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RESEARCH ARTICLE

Variants in *MTHFR* gene and neural tube defects susceptibility in China

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Abstract Neural tube defect (NTD) is a severe congenital birth abnormalities involving incomplete neural tube closure. 5, 10-methylenetetrahydrofolate reductase (*MTHFR*) gene plays key role in folate cycle and methylation cycle, which could affect the DNA synthesis, repair and methylation. In this study, we aim to investigate the correlation between *MTHFR* polymorphisms and NTD-affected pregnancy. There were 444 participants involved in our study. Tag-SNPs were identified in HapMap Databases. Blood samples were collected from all subjects to further extract the genomic DNAs by TaqMan Blood DNA kits. We also carried out a meta-analysis based on previous published studies to further examine the association between *MTHFR* polymorphisms and NTD. In case–control study analysis, two SNPs were identified to be associated with NTD risk. The 677 C > T genetic variant was correlated with increased risk of NTD-affected pregnancy. However, the 1298 A > C polymorphism was shown to lower the risk of NTD-affected pregnancy. The protective role of 1298 A > C polymorphisms was further supported by the result of meta-analysis. Our study revealed that the SNPs of 677C > T and 1298A > C in *MTHFR* were associated with NTD-affected pregnancy, in which 677C > T was a risk factor and in contrast 1298A > C was protective factor against NTD. Our results of meta-analysis also revealed the 1298A > C *MTHFR* polymorphism play protective role in NTD.

Keywords Neural tube defects · 5, 10-methylenetetrahydrofolate reductase · Polymorphism · Folic acid cycle

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Abbreviations

NTD	Neural tube defect
MTHFR	Methylenetetrahydrofolate reductase

Introduction

Birth defects are the major health threat and leading cause of death in infants under 1 year old. Neural tube defect (NTD) is one of severe congenital birth malformations involving incomplete development of the brain, spinal cord (Obeid et al. 2013), which occurs around the 28th day after conception (Liu et al. 2014a). NTD arises from failure of neural tube closure during the neurulation (Qi et al. 2014). The three main types of NTD include spina bifida, anencephaly and encephalocele. The incidence rate has been reported to be approximately 1 in 1000 newborns worldwide, and in China, NTD accounts for 20 to 25 % of birth defects (Zhang et al. 2013).

Previous studies have revealed that both environmental and genetic factors contribute to the pathogenesis of NTD (Li et al. 2011; Zhang et al. 2013). Though the precise mechanism is still unclear now, it was reported that NTD have association with the deficiency of folic acid (Welch 1983), a kind of water-soluble vitamin B, which is a coenzyme in one-carbon transfer reactions and plays important role in DNA synthesis and repair (Qi et al. 2014). Furthermore, the maternal supplement of folic acid can notably help prevent NTD, reducing the incidence rate by 50–75 % only if the pregnant women were given recommended doses ranging from 0.36 to 4 mg before and during the pregnancy (Berry et al. 1999). It is hypothesized that genetic variations in relation to folic acid metabolism contribute to the risk of NTD.

Some studies indicate that altered folic acid metabolism pathway may lead to NTD (Yates et al. 1987; van der Put

et al. 1997). Folic acid is initially transformed into a bioactive molecule in vivo, tetrahydrofolate, and then accomplishes the methylation cycle before participating in the folic acid metabolism. A variety of enzymes are involved in this pathway, such as the 5, 10-methylenetetrahydrofolate reductase (*MTHFR*), the methionine sythase reductase (*MTRR*) and vitamin B12-dependent methionine synthase (*MTR*) (Zhang et al. 2013), and in this paper, we mainly focus on *MTHFR*. *MTHFR* catalyzes the transformation from 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which serves as a methyl donor for the conversion of homocysteine to methionine (Liu et al. 2013). The *MTHFR* gene is located at chromosome 1p36.3 and is 2.2 kb in length with a total of 11 exons (Qiu et al. 2014). It is investigated that the single nucleotide polymorphisms (SNPs) of *MTHFR* gene have a strong association with NTD (Kirke et al. 2004; Carter et al. 2011; Pangilinan et al. 2012). Taking the most common polymorphism (677C > T) for instance, the 677C > T mutation substitutes the valine for the alanine at amino acid 222, which leads to thermolability and reduced activity of encoding enzyme in low level of folic acid (Lacasana et al. 2012). Other *MTHFR* polymorphisms may also affect central nervous system development/function. For example, *MTHFR* 1129C > T polymorphism can cause irreversible brain damage and mental retardation if not recognizes early in life (Strauss et al. 2007). The tag-SNPs of *MTHFR* were retrieved in our research, including rs2274976 (1781G > A), rs1801131 (1298A > C), rs4846048 (4485C > T) and rs1801133 (677C > T), from the HapMap database based on Han Chinese population. We aim to perform a case–control study to evaluate the correlation between the maternal *MTHFR* polymorphisms and NTD. Moreover, the previous studies on the association between *MTHFR* polymorphisms and NTD susceptibility in Asian ethnicity were identified to conduct a meta-analysis in order to comprehensively assess the impact of *MTHFR* polymorphisms on NTD.

Material and methods

Ethics statement

Our research was approved by Ethics Committee of First Affiliated Hospital of Xinjiang Medical University with written informed consents signed by all the participants (or their guardians) involved in this study.

Study population

A total of 444 participants, 144 NTD cases and 300 healthy controls, were recruited from the First Affiliated Hospital of Xinjiang Medical University, from May 2010 to June 2014. The 144 NTD cases were enrolled from the women that gave birth to infants with NTD. The women that gave birth to healthy infants were selected as controls. After reviewing the medical records of all subjects, we summarized a series of clinical characteristics of NTD cases and controls during the pregnancy. Based on written informed consents, we collected 5 ml venous blood specimen from each subject for DNA extraction and genotyping.

SNP selection

In this research, tag-SNPs of *MTHFR* were identified in CHB from International HapMap project databases (Hapmap Data Rel 24/Phase II Nov08, on NCBI B36, dbSNP b126) The retrieved SNP genotype data were further analyzed by Haploview 4.2 software (Broad Institute, Cambridge, MA, USA) with r^2 of 0.8 as a threshold and minor allele frequency greater than 0.05. The selected tag-SNPs by Haploview represent all the genetic variants of *MTHFR*.

DNA extraction and genotyping

We extracted the genomic DNAs with Qiagen DNA blood kit (Qiagen, Hilden, Germany) from whole blood samples acquired from participants, in accordance with manufacturer's instructions. The genotyping of SNPs were performed on Sequenom MassArray iPLEX platform (Sequenom Inc., San Diego, CA, USA) with matrix-assisted laser desorption ionizaiotn time of flight (MALDI-TOF) mass spectrometry. The design of PCR primers for amplification and extension were carried out with MassARRAY Assay Design Version 3.1 (Sequenom Inc., San Diego, CA, USA). PCR amplification was implemented in 5 μl volume with 10 ng genomic DNA, 100nM dNTP mix, 100nM each designed primers, 10×PCR buffer, 25 mM $MgCl_2$ and 5 U Taq DNA Polymerase. The reaction mixture were incubated at 94 °C for 15 min, then subjected to 45 cycles of denaturation (94 °C for 20 s), annealing (56 °C for 20 s), and extension (72 °C for 60 s), and terminated at 72 °C for 3 min. 1.7 U Shrimp alkaline phosphatase (SAP) was added to remove unincorporated dNTPs. Subsequently, the plate were placed at 37 °C for 20 min and then at 85 °C for 5 min. The PCR amplification products were mixed with iPLEX reaction mix components, which contained 10×iPLEX Buffer Plus, iPLEX Termination Mix, Primer mix and iPLEX enzyme (Table 1). The iPLEX reaction mix were initially denatured at 94 °C for 30 s, followed by 40 cycles at 94 °C for 5 s, [52 °C for 5 s and 80 °C for 5 s (repeated 5 times per cycle)]; and finally terminated at 72 °C for 3 min. After the resin treatment, the samples were spotted on a SpectroCHIP (Sequenom, Inc., San Diego, CA) and further measured by mass spectrometer. The data were collected and analyzed by SpectroTYPER 4.0 software (Sequenom, Inc., San Diego, CA).

Table 1 Primers of *MTHFR* gene polymorphisms for PCR amplification

Rs number	Position	Alias name	Primers for PCR amplification
rs2274976	Exon11	1781G > A	F: 5′-CATCCAGCCCACCGTAGT-3′
			R: 3′-ATGTAGGTGCTGTTGATGAAGG-5′
rs1801131	Exon7	1298A > C	F: 5′-TAGCCAATCCCTTGTCTCAA-3′
			R: 3′-TTCACTCACTACGACCTCACC-5′
rs4846048	3′UTR	4485C > T	F: 5′-GGCTGAAGCAGAGGAGTG-3′
			R: 3′-GGGATTCTAGTTGTCCGTA-5′
rs1801133	Exon4	677C > T	F: 5′- TCCCTATTGGCAGGTTAC-3′
			R: 3′-AGAAAGGGTAGGTCCACT-5′

F forward, *R* reverse

Statistical analysis

All statistical analyses were performed with SPSS 20.0 software. The *P* value less than 0.05 was considered as statistically significant. The student's *t* test and chi-square test were conducted to assess the difference in the clinical characteristics between NTD cases and controls. The genetic models, including homozygous comparison (MM vs. WW), dominant model (WM + MM vs. WW) and allele model (M vs. W), were established to compare the distribution of genotype and allele frequencies between NTD cases and healthy controls. Odds ratios (OR) with 95 % confidence intervals were used to the association between *MTHFR* polymorphisms and NTD susceptibility. In meta-analysis, heterogeneity between studies was analyzed by I^2 and Q-test. The fixed effect model was applied when I^2 was less than 0.05 and *P* value of Q-test was more than 0.05; otherwise, the random effects models should be implemented. Funnel plot were used to evaluate the publication bias..

Table 2 Characteristics of NTD mothers and controls

Maternal characteristics	NTD mother (*N*=144)	Control mother (*N*=300)	*P* value
Age (years) (n, %)			
≤20	37 (25.7 %)	62 (20.7 %)	0.482
20–30	102 (70.8 %)	228 (76.0 %)	
≥30	5 (3.5 %)	10 (3.3 %)	
Mean age ± SD	24.2±2.9	23.7±3.3	0.121
Gestational weeks (n, %)			
≤28	21 (14.6 %)	2 (0.7 %)	<0.001
28–37	82 (56.9 %)	205 (68.3 %)	
≥38	51 (35.5 %)	93 (31.0 %)	
Reproductive history (n, %)			
Primiparity	45 (31.3 %)	176 (58.7 %)	<0.001
Multiparity without defect offspring	82 (56.9 %)	115 (38.3 %)	
Multiparity with defect offspring	17 (11.8 %)	9 (3.0 %)	
Dietary habit (n, %)			
Vegetarian	45 (31.3 %)	67 (22.3 %)	0.043
Non-vegetarian	99 (68.7 %)	233 (77.7 %)	
Folate level (nM)	26.8±4.2	34.9±5.7	<0.001
Homocysteine level (μM)	10.9±1.7	10.7±1.6	0.228
Intake of folic acid (n, %)			
≥5 mg/day	31 (21.5 %)	98 (32.7 %)	0.016
<5 mg/day	113 (78.4 %)	202 (67.3 %)	
Hyperthermia during early pregnancy (n, %)			
Yes	15 (10.4 %)	22 (7.3 %)	0.271
No	129 (89.6 %)	278 (92.7 %)	
Medication during early pregnancy (n, %)			
Yes	17 (11.8 %)	19 (6.3 %)	0.048
No	127 (88.2 %)	281 (93.7 %)	
Alcohol drinking during pregnancy (n, %)			
Yes	7 (4.9 %)	26 (8.7 %)	0.152
No	137 (95.1 %)	274 (91.3 %)	
Tobacco smoking during pregnancy (n, %)			
Yes	3 (2.1 %)	14 (4.7 %)	0.184
No	141 (97.9 %)	286 (95.3 %)	

NTD neural tube defect, *SD* standard deviation

Result

Clinical characteristics of NTD cases and health control

In our study, 444 participants consisted of 144 NTD cases and healthy controls were recruited. The clinical characteristics were presented in Table 2. There was no significant difference in age between two groups. The age of participants mostly range from 20 to 30 in both groups. The average age in NTD cases and healthy controls were 24.2±2.9 and 23.7±3.3, respectively. Importantly, we find a significantly higher proportion of premature delivery in NTD cases compared with healthy controls ($P<0.001$). Concerning the reproductive history, more than half of healthy controls were primiparity. In contrast, over half of women in NTD group have previous multiparity without defect offspring. Compared with healthy control, the NTD cases group appeared to have higher proportion of previous multiparity with defected offsprings ($P<0.001$). In addition, over 30 % of women in NTD group were vegetarians, and the proportion of vegetarians in NTD cases was significantly higher than healthy controls ($P=0.043$). Moreover, lower levels of folate were detected in

NTD cases compared with healthy controls ($P<0.001$). The women giving birth to NTD offsprings were more likely to have daily intake of folic acid less than 5 mg ($P=0.016$). Furthermore, approximately 12 % of NTD cases were reported to have medication during early pregnancy, of which the proportion was higher than that in healthy controls. Besides, no significant difference in hyperthermia, alcohol drinking, tobacco smoking during early pregnancy and homocysteine level was observed between NTD cases and healthy controls.

Selection of tag-SNPs

Sixteen CHB-based tag-SNPs were retrieved from HapMap databases, with MAF>0.05 and $r^2=0.08$ as a threshold. As shown in the linkage disequilibrium of the sixteen tag-SNPs (Fig. 1), four tag-SNPs (rs4846048, rs2274976, rs1801131 and rs1801133) were identified by Haploview 4.0 (Broad Institute, Cambridge, MA) to represent all polymorphisms of *MTHFR*. As shown in Fig. 2, the SNPs of rs1801133, rs1801131 and rs2274976 were located in the exons, whereas the SNP of rs4846048 was situated in the 3′ untranslated region.

Association between *MTHFR* polymorphism in pregnant women and NTD susceptibility in fetus

The distribution of genotypes and alleles frequencies was presented in Table 3. We found a significant association between *MTHFR* polymorphisms and NTD risk in the 677C > T SNP. In homozygous comparison (TT vs. CC), the NTD cases have higher frequency of TT genotypes than healthy controls (OR= 2.75; 95 % CI: 1.53–4.95; $P=0.001$). Under the dominant model (CT + TT vs. CC), CT and TT carriers were associated with higher risk of conceiving NTD infants compared with pregnant women with CC wild type (OR=1.71; 95 % CI: 1.08–2.73; $P=0.022$). Under allele model (T vs. C), T allele was shown to be a risk factor of conceiving NTD infants for pregnant women (OR=1.60; 95 % CI: 1.20–2.12; $P=0.001$).

In contrast, the mutation of 1298A > C was associated with lower risk of being pregnant with NTD infants. In homozygous comparison (CC vs. AA), pregnant women with AA wild type have a higher risk of conceiving NTD infants compared with healthy controls (OR=0.48; 95 % CI: 0.24–0.95; $P=0.031$). Under the dominant model (AC + CC vs. AA), AA wild types were associated with higher risk of having NTD infants compared with AC and CC mutants. Under the allele model, A alleles carried by pregnant women were shown to be correlated with higher risk of NTD susceptibility in fetus.

As for rs2274976 and rs4846048 polymorphisms, both allelic as well as genotypic frequencies were similar between case and control mothers (all $P>0.05$).

Meta-analysis of the correlation between *MTHFR* polymorphisms in Asian pregnant women and NTD fetus risk

Due to the limited size of our samples, we performed a meta-analysis of correlation between *MTHFR* polymorphisms (677C > T and 1298A > C) in Asian pregnant women and

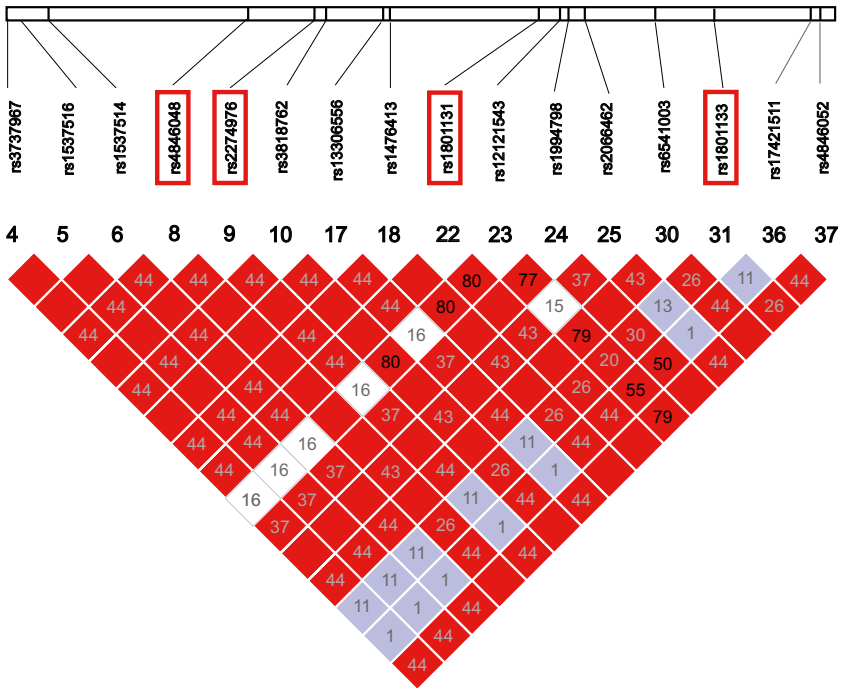

Fig. 1 Linkage disequilibrium of the 16 tag-SNPs

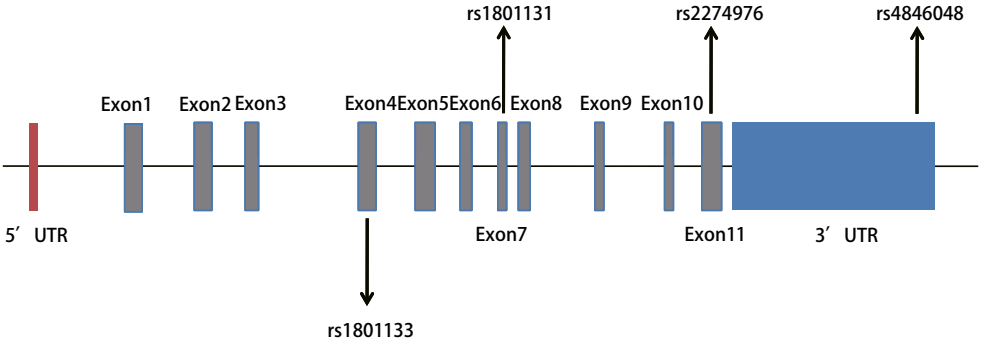

Fig. 2 Genetic locations of selected 4 tag-SNPs

NTD fetus risk based on previous studies. Eleven eligible case–control studies were identified according the inclusion criteria (Lee et al. 2000; Yu et al. 2000; Sadewa et al. 2004; Dalal et al. 2007; Shang et al. 2008; Harisha et al. 2010; Naushad and Devi 2010; Deb et al. 2011; Godbole et al. 2011; Kondo et al. 2014; Liu et al. 2014b). The basic information of the eleven studies was presented in Table 4. In this meta-analysis, NTD cases can be divided into two categories, upper (anencephaly and occipital encephalocele) and lower defects (thoracolumbar or lumbosacral spina bifida). The data extracted from the 11 studies were summarized in Table 5.

Concerning the 677C > T polymorphisms, we found no significant difference in genotype distribution between total NTD cases and healthy controls under the five genetic models. Subtype analysis revealed no association between the upper or lower NTD cases and 677C > T SNPs as well. However, 1298A > C polymorphisms were significantly associated with lower risk of conceiving NTD infants in Asian pregnant women under five genetic models (all $P<0.01$). But there was also no apparent difference observed in genotypes distribution between NTD subtype cases and healthy controls.

Table 3 Maternal *MTHFR* polymorphisms and NTDs risk in the offspring

Genotype	NTD mother (N=144)	Control mother (N=300)	OR (95 % CI)	P value
rs1801133 (677C > T)				
CC	31	96	Ref.	
TT	40	45	2.75 (1.53–4.95)	**0.001**
CT + TT	113	204	1.71 (1.08–2.73)	**0.022**
C allele	135	351	Ref.	
T allele	153	249	1.60 (1.20–2.12)	**0.001**
rs1801131 (1298A > C)				
AA	76	126	Ref.	
CC	13	45	0.48 (0.24–0.95)	**0.031**
AC + CC	68	174	0.65 (0.44–0.97)	**0.033**
A allele	207	381	Ref.	
C allele	81	219	0.68 (0.50–0.92)	**0.014**
rs2274976 (1781G > A)				
GG	88	177	Ref.	
AA	20	38	1.06 (0.58–1.93)	0.852
AA + GA	56	123	0.92 (0.61–1.38)	0.672
G allele	212	439	Ref.	
A allele	76	161	0.98 (0.71–1.34)	0.889
rs4846048 (4485C > T)				
CC	72	133	Ref.	
TT	17	43	0.73 (0.39–1.37)	0.327
TT + TC	72	167	0.80 (0.53–1.19)	0.262
C allele	199	390	Ref.	
T allele	89	210	0.83 (0.61–1.12)	0.227

NTD neural tube defects, *OR* odds ratio, *CI* confidence interval

Significant values are highlighted

Table 4 Included studies that examined risk of NTDs in relation to 677C > T and 1298A > C

First author	Year	Country	Study population	NTD children outcomes	Sample size		Studied SNP	Rs number	OR (95 % CI) (Allelic model)
					Case	Control			
Lee BH	2000	Korea	NTD's mother	Meningomyelocele	30	43	C677T	rs1801133	0.60 (0.30–1.19)
Yu J	2000	China	NTD's mother	NTD cases	42	24	C677T	rs1801133	2.24 (1.09–4.62)
Sadewa AH	2004	Indonesia	NTD's mother	Encephalocele	8	47	C677T	rs1801133	1.54 (0.29–7.99)
							A1298C	rs1801131	1.16 (0.39–3.48)
Dalal A	2007	India	NTD's mother	Spina bifida	83	60	C677T	rs1801133	1.41 (0.75–2.64)
							A1298C	rs1801131	0.71 (0.43–1.17)
Shang Y	2008	China	NTD's mother	Spina bifida	38	80	C677T	rs1801133	0.71 (0.41–1.25)
Harisha PN	2010	India	NTD's mother	Encephalocele, meningomyelocele, spina bifida, etc.	45	102	C677T	rs1801133	2.16 (1.01–4.65)
Naushad SM	2010	South India	NTD's mother	Anencephaly, encephalocele,	50	80	C677T	rs1801133	2.69 (1.34–5.39)
				meningocele, meningomyelocele			A1298C	rs1801131	0.61 (0.36–1.47)
Deb R	2011	North India	NTD's mother	Anencephaly, encephalocele, myelomeningocele, spina bifida	111	222	C677T	rs1801133	0.88 (0.58–1.35)
Godbole K	2011	India	NTD's mother	Anencephaly, encephalocele,	305	684	C677T	rs1801133	0.96 (0.71–1.28)
				spina bifida, spinal dysraphism			A1298C	rs1801131	0.79 (0.65–0.97)
Kondo A	2014	Japan	NTD's mother	Spina bifida	115	4517	C677T	rs1801133	0.86 (0.66–1.14)
Liu J	2014	China	NTD's mother	Anencephaly, encephalocele,	573	572	C677T	rs1801133	1.16 (0.98–1.37)
				spina bifida			A1298C	rs1801131	0.91 (0.72–1.15)
Current study	2014	China	NTD's mother	Anencephaly, encephalocele,	144	300	C677T	rs1801133	1.60 (1.20–2.12)
				spina bifida, spinal dysraphism			A1298C	rs1801131	0.68 (0.50–0.92)

Table 5 Meta-analysis results of the association between the *MTHFR* polymorphisms and NTD risk

SNP/Groups	M allele vs. W allele (Allelic model)				WM + MM vs. WW (Dominant model)				MM vs. WW + WM (Recessive model)				MM vs. WW (Homozygous model)				WM vs. WW (Heterozygous model)			
	OR	95%CI	*P*	P_h	OR	95%CI	*P*	P_h	OR	95%CI	*P*	P_h	OR	95%CI	*P*	P_h	OR	95%CI	*P*	P_h
677C > T																				
Total	1.17	0.95–1.44	0.135	0.001	1.16	0.91–1.48	0.229	0.023	1.36	0.90–2.02	0.143	0.013	1.48	0.90–2.45	0.121	0.003	1.08	0.92–1.26	0.351	0.187
Upper	0.88	0.58–1.37	0.584	0.098	0.87	0.43–1.74	0.689	0.042	1.05	0.79–1.40	0.730	0.688	1.26	0.86–1.84	0.235	0.276	1.18	0.85–1.63	0.316	0.072
Lower	1.02	0.68–1.54	0.914	0.006	1.08	0.68–1.73	0.740	0.045	1.02	0.78–1.32	0.898	0.088	1.04	0.75–1.43	0.827	0.092	01.11	0.86–1.43	0.442	0.266
1298A > C																				
Total	0.79	0.70–0.90	**0.001**	0.567	0.76	0.65–0.90	0.001	0.790	0.68	0.51–0.90	0.008	0.385	0.60	0.45–0.82	**0.001**	0.290	0.79	0.67–0.94	0.009	0.964
Upper	0.93	0.72–1.21	0.605	0.904	0.88	0.65–1.19	0.404	0.729	1.31	0.59–2.93	0.509	0.527	1.39	0.60–3.23	0.438	0.685	0.85	0.62–1.15	0.284	0.601
Lower	0.82	0.63–1.07	0.141	0.174	0.81	0.60–1.09	0.162	0.239	0.64	0.25–1.67	0.360	0.153	0.58	0.22–1.53	0.270	0.106	0.82	0.60–1.11	0.192	0.415

P_h: *P* value of heterogeneity test; Upper: anencephaly and occipital encephalocele; Lower: thoracolumbar and lumbosacral spina bifida; *OR* odds ratio, *CI* confidence interval
Significant values are highlighted.

Discussion

In this study, we focused on the association between polymorphisms of *MTHFR* and NTD fetus risk in Chinese pregnant women. A total of 444 subjects including 144 NTD cases and 300 healthy controls were enrolled in our study. Comparing the clinical characteristics between NTD cases and healthy controls, there were significant differences in the gestational weeks, reproductive history, dietary habit, folate level, daily intake of folic acid and medication during early pregnancy between two groups, which suggested that these factors may contribute to the occurrence of NTD in fetus. Meanwhile, our study identified two selected tag-SNPs (rs1801133 and rs1801131) were associated with NTD fetus risk in Chinese pregnant women. The genetic variants of rs1801133 were risk factors for NTD fetus, and oppositely the polymorphisms of rs1801131 were protective factors against NTD fetus. In addition, our meta-analysis indicated that the SNPs of rs1801131 may lower the risk of conceiving NTD fetus in Asian pregnant women in comparison to the wild type of rs1801131.

In previous studies, the multiparity was considered as one of the risk factors for NTD (Bianca et al. 2002). Consistent with previous studies, the women giving birth to NTD infants were more likely to be multiparous than healthy controls in our study. NTD were more common in premature infants than in term infants (Honein et al. 2009). Our study indicated that preterm delivery rate is higher in NTD cases than in healthy controls. A number of studies have already demonstrated that the periconceptional folic acid supplementation can substantially lower the risk of NTD-affected pregnancies (Werler et al. 1993). Folic acid is metabolized into bioactive form folate (tetrahydrofolate) in vivo. Folate was essential for DNA synthesis, repair and methylation (Simpson et al. 2010), especially important for the rapid cell division and proliferation in embryo. In our results, we demonstrated the NTD cases have lower folate level and daily intake of folic acid. Vegetarian diet is known to be deficient in vitamin B12, which may lead to the imbalance of methylation cycle and affect the DNA synthesis (Fig. 3) (Weiss et al. 2004). Hence, the pregnant women with vegetarian diet may be at higher risk of NTD-affected pregnancy (Refsum 2001). Periconceptional medication use has been associated with increased NTD risk in fetus, such as valproic acid for epilepsy (Lindhout and Schmidt 1986), ovulation-inducing medication (Greenland and Ackerman 1995), oral contraceptives (Kasan and Andrews 1980). Our data also revealed a higher proportion of NTD cases have vegetarian diet and periconceptional medication use than normal controls.

As shown in Fig. 3, *MTHFR* play crucial role in the conversion from 5, 10 methylene THF to 5 methyl THF, which subsequently affects the conversion from homocysteine to methionine and lead to low level of methionine. Animal studies suggested the methionine is required for neural tube

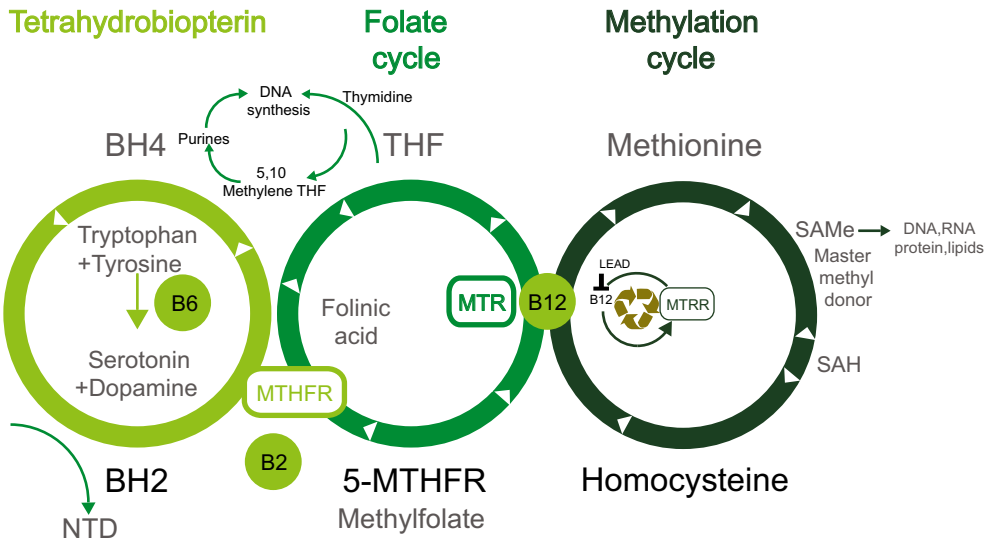

Fig. 3 Schematic diagram of folate and methylation cycle

closure in rat embryos (Coelho et al. 1989). Supplementation of methionine can substantially reduce the occurrence of NTD in Axd mice, which are prone to develop NTD (Essien and Wannberg 1993). Therefore, the conversion from homocysteine to methionine could be a critical step for neural tube closure. The mutation in *MTHFR* may affect the methionine level and contribute to the development of NTD. The 677 C > T mutation in *MTHFR* was first reported in 1995 and the *MTHFR* mutants encode the thermolabile form of *MTHFR* with reduced enzyme activity (van der Put et al. 1995). A number of studies have investigated the association between the 677 C > T polymorphisms of *MTHFR* and NTD. However, there was no consistent conclusion based on previous studies. Mornet et al. reported no evident association between 677 C > T variants of *MTHFR* and NTD (Mornet et al. 1997). De Villarreal et al. indicated that the 677 C > T mutation in *MTHFR* is important risk factor for NTD (Martinez de Villarreal et al. 2001). The inconsistent results may be attributed to many factors, such as sample size, ethnicity etc. In our study, we found that the 677 C > T polymorphisms were significantly associated with increased risk of NTD offspring. In order to comprehensively evaluate the impact of *MTHFR* polymorphisms on NTD-affected pregnancies, we performed a meta-analysis based on previous studies. It showed no significant association between 677 C > T polymorphisms and NTD in Asian ethnicity. The 1298 A > C mutation is another common genetic variation in *MTHFR*, which also result in the reduction of enzyme activity to lesser extent (van der Put et al. 1998). Van der Put det al. showed that the 1298 A > C may play a minor role in NTD susceptibility and the relative NTD risk of 1298 A > C mutation is smaller than the 677 C > T. Several studies suggested that the heterozygous and homozygous genotypes of 1298 A > C in *MTHFR* are associated with NTD (De Marco et al. 2002; Boduroglu et al. 2005). Nonetheless, some published studies were unable to find the association between 1298 A > C mutation and NTD (Stegmann et al. 1999; Volcik et al. 2000; Richter et al. 2001; Cunha et al. 2002). Interestingly, other reports revealed that the 1298 A > C polymorphisms were associated with lower risk of NTD. In our study, we find the 1298 A > C polymorphism may serve as protective factors in Asian women against the NTD-affected pregnancy. There are probably a number of factors that may contribute to the conflicting conclusions between studies, such as sample size, different selection criteria, ethnicity, etc. In our study, we showed that the 1298 A > C polymorphisms served as protective factors against NTD-affected pregnancy. Our results of meta-analysis based on previous studies also confirm the protective role of A > C polymorphisms in NTD.

In conclusion, our study revealed that the SNPs of 677 C > T and 1298 A > C in *MTHFR* were associated with NTD-affected pregnancy, in which 677 C > T was a risk factor and in contrast 1298 A > C was a protective factor against NTD. The meta-analysis in our study also support the protective role of 1298 A > C, but it failed to find the significant association between 677 C > T and NTD-affected pregnancy.

Competing Interests The authors declare that they have no competing financial interests.

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