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Combined folate gene *MTHFD* and *TC* polymorphisms as maternal risk factors for Down syndrome in China

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ABSTRACT. We examined whether polymorphisms in the methylenetetrahydrofolate dehydrogenase (*MTHFD*) and transcobalamin (*TC*) genes, which are involved in folate metabolism, affect maternal risk for Down syndrome. We investigated 76 Down syndrome mothers and 115 control mothers from Bengbu, China. Genomic DNA was isolated from the peripheral lymphocytes. Polymerase chain reaction and restriction fragment length polymorphism were used to examine the polymorphisms of *MTHFD* G1958A and *TC* C776G. The frequencies of the polymorphic alleles were 24.3 and 19.1% for *MTHFD* 1958A, 53.9 and 54.2% for *TC* 776G, in the case and control groups, respectively. No significant differences were found between two groups in relation to either the allele or the genotype frequency for both polymorphisms. However, when gene-gene interactions between these two polymorphisms together with previous studied C677T and A1298C polymorphisms in the methylenetetrahydrofolate reductase (*MTHFR*)

gene were analyzed, the combined *MTHFR* 677CT/TT and *MTHFD* 1958AA/GA genotype was found to be significantly associated with the risk of having a Down syndrome child [odds ratio (OR) = 3.11; 95% confidence interval (95%CI) = 1.07-9.02]. In addition, the combined *TC* 776CG and *MTHFR* 677TT genotype increased the risk of having a child with Down syndrome 3.64-fold (OR = 3.64; 95%CI = 1.28-10.31). In conclusion, neither *MTHFD* G1958A nor *TC* C776G polymorphisms are an independent risk factor for Down syndrome. However, the combined *MTHFD*/*MTHFR*, *TC*/*MTHFR* genotypes play a role in the risk of bearing a Down syndrome child in the Chinese population.

Key words: Down syndrome; Folate; *MTHFD*; *Transcobalamin gene*; Polymorphisms

INTRODUCTION

Down syndrome (DS), or trisomy 21, is a complex autosomal disease with growth and mental retardation resulting from the presence and expression of 3 copies of the genes located on chromosome 21. In most cases, the extra chromosome is from the oocyte. Despite the fact that DS is caused by nondisjunction of chromosome 21 during meiosis I or II (Antonarakis et al., 1992), little is known about the mechanisms of the failure of chromosome segregation. So far, advanced maternal age is the only well-established risk factor for DS. However, almost 80% of children with this disease are born from mothers at an age of 35 or younger (Eskes, 2006). A study has shown that young mothers of DS individuals are more prone to occurrence of chromosome segregation error for both chromosomes 13 and 21 in peripheral blood cells compared to controls (Migliore et al., 2006). These facts indicate that there are other predisposition factors in these mothers besides the aging of their eggs.

There is evidence that some mothers of infants with DS have abnormal folate and methyl metabolism, as well as mutations in folate genes, which are features that are also seen in neural-tube defects (NTD) (James et al., 1999; Hobbs et al., 2000; Botto and Yang, 2000). In 2003, Barkai et al. found a higher frequency of DS infants in families with a risk of NTD, and vice versa. This evidence indicated that the same genetic determinants of folate and methyl metabolism influenced both diseases, at least in proportion of cases. Some studies have suggested that abnormal folate and methyl metabolism may be associated with genomic DNA hypomethylation and abnormal purine synthesis (Scala et al., 2006; Coppedè, 2009). On the other hand, genomic DNA hypomethylation has been causally related to both chromosomal instability and abnormal segregation (Wang et al., 2004; Kim et al., 2011). On the basis of this evidence, several studies from distinct geographic regions have evaluated the role of polymorphisms of folate genes as maternal risk factors for DS (da Silva et al., 2005; Scala et al., 2006; Coppedè et al., 2006; Wang et al., 2008; Brandalize et al., 2010). The most common genes studied in different populations include the methylenetetrahydrofolate reductase (*MTHFR*) gene, whose product catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methytetrahydrofolate, the methyl donor for the remethylation of homocysteine (Hcy) to methionine, and the methionine synthase reductase (*MTRR*) gene, encoding an enzyme that maintains the methionine synthase enzyme in an active state for the remethylation of Hcy to methionine.

In the folate metabolic pathway, the methylenetetrahydrofolate dehydrogenase (*MTHFD*) gene encodes a trifunctional enzyme that catalyzes the conversion of tetrahydrofolate to 10-formyl, 5,10-methenyl, and 5,10-methylenetetrahydrofolate. The *MTHFD* G1958A polymorphism reduces the enzyme's activity and stability, and has been associated with an increased risk of NTD (De Marco et al., 2006). Transcobalamin (TC), a cobalamin-transporting protein, plays an important role in vitamin B12 cellular uptake and metabolism. *TC* C776G, a common genetic polymorphism, may affect the function of TC, and then negatively affect vitamin B12 metabolism (von Castel-Dunwoody et al., 2005). Thus, genetic variants in the *TC* gene possibly have an influence on cellular methylation reactions. The aim of this study was to investigate the relationship of *MTHFD* G1958A and *TC* C776G in the risk of having a child with DS, as well as the interactions between these polymorphisms and the *MTHFR* C677T, A1298C polymorphisms, previously studied by our group.

MATERIAL AND METHODS

Study population and specimen collection

Peripheral blood samples were collected from 76 mothers (19-44 years of age at the time of conception) who had given birth to a DS child (full trisomy 21) and 115 age-matched mothers who had at least one healthy child and had experienced no miscarriages or abnormal pregnancies. Case mothers were recruited from the Central Laboratory, First Affiliated Hospital of Bengbu Medical College, Bengbu, China. For the purposes of our analyses, case and control mothers were limited to Han ethnicity, resided in the same geographic area, and had a similar social background, to reduce ethnic and geographic variation. The regional Ethics Committee previously approved all the research protocols. Blood samples (5 mL) were collected in EDTA tubes from all participants. Genomic DNA was isolated from lymphocytes in whole blood using the Ezup Column Blood Genomic DNA Purification kit (Sangon Biotech, Shanghai, China), according to the manufacturer protocol, and was stored at -20°C until genotype analysis was performed.

Genotype analyses

MTHFD G1958A and *TC* C776G genotypes were analyzed by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) according to the protocol described by Neagos et al. (2010) and Biselli et al. (2008a), respectively. The primers for amplification of *MTHFD* G1958A polymorphism were: forward 5'-CCT GGT TTC CAC AGG GCA CTC-3', and reverse 5'-CCA CGT GGG GGC AGA GGC CGG AAT ACC GG-3'. PCR conditions were denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 1 min for a total of 35 cycles with an initial denaturation at 94°C for 4 min and a final extension of 7 min. An amplification product 310 bp long was obtained. After digestion of the PCR product with restriction enzyme *Msp*I, the A allele was detected by the appearance of 282- and 28-bp fragments while 196-, 86- and 28-bp fragments for G-allele. Digestion fragments were then subjected to electrophoresis on a 3% agarose gel.

The primers for amplification of the *TC* C776G polymorphism were: forward 5'-GCT CAA ACT TCA ACC CTG GTC-3', and reverse 5'-GTG CCA GAC AGT CTG GGA

AG-3'. PCR conditions were denaturation at 94°C for 30 s, annealing at 59°C for 30 s and elongation at 72°C for 1 min for a total of 35 cycles with an initial denaturation at 94°C for 4 min and a final extension of 7 min. PCR amplification generated a 439-bp fragment. After *Scr*fI digestion of the PCR product, a 276-bp fragment was visible on the agarose gel in the presence of the G allele while the C allele was detected by the presence of 118- and 158-bp (16-, 17- and 130-bp fragments visible, in both situations representing the internal control of *Scr*fI digestion).

Statistical analysis

Allele frequencies were calculated for each genotype, and the differences in allele frequencies between the mothers of DS children and control mothers were determined using a chi-square test. Hardy-Weinberg equilibrium for each polymorphism in cases and controls was also tested by chi-square test. The odds ratios (ORs) were calculated for both the heterozygous and homozygous mutant genotypes compared with the wild types, respectively. These ORs were used as an estimate of relative risk. The "two-by-four" approach was used to explore possible interactions of mutant genotypes as described elsewhere (Scala et al., 2006). The interactions between combination genotypes of *MTHFD* G1958A, *TC* C776G, *MTHFR* C677T and A1298C were evaluated by calculation of the odds ratio for the presence of both mutant genotypes compared with the absence of both. The Fisher exact test was used when appropriate. All statistical analyses were performed using the SPSS software, version 17.0.

RESULTS

Allele frequencies

Table 1 gives the *MTHFD* and *TC* allele frequencies for the DS and control mothers. The distribution of the *MTHFD* and *TC* genotypes were found to be in Hardy-Weinberg equilibrium in both groups. The frequencies of the *MTHFD* 1958A allele was 24.3% in DS mothers and 19.1% in control mothers ($P > 0.05$). For the *TC* gene, the mutant allele frequency was 53.9% in case mothers and 54.2% in the control mothers ($P > 0.05$). No statistically significant differences in allele frequencies for either gene were observed between case and control mothers (Table 1).

Table 1. Allele frequencies of *MTHFD* G1958A and *TC* C776G polymorphisms in mothers of Down syndrome (DS) children and controls.

Polymorphism	Allele	DS mother alleles (%)	Control mother alleles (%)	χ^2	P
MTHFD	G	115 (75.7)	186 (80.9)	-	-
	A	37 (24.3)	44 (19.1)	1.49	0.223
TC	C	70 (46.1)	99 (45.8)		-
	G	82 (53.9)	117 (54.2)	0.002	0.967

Genotype analysis and risk of DS

As shown in Table 2, the GA heterozygous genotype and combination of het-

erozygous and homozygous genotype (GA and AA) frequencies of *MTHFD* at site 1958 were higher among case mothers compared to control mothers. However, there were no significant differences in genotype frequencies between the two groups. The frequencies of *TC* C776G genotypes (CC, CG and GG) were respectively 18.4, 55.3 and 26.3% among DS mothers and 25.9, 39.8 and 34.3% among control mothers. The heterozygous mutation frequency was higher among case mothers than among control mothers. However, the difference in heterozygous mutation frequency between the two groups was not at a statistically significant level. The combination of heterozygous and homozygous *TC* C776G polymorphism (CG or GG) did not show significant differences between the two groups (Table 2).

Table 2. Genotype frequencies of *MTHFD* G1958A and *TC* C776G polymorphisms in mothers of Down syndrome (DS) children and controls.

Genotype	DS mothers [N, (%)]	Control mothers [N, (%)]	OR (95%CI)	P
MTHFD	76 (Total)	115 (Total)		
GG	42 (55.3)	76 (66.1)	Reference	
GA	31 (40.8)	34 (29.6)	1.65 (0.89-3.05)	0.110
AA	3 (3.9)	5 (4.3)	1.09 (0.91-1.11)	0.913
GA+AA	34 (43.7)	36 (33.9)	1.71 (0.94-3.12)	0.080
TC	76 (Total)	108 (Total)		
CC	14 (18.4)	28 (25.9)	Reference	
CG	42 (55.3)	43 (39.8)	1.95 (0.91-4.22)	0.086
GG	20 (26.3)	37 (34.3)	1.08 (0.47-2.51)	0.856
CG+GG	62 (81.6)	80 (74.1)	1.55 (0.75-3.19)	0.232

Interactions between *MTHFD*, *TC* and *MTHFR* genotypes

To evaluate potential gene-gene interactions, all possible combinations between mutant genotypes of *MTHFD*, *TC* and *MTHFR* (previous study) genes were analyzed by a two-by-four table to obtain the combined risk due to the presence of two different mutant genotypes in the same women. As shown in Table 3, none of the combined genotypes of *MTHFD* and *TC* genes was associated with the risk of having a child with DS. However, the interaction analysis between the both gene polymorphisms described above together with the previous studied *MTHFR* C677T and A1298C polymorphisms showed that the combination of genotypes *MTHFD* 1958GA/AA and *MTHFR* 677CT/TT was associated with a significant increased risk of having a DS infant compared with the *MTHFD* 1958GG and *MTHFR* 677CC genotypes (OR = 3.11; 95%CI = 1.07-9.02; P = 0.032) (Table 4). The presence of genotypes with *TC* 776CG and *MTHFR* 677TT combined increased the risk for having a DS child 3.64-fold (OR = 3.64; 95%CI = 1.28-10.31; P = 0.012) (Table 5). No interactions were found between the remaining genotypes analyzed.

Table 3. Interactions between *MTHFD* G1958A and *TC* C776G genotypes in mothers of Down syndrome (DS) children (N = 76) and controls (N = 108).

MTHFD 1298GA or AA	*TC* 776GG	DS mothers [N, (%)]	Control mothers [N, (%)]	OR (95%CI)	P
+	+	11 (14.5)	13 (12.0)	1.23 (0.49 -3.08)	0.657
-	+	9 (11.8)	23 (21.3)	0.57 (0.23-1.39)	0.211
+	-	23 (30.3)	24 (22.2)	1.39 (0.68-2.87)	0.368
-	-	33 (43.4)	48 (44.5)	Reference	-

Table 4. Interactions between *MTHFD* G1958A and *MTHFR* C677T genotypes in mothers of Down syndrome (DS) children (N = 74) and controls (N = 114).

MTHFD 1298GA or AA	*MTHFR* 677CT or TT	DS mothers [N, (%)]	Control mothers [N, (%)]	OR (95%CI)	P
+	+	28 (37.9)	27 (23.7)	3.11 (1.07-9.02)	0.032
-	+	34 (45.9)	57 (50.0)	1.79 (0.65-4.95)	0.258
+	-	6 (8.1)	12 (10.5)	1.50 (0.39-5.77)	0.554
-	-	6 (8.1)	18 (15.8)	Reference	-

Table 5. Interactions between *TC* C776G and *MTHFR* C677T genotypes in mothers of Down syndrome (DS) children (N = 73) and controls (N = 105).

TC 776CG	*MTHFR* 677TT	DS mothers [N, (%)]	Control mothers [N, (%)]	OR (95%CI)	P
+	+	13 (17.8)	7 (6.7)	3.64 (1.28-10.31)	0.012
-	+	8 (10.9)	16 (15.2)	0.98 (0.37-2.61)	0.966
+	-	28 (38.4)	35 (33.3)	1.57 (0.78-3.15)	0.207
-	-	24 (32.9)	47 (44.8)	Reference	-

DISCUSSION

Maternal age is the only well-established risk factor for DS, and after age 35 the risk of bearing a child with DS increases accordingly. The occurrence of DS independent of maternal age indicates that there are other risk factors for this syndrome. James et al. (1999) reported that abnormal Hcy and folate metabolism present in mothers of children with DS and the *MTHFR* C677T polymorphism were independent risk factors for having a child with DS. This study hypothesized that the C677T mutation may predispose to aberrant DNA methylation and increased risk of meiotic nondisjunction.

Several studies have been conducted on the role of folate gene polymorphisms and metabolism in the pathogenesis of DS to provide evidence and new clues (da Silva et al., 2005; Scala et al., 2006; Wang et al., 2008; Biselli et al., 2008b; Coppedè et al., 2006, 2009; Neagos et al., 2010; Brandalize et al., 2010). The genes investigated as maternal risk factors for DS include methionine synthase, reduced folate carrier 1 and cystathionine-beta-synthase besides *MTHFR* and *MTRR*. However, the results are still conflicting. The varied results may be explained by differences in the nutritional habits and genetic background of distinct populations. The allele frequencies have been shown to vary greatly between different populations. In our recent study, we observed that *MTHFR* 677T allele was more prevalent among mothers with DS children than among control mothers (Liao et al., 2010). The result indicated that polymorphisms of folate metabolism genes may play a role in the risk of DS pregnancies in the Chinese population. The focus of the present study was to identify key risk factors involved in the folate metabolic pathway for DS in mothers who had at least one affected child. To minimize ethnic bias, all participants were restricted to Han ethnicity in middle China. To our knowledge, this is the first study that has analyzed the relationship between the *MTHFD* G1958A and *TC* C776G polymorphisms and the birth risk of a DS child in the Chinese population.

Our results did not show an association of *MTHFD* G1958A polymorphism with the risk of having an affected child. The results of the present research are in agreement with a previous study and indicate that *MTHFD* 1958A allele alone is not associated with an increased risk of having a DS child (Scala et al., 2006; Neagos et al., 2010). In our study, we found that the frequencies of the GG, GA and AA were 55.3, 40.8 and 3.9% among DS mothers and 66.1,

29.6 and 4.3% among control mothers, respectively. AA homozygous genotype frequency at this site was lower in the Chinese population than in Italian and Romanian populations reported by Scala et al. (2006) and Neagos et al. (2010), respectively. Low 1958AA homozygous genotype frequency (5.4% in controls) was also reported in Chinese population by Wang et al. (2007a). *MTHFD* G1958A polymorphism may result in disturbance of the folate-mediated Hcy pathway and has been observed with increased risk of birth defects such as NTD and unexplained second semester pregnancy loss (Parle-McDermott et al., 2005; De Marco et al., 2006). However, in the present study, this mutant was not associated with an increased risk for giving birth to a DS child. In light of the lower frequency of the mutant homozygous genotype observed in this study, a large sample study should be conducted in the Chinese population to confirm this result. Nevertheless, a role of *MTHFD* G1958A polymorphism in the risk of bearing a DS child cannot be excluded since the combined *MTHFD* 1958AA/*RFC1* 80GG genotype was observed as a maternal risk factor of having a DS child by Scala et al. (2006).

von Castel-Dunwoody et al. (2005) reported that the *TC* C776G polymorphism negatively affected the concentration of the TC-vitamin B12 complex and altered the cellular availability of vitamin B12, thus exacerbating the effects of low vitamin B12 status. There is evidence for an association between *TC* C776G polymorphism and the increased risk of bearing a child with NTD (Afman et al., 2002; Guéant-Rodriguez et al., 2003). However, no association was observed between the *TC* C776G polymorphism and the maternal risk of having a child with DS in the present study. This result was consistent with previous research from Brazil (Biselli et al., 2008a; Fintelman-Rodrigues et al., 2009). However, we found that the heterozygous mutation frequency was higher among case mothers (55.3%) than among control mothers (39.8%), although not at a statistically significant level (P = 0.086). In addition, the frequency of the GG homozygous genotype, the mutant genotype, was higher in the Chinese population (34.3% in control mothers) than in the Brazilian population (9.7% in control mothers) (Biselli et al., 2008a). We also observed that allele and genotype frequencies in our study were quite similar to those seen from another group in the Chinese population. High frequency of GG homozygous genotype was found by Chen et al. (2008) (39.3% in ethnic Han from Central China). The results suggest that transport and metabolic pathways of vitamin B12 may vary greatly in different populations.

Several investigators have reported that individual polymorphism may be insufficient to cause an increased incidence of birth of children with DS, but that a genotype in which two or more polymorphisms in distinct enzymes of the pathways may lead to increased incidence (Acácio et al., 2005; Coppedè et al., 2006; Biselli et al., 2008b). We explored gene-gene interactions and found the first evidence of a positive interaction between the polymorphic variants of the *MTHFR*, *MTHFD* and *TC* genes. The combined *MTHFR* 677CT/TT and *MTHFD* 1958AA/GA genotype was significantly associated with the risk of having a DS child compared with the combined 677CC and 1958GG genotype (OR = 3.11; 95%CI = 1.07-9.02; P = 0.032). In addition, the presence of the combined *TC* 776CG and *MTHFR* 677TT genotype increased the risk of having a child with DS 3.64-fold (OR = 3.64; 95%CI = 1.28-10.31; P = 0.012). Zetterberg et al. (2003) reported that combined *MTHFR* 677TT/*TC* 776GG and combined *MTHFR* 677TT/*TC* 776CG genotypes gave an OR for spontaneous abortion of 3.8 (95%CI = 1.4-9.9, P = 0.005). In a previous study, we found that the *MTHFR* 677-1298 T-C haplotype was present in the Chinese population and played a role in the risk of having a DS child (Liao YP, Zhang D and Zhou W, unpublished results). These results provided further

evidence for a major role of gene-gene interactions in the assessment of genetic susceptibility to DS. In the last twenty years, few studies have found the *MTHFR* C677T polymorphism as an independent risk factor for bearing DS children (James et al., 1999; Hobbs et al., 2000; da Silva et al., 2005; Wang et al., 2007b, 2008), and most studies conducted in this field have shown a positive view about the cooperation of *MTHFR* C677T or A1298C polymorphism with other factors including gene polymorphisms and vitamin intake involved in folate and methyl metabolism (O'Leary et al., 2002; da Silva et al., 2005; Acácio et al., 2005; Rai et al., 2006; Biselli et al., 2008b; Meguid et al., 2008; Coppedè et al., 2006, 2009; Brandalize et al., 2009, 2010), where some of them have been in Chinese populations (Wang et al., 2007b, 2008; Liao et al., 2010). It seems that the *MTHFR* gene mutation acts as a key factor in the assessment of risk to DS.

In conclusion, the results of the present study indicate that either *MTHFD* G1958A or *TC* C776G polymorphism is not an independent risk factor for bearing a DS child. However, combined genotypes including *MTHFR*, *MTHFD* and *TC* gene may play a role in DS pregnancies in Chinese women. These results are in agreement with the notion that the presence of two or more mutant alleles in the genome could increase the risk of bearing a DS child. Both epidemiological investigations and experimental studies have shown that impaired folate and methyl metabolism can result in DNA hypomethylation and abnormal chromosome segregation. A role for abnormal folate and methyl metabolism in the risk of DS pregnancies cannot be excluded. Further studies should be designed to obtain direct proof of meiotic nondisjunction resulting from aberrant folate and methyl metabolism and to explain the epidemiological data in several studies.

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