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Polymorphisms in *MTHFD1* Gene and Susceptibility to Neural Tube Defects: A Case-Control Study in a Chinese Han Population with Relatively Low Folate Levels

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Background: The polymorphism of methylenetetrahydrofolate dehydrogenase (MTHFD1) has been reported as a risk factor for neural tube defects (NTDs). In the present study, we aimed to investigate whether the single-nucleotide polymorphisms (SNPs) of MTHFD1 gene are associated with NTDs in a Chinese population and to determine their mechanism of action.

Material/Methods: MTHFD1 gene was scanned in a total of 270 NTDs cases and 192 healthy controls by using next-generation sequencing (NGS) method. After quality control procedures, 208 selected SNP sites in MTHFD1 gene were enrolled for follow-up statistical association analyses. Functional analyses were also performed for significant SNPs through bioinformatics analysis. Folic acid levels of brain tissue in available NTDs cases and healthy controls (113 and 123, respectively) were measured. Statistical and bioinformatics analyses were performed to investigate the relationship between SNPs in MTHFD1 and susceptibility to NTDs.

Results: Statistical analysis showed that 2 independent SNPs, rs1956545 and rs56811449, confer the risk of NTDs (P value=0.0195, OR (odds ratio)=1.41, 95% CI (confidence interval)=1.06–1.88; P value=0.0107, OR=0.56, 95% CI=0.36–0.87). The haplotype GGGG, which consists of 4 SNPs (rs2236225, rs2236224, rs1256146, and rs6573559), is also associated with risk of NTDs (P value=0.0438, OR=0.7180, 95% CI=0.5214–0.9888). The risk allele C of rs1956545 is also associated with decreased folic acid levels in the brain (P value=0.0222, standard beta=–0.2238, 95% CI=–0.4128 – –0.0349) according to analysis in the subset of NTDs cases and healthy controls. Bioinformatics analysis indicates that rs1956545 and rs56811449 are within ENCODE regulatory regions, the open chromatin regions of blastula Trophoblast cell line, and histone-marked region of brain astrocyte cell line.

Conclusions: The polymorphism of SNP *loci* rs1956545 and rs56811449 as well as a haplotype in MTHFD1 gene could serve as an indicator for the occurrence of NTDs in Chinese population and some specific genotypes of the *loci* may have lower risk of developing NTDs.

MeSH Keywords: Folic Acid Deficiency • Neural Tube Defects • Polymorphism, Single Nucleotide

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Background

Congenital malformations are major factors causing infant mortality in developed countries, and also lead to long-term health problems in those who survive. One of the most common malformations is neural tube defects (NTDs), which are characterized by central nervous system abnormalities, and affect 0.5–2 per 1000 pregnancies worldwide [1]. However, the incidence of NTDs varies dramatically among countries and regions. Shanxi Province, located in Northern China, has reported the highest birth prevalence of NTDs in the world [2].

NTDs arise during the embryogenesis process when the closure of neural tube is disrupted [3]. Generally, NTDs encompass kinds of morphologically distinct malformations. Most defects of NTDs are referred to as open NTDs, while a number of closed or skin-covered conditions have also been reported. For open NTDs, categories can fall into 3 general types: anencephaly, which is characterized by absence of the cranial vault and severe defects in the cerebral hemispheres; spina bifida (meningomyelocele), which is characterized by defects in the neural arches; and craniorachischisis, which is characterized by failure of neural tube closure [3].

Development of NTDs is a multi-step process controlled by genes, and influenced by a variety of environmental factors. Despite years of intensive epidemiological, clinical, and experimental research, the underlying etiology of NTDs stays unrevealed. Several genes have been reported to be involved in the development of NTDs, including those encoding folate receptors, cystathionine-b-synthase, methionine synthase, methionine synthase reductase, and reduced folate carrier-1 [4–6]. However, most of these studies have not been confirmed and warrant further investigation.

In addition to studies on NTDs susceptibility gene, environmental factors are also a well-known research direction in NTDs. Folic acid is one of the most important environmental factors related to NTDs occurrence. Epidemiologic studies found that periconceptional folate supplementation could greatly reduce a woman's risk of having an NTD baby, by as much as 70% in some populations [7]. Our previous study revealed that low maternal folate serum concentration was related to the increased risk of NTD in a high-risk population in Shanxi province [2]. However, the strong link between folic acid and NTDs, the genetic mechanisms through which folic acid improves neural tube development, remains unknown.

Given the crucial role of folic acid in NTDs, the members catalyzing these reactions has drawn much recent attention; one of the most important gene families is the methylenetetrahydrofolate dehydrogenase (MTHFD1) family in eukaryotes. The first member of this family identified in NTDs was the cytoplasmic MTHFD1 protein, which is a trifunctional protein catalyzing 3 sequential reactions in the interconversion of 1-carbon derivatives of tetrahydrofolate (THF) [8]. According to a previous study, 2 amino acid substitutions identified in *MTHFD1* turned out to be present in patients with NTDs [9], and Brody et al. [10] also reported that polymorphism in 1 locus of *MTHFD1* gene was a maternal risk factor for NTD risk in the Irish population.

Ethnicity and other risk factors, such as, maternal age, smoking, and alcohol drinking, commonly contribute to occurrence of NTDs. The risk of birth defects of Han Chinese was reported to be higher than that of ethnic Mongols [11]. In the present study, we aimed to investigate the polymorphism of *MTHFD1* gene in the Chinese high-risk population of NTDs, and also determined the folate concentrations in brain tissue of NTDs to explore the link between *MTHFD1* gene polymorphism and fetal folate level.

Material and Methods

Patients

We collected 270 stillborn NTD samples at the Capital Institute of Pediatrics (Beijing, China) from January 2002 to December 2004. The prevalence of the NTD in the area was 199.38/10 000, based on the local epidemiologic data. The enrolled samples were diagnosed for NTD with ultrasonography. We collected 192 fetuses aborted for nonmedical reasons in the same area and used them as control participants. The study was approved by the Capital Institute of Pediatrics (Beijing, China) Hospital ethics committee. The ethics committee approved the screening, inspection, and data collection of the patients, and all subjects signed a written informed consent form. All work was undertaken following the provisions of the Declaration of Helsinki.

Determination of the folic acid level in brain tissue

The level of folic acid was determined using a competitive receptor binding immunoassay (Chemiluminescent Immunoenzyme Assay Access Immuno-assay system II: Beckman Coulter, Krefeld, Germany) according to the standard protocol. Briefly, 15 mg of embryonic brain tissue samples was homogenized with 1 ml of extraction buffer (TRIS-buffered saline, A16792, BECKMAN, Germany). The homogenized tissue was ultra-sonicated for 3 min with 10-s ultra-sonication and 10-s interval (Bioruptor pico, Diagenode, Belgium). The samples were then centrifuged at 4°C, 12 000 rpm for 3 min, then 200 µl of supernatant was added to the sample cups for folate detection.

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Table 1. Genotype and alleles composition of SNPs rs1956545 and rs56811449 of *MTHFD1* in 270 patients and 192 controls.

SNP	Chr.	Position[a]	Allele[b]	Genotype frequency in case	Genotype frequency in control	Allele frequency in case	Allele frequency in control	OR (95% CI)[c]	P value
rs1956545	14	64852905	C/T	TT: 71 (27.4%)	TT: 64 (34.6%)	C: 238 (45.9%)	C: 142 (38.4%)	1.41 (1.06,1.88)	0.0195
				TC: 138 (53.3%)	TC: 100 (54.1%)	T: 280 (54.1%)	T: 228 (61.6%)		
				CC: 50 (19.3%)	CC: 21 (11.4%)				
rs56811449	14	64884076	T/C	CC: 223 (83.2%)	CC: 141 (74.2%)	T: 45 (8.4%)	T: 51 (13.4%)	0.56 (0.36,0.87)	0.0107
				CT: 45 (16.8%)	CT: 47 (24.7%)	C: 491 (91.6%)	C: 329 (86.6%)		
				TT: 0 (0%)	TT: 2 (1.0%)				

[a] For Hg19; [b] Minor/major; [c] Gender-adjusted.

DNA extraction, hybridization and sequencing

The whole genome DNA of each sample was extracted using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to standard protocol. Then the DNA was purified using Invitrogen Qbit Spectrophotometer and sheared using the Covaris™ system. A library was constructed using Agilent Custom SureSelect Enrichment Kit. Custom capture oligos were designed using the SureDesign website of Agilent Technologies. Hybridization reactions were carried out by: incubating the hybridization mixture for 16 or 24 hours at 65°C with a heated lid at 105°C in an AB 2720 Thermal Cycler (Life Technologies Corporation, USA). After the reaction, the hybridization mixture was captured and washed with magnetic beads (Invitrogen, USA) and SureSelect Target Enrichment Kit (Agilent technologies, Inc., USA).

The production was then enriched with the following cycle conditions: 98°C for 30 s; 10 cycles of 98°C for 10 s; 60°C for 30 s; and 72°C for 30 s; 72°C for 5 min. Twelve libraries were pooled in total, and then bridge amplification on cBot (Illumina, Inc, San Diego, CA) was performed following the standard manufacturer's protocols. After hybridization of the sequencing primers, base incorporation was carried out on a Genomic analyzer II Sequencer (Illumina, Inc, San Diego, CA) following the manufacturer's standard sequencing protocols, for 101 cycles of sequencing per read to generate paired-end reads, including 100 bps at each end and 6 bps of the index tag.

Analysis of polymorphism of *MTHFD1* sequences

Sequences were aligned and edited using BWA software [12] in hg19 database. Primer sequence was removed after alignment. Varscan SNP and indel calling were conducted with loose standard (min-coverage=1, min alternative allele reads=1, min-var-freq >0.03); SNV calling (Samtools pileup (MAPQ 30) and Varscan SNP and indel calling were conducted with strict standard (min-coverage=2, min alternative allele reads=2, min-var-freq >0.1). Data for subsequent genotype calling were analyzed using ANNOVAR software. The sequence alignment against the reference genomic sequence in hg19 and the single-nucleotide variation was annotated in HGVs. Poor confidence "variation" was excluded by visual inspection of sequence alignment and read coverage data.

Statistical analysis

For quality controls (QC) for SNVs, we only keep the variants with call rate >0.8, minor allele frequency (MAF) >0.01 (for LD (linkage disequilibrium analysis we further require MAF >0.05) and Hardy-Weinberg equilibrium test *P* value >0.05 in controls. The association test for the estimation of the risk of NTDs related to the polymorphism of *MTHFD1* gene was performed by using an additive model in logistic regression with sex as the covariate. *P* value, adjusted odds ratio (OR), and 95% confidence interval (CI) were calculated by use of the above model. Hardy-Weinberg equilibrium was assessed by chi-square test. LD blocks and haplotypes were estimated by using the confidence interval approach [13] implemented in Haploview [14]. Haplotype-NTDs association was tested by using the chi-square test (Haploview). The association between SNP/haplotype and brain folic acid level was assessed by linear regression (additive model) with adjustment of sex and performed for NTDs case group and control group. All calculations were perform in PLINK v1.07 [15] with a significance level of 0.05. Bioinformatics analysis to explore functions of significant SNPs was carried out by mapping the SNPs to Ensembl coding annotation data [16] and ENCODE non-coding annotation data [17].

Results

No significant difference was detected regarding sex ratio between the NTD patient group and control group (*P* value >0.05

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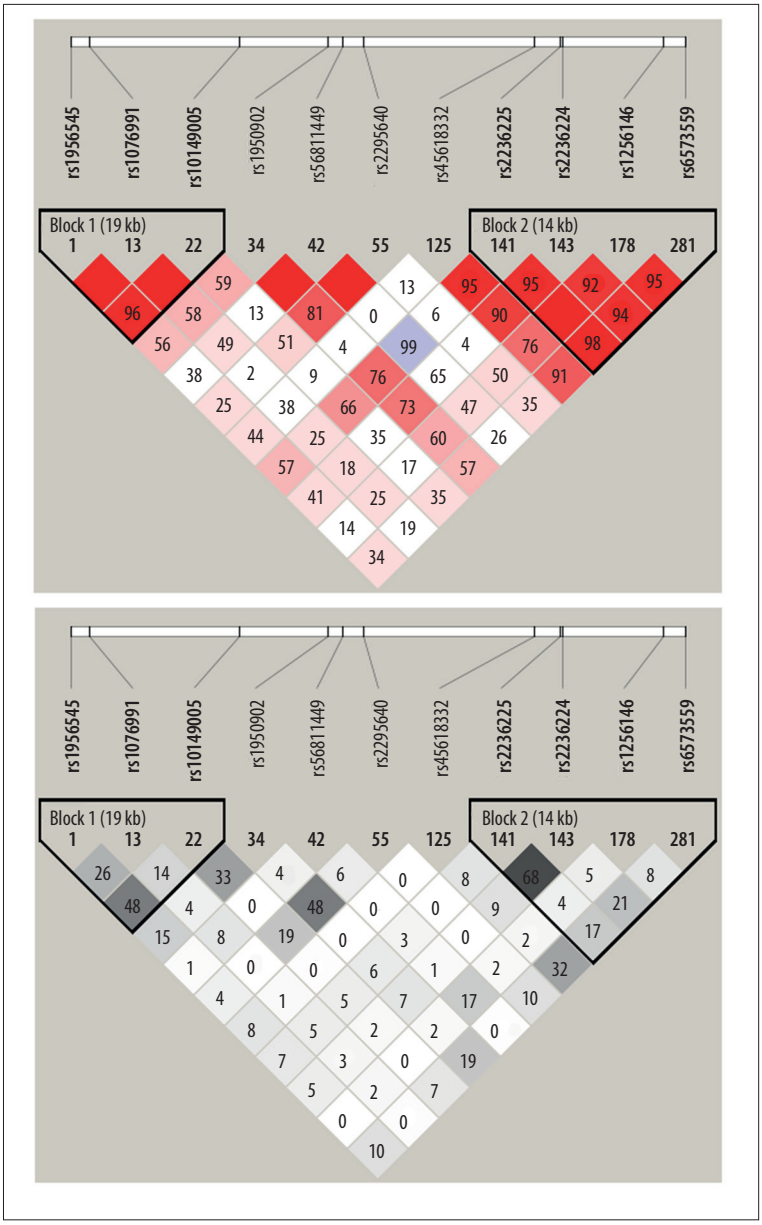

Figure 1. LD information and LD blocks of *MTHFD1* gene region. Upper: color and LD value are shown with D'. Lower: color and LD value are shown with r^2. LD block were estimated by Confidence Intervals implemented in Haploview.

for regression model). Based on the polymorphism analysis, 208 SNV sites were identified in the *MTHFD1* gene and included in the subsequent analyses. After QC, 41 SNPs remained. Two independent SNPs rs1956545 and rs56811449 (*r2*=0.01) are significantly associated with susceptibility of NTDs (Table 1). In detail, the minor allele C of rs1956545 could dramatically increase the occurrence rate of NTDs (*P* value=0.0195, OR=1.41, 95% CI=1.06–1.88) while the minor allele T of rs56811449 decreases the occurrence rate of NTDs (*P* value=0.0107, OR=0.56, 95% CI=0.36–0.87). Eleven SNPs remained for LD analysis, and the LD information and LD blocks of *MTHFD1* gene region are shown in Figure 1. The haplotype GGGG consisting of 4 SNPs (rs2236225, rs2236224, rs1256146, and rs6573559) is also associated with risk of NTDs (*P* value=0.0438, OR=0.7180, 95% CI=0.5214–0.9888). Table 2 shows the details. This haplotype does not contain the 2 associated SNPs mentioned above.

In NTDs cases, the risk allele C of rs1956545 is associated with the decrease of folic acid level of brain (*P* value=0.0222, standard beta=–0.2238, 95% CI=–0.4128 – –0.0349) (Table 3). Figure 2 shows the variability of folic acid level among 3 genotypes in NTDs cases. Another SNP, rs10149005 (r^2=0.48 with rs1956545), is also associated with brain folic acid level (*P* value=0.03087, standard beta=–0.2048, 95% CI=–0.3883 – –0.02127).

Rs1956545 is located at the upstream region of *MTHFD1* gene, while rs56811449 and rs10149005 are in the intronic

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Table 2. Results of haplotype-based association test of the haplotypes consisting of 4 SNPs (rs2236225, rs2236224, rs1256146, and rs6573559).

Haplotype	Frequency in case	Frequency in control	Chi-square statistics	OR (95% CI)	P value
GGGT	0.387	0.354	1.022	1.1521 (0.8781, 1.5115)	0.312
AAGG	0.221	0.219	0.002	1.0117 (0.7376, 1.3876)	0.9652
GGGG	**0.184**	**0.239**	**4.064**	**0.7180 (0.5214, 0.9888)**	**0.0438**
GGAG	0.129	0.122	0.083	1.0659 (0.7175, 1.5836)	0.7727
GAGG	0.057	0.045	0.63	1.2828 (0.7010, 2.3474)	0.4273

Table 3. Summary of association test between SNPs and folic acid level of brain of NTDs cases.

SNP/haplotype	Chr.	Position[a]	Allele[b]	Standard beta (95% CI)		Statistics	P value
rs1956545	14	64852905	C/T	–0.2238	(–0.4128, –0.0349)	–2.322	0.02222
rs56811449	14	64884076	T/C	0.04884	(–0.1385, 0.2362)	0.511	0.6104
rs10149005	14	64872230	G/C	–0.2048	(–0.3883, –0.02127)	–2.187	0.03087

[a] For Hg19; [b] Minor/major.

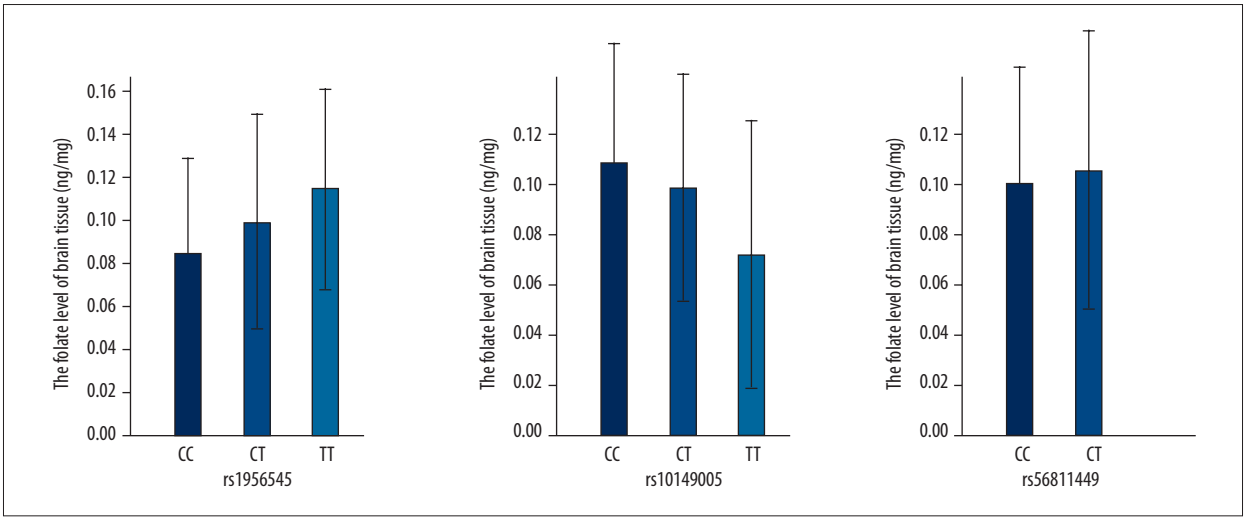

Figure 2. The folate level of brain tissue among 3 genotypes in NTDs cases. rs10149005 and rs1956545 are associated with folic acid level of brain ($P<0.05$). The folate level was decreased significantly associated with the risk allele C of rs1956545 (P value=0.02222).

regions of *MTHFD1* gene. Based on ENCODE data, rs1956545 and rs56811449 are mapped to the open chromatin regions of blastula Trophoblast cell line HTR8svn and histone-marked region of brain astrocyte cell line NH-A. Rs56811449 is also mapped to the open chromatin region of spinal cord astrocyte cell line HA-sp. Table 4 shows the details, indicating that these 2 SNPs may influence expression of *MTHFD1* gene.

Discussion

The incidence of NTDs is reported to vary depending on geographic location, ethnicity, season, sex of the affected newborns, and socioeconomic status of the parents [18,19]. The reason for this variation is unclear, but epidemiological studies have provided opportunities to identify risk factors of NTDs, to which susceptibility may be modified by genetic predisposition [20–22]. In this study, we performed a large-scale study

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Table 4. Details of ENCODE regulatory region information of rs1956545 and rs56811449.

SNP	Position[a]	ENCODE regulatory Region[b]	Regulatory region type	Marker	Cell type	Cell line	Data source
rs1956545	chr14: 64852905	chr14: 64852428-64852912	Open chromatin	DNase I	blastula Trophoblast	HTR8svn	wgEncodeOpenChromDnase
		chr14: 64852901-64852916	Open chromatin	FAIRE	blastula Trophoblast	HTR8svn	wgEncodeOpenChromFaire
		chr14: 64849634-65007690	Histone marked region	H3K36me3	brain astrocyte	NH-A	wgEncodeBroadHistone
		chr14: 64845573-64885378	Histone marked region	H3K79me2	brain astrocyte	NH-A	wgEncodeBroadHistone
		chr14: 64849411-64885322	Histone marked region	H4K20me1	brain astrocyte	NH-A	wgEncodeBroadHistone
rs56811449	chr14: 64884076	chr14: 64883742-64884201	Open chromatin	DNase I	blastula Trophoblast	HTR8svn	wgEncodeOpenChromDnase
		chr14: 64883724-64884119	Open chromatin	FAIRE	blastula Trophoblast	HTR8svn	wgEncodeOpenChromFaire
		chr14: 64883960-64884110	Open chromatin	DNase I	spinal cord astrocyte	HA-sp	wgEncodeUwDgf
		chr14: 64883686-64884524	Histone marked region	H3K4me2	brain astrocyte	NH-A	wgEncodeBroadHistone
		chr14: 64883851-64884165	Histone marked region	H3K9ac	brain astrocyte	NH-A	wgEncodeBroadHistone
		chr14: 64883688-64884224	Histone marked region	H3K27ac	brain astrocyte	NH-A	wgEncodeBroadHistone
		chr14: 64849634-65007690	Histone marked region	H3K36me3	brain astrocyte	NH-A	wgEncodeBroadHistone
		chr14: 64873070-64922569	Histone marked region	H3K4me1	brain astrocyte	NH-A	wgEncodeBroadHistone
		chr14: 64883132-64884795	Histone marked region	H3K4me1	brain astrocyte	NH-A	wgEncodeBroadHistone
		chr14: 64881007-64915924	Histone marked region	H3K4me3	brain astrocyte	NH-A	wgEncodeBroadHistone
		chr14: 64845573-64885378	Histone marked region	H3K79me2	brain astrocyte	NH-A	wgEncodeBroadHistone
		chr14: 64849411-64885322	Histone marked region	H4K20me1	brain astrocyte	NH-A	wgEncodeBroadHistone

[a, b] For Hg19.

to evaluate the polymorphism of *MTHFD*1 gene in a high-risk Northern Chinese population. This study demonstrated that the polymorphism of rs1956545 and rs56811449, as well as haplotype GGGG (rs2236225, rs2236224, rs1256146, and rs6573559), in *MTHFD*1 gene were significantly associated with the susceptibility of NTDs. The risk allele C of rs1956545 is also associated with the decrease of folic acid level of fetal brain according to analysis in the subset of NTDs cases and healthy controls.

Recent research, including randomized and community-based trials, demonstrated that maternal periconceptional supplementation with folic acid alone or multivitamins containing folic acid can reduce the risk of NTDs in offspring [23–25], but the mechanism by which folic acid prevents NTDs still remains complex. The synthesis of folic acid plays an important role in DNA methylation, DNA synthesis, cell division, and tissue growth, especially in rapidly developing cells [26]. Thus, a defect in folic acid metabolism could result in impaired DNA synthesis or DNA methylation involved in the neurulation process.

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Folate-dependent one-carbon (1C) metabolism is highly compartmentalized in eukaryotes, and mitochondria play a critical role in the process [27]. In the eukaryotes, the reactions are catalyzed by members of the MTHFD1 family [28]. As the first member characterized in this family, the MTHFD1 protein incorporates formate released from mitochondria into the cytoplasmic 1C THF pool as 10-formyl-THF (CHO-THF), which is required for *de novo* purine biosynthesis. An *Mthfd1* variant in a mouse model causes embryonic lethality through complete disruption of formyl-THF synthetase activity, suggesting that folate-activated formate is indispensable during embryonic development [29]. Maternal *Mthfd1*$^{gt/+}$ mice (1958G/A) exhibits fetal growth restriction and impaired fertility but not NTDs, due to reduced folate and choline status, whereas this exacerbated maternal folate deficiency may be a potential mechanism through which polymorphisms of MTHFD1 gene confer risk of human NTDs [30]. To identify the function of *MTHFD*1 gene involved in the mitochondrial formate production during the development of NTDs, we investigated the polymorphism of *MTHFD1* gene in a Chinese Han population, and found that 2 independent SNPs, rs1956545 and rs56811449, in *MTHFD1* gene were significantly related to risk of developing NTDs.

In the present study, we found that the minor allele C of rs1956545 significantly increased the occurrence rate of NTD and was also significantly associated with the decrease of folate level among NTD fetus. The result suggests that the allele C of rs1956545 may increase the susceptibility of NTDs by affecting fetal folate metabolism. Further bioinformatics analysis showed that rs1956545 was located at the upstream region of *MTHFD1* gene, which is mapped to the open chromatin regions, suggesting that the allele C might alter the transcription factor accessibility; therefore, it may contribute to the dysfunction of this gene, finally resulting in disordered folate metabolism. Carroll et al. also reported that a promoter polymorphism (rs1076991C.T) in *MTHFD1* gene was associated with NTD risk [31]. Although the association of allele C with the decreased folate level was not significant in the control group, a declining trend was obvious. A study with a larger sample size is needed to confirm the association between the rs1956545 genotype and folate level of the fetus. Other genes involved in folate metabolism might play a synergistic effect in controlling folate level.

In our study, we also found that the allele T of rs56811449, which was in the intronic regions of *MTHFD1* gene, decreased the occurrence rate of NTDs. In silico analysis revealed that this SNP was mapped to the open chromatin regions and histone marked region of neurogenic cell, suggesting that the risk genotype of rs56811449 would change chromatin accessibility, resulting in decreasing gene expression.

It was reported that rs2236225 polymorphism has a significant role in increasing NTDs risk in the Italian and Irish population [32]. However, in this study, we did not detect individual association of rs2236225 genotype/allele frequency with NTDs susceptibility in Chinese Han population, but haplotype GGGG consisting of rs2236225, rs2236224, rs1256146, and rs6573559 was associated with risk of NTDs. We suggest that the discrepancy might due to ethnic differences.

Conclusions

The current case-control study demonstrated that the polymorphism of SNP *loci* rs1956545 and rs56811449, as well as a haplotype, in *MTHFD*1 gene were significantly associated with the susceptibility of NTDs. The C allele of rs1956545 was related to a decreasing folate level in NTD fetuses. Although we concluded that these polymorphisms of SNP locus in *MTHFD1* gene could help to diagnose NTDs in Chinese Han population and some specific genotype of this locus may have lower risk of developing NTDs, further functional studies are needed to elucidate the mechanism involved in the influence of polymorphism of *MTHFD1* gene on NTDs.

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