequencies of the minor alleles were lower in PTB cases than in controls, which the frequencies were 0.066 in PTB cases and 0.095 in controls. G and T allele frequencies of rs1537515 were the same with rs1537516 (P=0.011; OR: 0.666; 95% CI, 0.49-0.91). Rs45451599 was not found associated with PTB (P=0.52; OR: 0.76; 95% CI, 0.33-1.74). The 18-25 nucleotides in length of microRNAs (miRNAs) which can regulate gene expressions are involved in binding partial complementary sequences within 3'-UTR. The two loci are at 3'-UTR of *MTHFR* mRNA. Rs1537516 is a potential target of miR-1304-3p, while rs1537515 is miR-1224-3p and miR-3150-5p.

Conclusions: In conclusion, rs1537515 and rs1537516 within the 3'-UTR of the *MTHFR* gene may be associated with susceptibility to PTB.

Keywords: Preterm birth (PTB); single nucleotide polymorphism (SNP); folic acid metabolism; folate; *methylenetetrahydrofolate reductase (MTHFR)*

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Introduction

Preterm birth [(PTB), delivery before 37 weeks in humans] is the leading cause of neonatal mortality and morbidity, with an incidence between 5-12% (1). In Suzhou, the incidence is 6%. Etiology is complex, including environment and genetic factors involved in both maternal and fetal aspects. Many studies revolved that both maternal and fetal genetic factors contributed to gestational age (2-4). PTB has significant genetic contributions that genetic factors account for 25-

40% of variation in gestational duration (5). Recent PTB large-scale genetic studies were published (6). Identifying genetic contributions will help to genetic screening tests and to predict to PTB.

Folic acid metabolism is closely related to many important genetic and epigenetic pathways. It can affect DNA synthesis, amino acid metabolism, methyl donor contents, homocysteine (Hcy) concentration that could give rise to congenital heart disease (CHD) (7) and PTB.

Folate deficiency during pregnancy is one of the risk factors for anaemia, low birth weight and PTB. In a cohort of 34,480 singleton pregnancies, preconceptional folate supplementation is associated with a 50-70% reduction in the incidence of spontaneous PTB (8). Growing evidences show that polymorphisms of genes related to folic acid metabolism pathways are involved in PTB. In recent years, several studies focus on association study of single nucleotide polymorphisms (SNPs) with folic acid metabolism and it is reported that SNPs in the coding sequence of several genes are associated with PTB (9-12).

Methylenetetrahydrofolate reductase (MTHFR) is an enzyme mainly involved in folic acid metabolism pathways responsible for the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant form of folate in plasma involved in DNA methylation pathways or purine synthesis (13). Polymorphisms locating in the exons of this gene can result in the transition of amino acid that finally lead to the reduction of enzyme activity. For instance, common polymorphism 677C > T homozygous variant can reduce its activity by 70% compared with wild type (14). Chen *et al.*'s study shows that the infant *MTHFR* CT and TT genotypes are responsible for mother's PTB and low birth weight (15). At present there are few reports of the association of genetic variation at non-coding area including 3'-untranslated region (3'-UTR) of *MTHFR* with PTB. 3'-UTR, a non-coding region of mRNA, is a target of microRNA (miRNA). miRNAs can regulate the genes translation through sequence-specific binding to 3'-UTR, thus will result in inhibition of translation or degradation of target mRNAs which can reduce the activity of enzyme (16). Recent studies show that polymorphisms in miRNA and 3'-UTR are related with tumors synthesis (17-19) and CHD (7).

We selected three SNPs at 3'-UTR of *MTHFR* that may disrupt binding capacity of miRNA using miRNA prediction tools (miRnadb, TargetScan and PicTar). Then we performed genotyping of the three SNPs in a case-control study of PTB to identify whether or not they were involved in the susceptibility to PTB.

Materials and methods

Study subjects

A total of 1,135 subjects were enrolled with informed consent, including 480 cases of preterm babies and 655 normal in-term babies who were born in the Suzhou Maternal-child Medical Center from 2012 to 2013. DNA

from all samples was extracted from blood spots using NP968 automatic nucleic acid extraction apparatus (Xi'an Tianlong Science and Technology Co. Ltd., China), which was originally used for neonatal screening for inborn errors of metabolism. The study was approved by the Ethic Committee for Human Research, Suzhou Hospital Affiliated to Nanjing Medical University.

SNPs selection

Three prediction tools were used for bioinformatic analysis of the miRNA binding sites at the 3'-UTR of *MTHFR*: miRnadb, PicTar and TargetScan (20-22). miRNA 22, miRNA24, miR-1304-3p, miR-1224-3p and miR-3150-5p were candidate miRNAs for further study. Nine SNPs at the 3'-UTR of *MTHFR* including rs145253240 (minor allele frequency; MAF 0.05%), rs45451599 (MAF 0.6%), rs45574135 (MAF unknown), rs186662631 (MAF 0.2%), rs191876689 (MAF <0.01), rs45445997 (MAF 0.2%), rs1537515 (MAF 8.4%), rs1537516 (MAF 8.4%) and rs182398073 (MAF 0.5%) were candidate SNPs. A small cohort of samples including 50 PTB cases and 50 controls were genotyped. Only three SNPs loci, rs1537516, rs1537515 and rs45451599 showed heterozygotic or homozygotic genotypes. So these three SNPs were finally selected for further study.

Genotyping

To understand the potential role of SNPs in genes involved in folate metabolism in PTB, three candidate SNPs in *MTHFR* were genotyped using the SNaPshot method. The protocol for the SNaPshot method has been published elsewhere and was used with some modifications (23,24). The primers were synthesized by the Shanghai Genearray Company in China. The primers were synthesized by the Genearray Company (Shanghai, China).

Statistics analysis

The χ^2 test was performed, using SPSS software (17.0) for Windows. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated when $P < 0.05$ was considered statistically significant.

Results

We screened three candidate SNPs (rs45451599,

Table 1 ORs and 95% CIs for 3 polymorphisms in PTB babies and in-term control

General information/genotypes	Preterm delivery (N=480), n (%)	Term delivery (N=655), n (%)	OR	95% CI	P values
Rs45451599					
AA	471 (98.1)	639 (97.6)	–	–	–
AG [#]	9 (1.9)	16 (2.6)	0.76	0.33-1.74	0.52
G ^{##}	0 (0.0)	0 (0.0)	–	–	–
AG + GG	9 (1.9)	16 (2.6)	0.76	0.33-1.74	0.52
Rs1537516					
CC	418 (87.1)	534 (81.5)	–	–	–
CT [#]	61 (12.7)	117 (17.9)	0.67	0.47-0.93	0.017
TT ^{##}	1 (0.2)	4 (0.6)	–	–	–
CT + TT	62 (12.9)	121 (18.5)	0.65	0.46-0.91	0.012
Rs1537515					
GG	418 (87.1)	534 (81.5)	–	–	–
GT [#]	61 (12.7)	117 (17.9)	0.67	0.47-0.93	0.017
TT ^{##}	1 (0.2)	4 (0.6)	–	–	–
GT + TT	62 (12.9)	121 (18.5)	0.65	0.46-0.91	0.012

[#], heterozygotes; ^{##}, homozygotes; OR, odd ratio; CI, confidence intervals; PTB, preterm birth.

rs1537515 and rs1537516) at 3'-UTR of *MTHFR* to determine their association with PTB in 480 PTB babies and 655 term babies using the SNaPshot method. All SNaPshot extension primers were designed in the reverse orientation of NCBI Reference Sequence NC_000001.10. Mutations are shown in B and C marked with an asterisk. There were 281 male and 199 female infants in the PTB group, and 379 male and 276 female infants in the control group. The P value of infants' gender was 0.842. The genotype frequencies of the three SNPs were in agreement with Hardy-Weinberg equilibrium. Data regarding genotype distributions of three SNPs and χ^2 analysis is shown in *Table 1*. There are mutation frequencies in two SNPs (rs1537515 and rs1537516) showed significant differences between PTB group and control group, 17.9% (117/655) GT heterozygote of rs1537515 were found in the control group, which was significantly higher than that in the PTB group [12.7% (61/480)], suggesting that GT heterozygote of rs1537515 may be a protective factor for PTB (OR: 0.65; 95% CI, 0.46-0.91; and P=0.012). Similarly, CT heterozygote of rs1537516 may be a protective factor for PTB. However, we found genotype distribution of rs45451599 showed no significant differences between the PTB group and the control group, although the AG heterozygote of rs45451599 was 2.6% in the control group, higher than

that in the preterm group (1.9%), which suggested that this SNP has no correlation with PTB.

Furthermore, we also found a consistent protection effect against preterm from the T allele of rs1537516 and rs1537515, compared to the G allele of rs1537515 and C allele of rs1537516 in 34-37 week babies group (per allele OR: 0.677; 95% CI, 0.49-0.94; P=0.021) (*Table 2*). However, in <32-week group and 32-33-week group, we found no significant correlation between T allele of rs1537516 and rs1537515 with preterm, P>0.05. This contradiction could be explained by the different factors for different gestational age preterm.

Discussion

It is reported that preconception supplementation of folic acid can make spontaneous PTB 50-70% reduction in the study of 34,480 pregnant women (8). But its potential pathogenic mechanism is unknown. The molecular epidemiological studies have confirmed that SNPs in folic acid metabolism related genes lead to reduction of the enzyme activity and thus explain the individuals carrying the SNPs with susceptibility to PTB. *MTHFR* is a key enzyme for intracellular folate homeostasis and metabolism. It is reported that two common *MTHFR* polymorphisms, *MTHFR*-677C > T and *MTHFR*-1298A > C, may be

Table 2 Allele frequencies comparison of the two SNP sites

Gestational age	Allele	Preterm (%)	Control (%)	P	OR	95% CI
<32 weeks	Wild	30 (93.75)	1,185 (90.46)	0.530	0.632	0.15-2.68
	Mutation	2 (6.25)	125 (9.54)			
32-34 weeks	Wild	111 (94.07)	1,185 (90.46)	0.195	0.598	0.27-1.31
	Mutation	7 (5.93)	125 (9.54)			
34-37 weeks	Wild	756 (93.33)	1,185 (90.46)	0.021	0.677	0.49-0.94
	Mutation	54 (5.67)	125 (9.54)			
Total	Wild	897 (93.44)	1,185 (90.46)	0.011	0.666	0.49-0.91
	Mutation	63 (5.56)	125 (9.54)			

SNP, single nucleotide polymorphism; OR, odd ratio; CI, confidence intervals.

associated with PTB by decreasing the production of the enzyme of *MTHFR* (12,15). However, these studies focused on SNPs in the coding region and non-coding region have been largely ignored.

It has been proposed that 3'-UTR sequence is recognized via transcriptional and post-transcriptional regulatory pathways. This regulatory region plays an important role in the control of genes expressions. Many recent studies on 3'-UTR function have been reported, and SNPs at 3'-UTR are predicted to play roles in gene expression (25,26) and susceptibility of diseases (27-31), especially in CHD (22). As CHD and PTB shares common folic acid metabolism pathways, it is necessary to research PTB and 3'-UTR. But to our knowledge, there is few articles focusing on the role of *MTHFR* 3'-UTR in PTB. In that sense, it is necessary to extend from *MTHFR* coding region to non-coding area. In this article, we analyzed the association between three SNPs locating at 3'-UTR in *MTHFR* and PTB.

The 3'-UTR of *MTHFR* has solid predictions for extremely conserved miRNA binding sites, so SNPs in the miRNA target sites may have influence on the gene expression (32). rs1537515 is one of the SNPs in the miRNA target sites, locating in the binding sites of hsa-miR-1224-3p and miR-3150-5p according to software predictions. Based on its special position, rs1537515 attracts us to research its effect on PTB, although there is no study on the association between those miRNAs and *MTHFR* expression. Firstly, we analyzed genotyping distributions of rs1537515 in Han Chinese population, and the results revealed that the genotyping distributions of rs1537515 were GG 81.5%, GT 17.9% and TT 0.6%. The frequencies of the heterozygous genotype GT of rs1537515 was 12.7% in PTB group, significantly lower than 17.9% in control group. The results indicated that

GT of rs1537515 was a protective genetic factor for PTB. As expected, the comparison of allele frequencies showed that wild allele played the leading role. Although, it's unknown that how to protect from PTB by GT of rs1537515, it gives a new clue to the study of genetic susceptibility to PTB.

Rs1537516 is another SNP in the miRNA target sites, locating at the binding sites of hsa-miR-1304-3p according to prediction. Rs1537516 is 40bp distance from rs1537515. Due to perfectly correlate with rs1537515, it's proposed that rs1537516 and rs1537515 have the similar regulatory function on *MTHFR* expression. Indeed, similarly to rs1537515, the frequencies of the heterozygous genotype CT of rs1537516 was 12.7% in PTB group, significantly lower than 17.9% in control group. Our study revealed that CT of rs1537516 was a protective genetic factor for PTB. Same with rs1537516, the comparison of allele frequencies of this site played the leading role in PTB.

Rs45451599 is in miR-24 binding site, however, no significant differences in rs45451599 genotypes were observed among the PTB babies (9/480, 1.9%) and controls (16/655, 2.6%). This may be the result of the small number of AG variants in this case-control study in Han Chinese populations, reducing the power necessary for detectable differences. However, further studies should be performed in different ethnic groups to confirm this result.

Conclusions

In conclusion, this research reveals an association between SNPs at 3'-UTR of *MTHFR* gene in folate metabolic pathways which may affect the binding capacity of hsa-miR-1304-3p, miR-1224-3p and miR-3150-5p and subsequently increases folate pools and involves in the prevention of

PTB. But further functional study should be done for confirmation the significance of these two SNPs in folate metabolic pathways.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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