Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Polymorphisms in genes involved in folate metabolism as maternal risk factors for Down syndrome in China*

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Received Nov. 24, 2007; revision accepted Jan. 7, 2008

Abstract: Objective: To explore the relationship between genetic polymorphisms in methylenetetrahydrofolate reductase (MTHFR), methionine synthase reductase (MTRR), the central enzymes in folate metabolism that affects DNA methylation and synthesis, and the risk of Down syndrome in China. Methods: Genomic DNA was isolated from the peripheral lymphocytes of 64 mothers of children with Down syndrome and 70 age matched control subjects. Polymerase chain reaction and restriction fragment length polymorphism were used to examine the polymorphisms of MTHFR 677C→T, MTRR 66A→G and the relationship between these genotypes and the risk of Down syndrome was analyzed. Results: The results show that the MTHFR 677C→T polymorphism is more prevalent among mothers of children with Down syndrome than among control mothers, with an odds ratio of 3.78 (95% confidence interval (*CI*), 1.78~8.47). In addition, the homozygous MTRR 66A→G polymorphism was independently associated with a 5.2-fold increase in estimated risk (95% *CI*, 1.90~14.22). The combined presence of both polymorphisms was associated with a greater risk of Down syndrome than the presence of either alone, with an odds ratio of 6.0 (95% *CI*, 2.058~17.496). The two polymorphisms appear to act without a multiplicative interaction. Conclusion: MTHFR and MTRR gene mutation alleles are related to Down syndrome, and CT, TT and GG gene mutation types increase the risk of Down syndrome.

Key words: Tetrahydrofllate dehydrogenase, Ligases, Down syndrome, Folate
doi:10.1631/jzus.B0710599 **Document code:** A **CLC number:** R394

INTRODUCTION

Trisomy 21, or Down syndrome, is the most common genetic cause of human mental retardation, with an incidence of 1/600~1/1000 live births (Smith and Berg, 1995). In addition, trisomy 21 is a major cause of premature pregnancy failure. It is estimated that 1/150 conceptions have trisomy 21 and that 80% of these are lost during early pregnancy (Boué *et al*., 1975; Hassold and Jacobs, 1984; Freeman *et al*., 1991); The nondisjunction event that results in two copies of chromosome 21 takes place in anaphase of meiosis I during oocyte maturation before ovulation, and/or in anaphase of meiosis II around the time of fertilization in the adult female (Ramírez *et al*., 2007). Despite the prevalence and health consequences of Down syndrome, the biochemical and molecular basis for meiotic nondisjunction is not understood.

Folic acid is essential for the de novo synthesis of nucleotide precursors for normal DNA synthesis, and is also essential for normal cellular methylation reactions. Chronic folate/methyl deficiency in vivo and in vitro has been associated with abnormal DNA methylation (Pogribny *et al*., 2004; James *et al*. 2003), DNA strand breaks (Duthie *et al*., 2002), altered chromosome recombination (Zijno *et al*., 2003), and aberrant chromosome segregation (Parry *et al*., 2002). On the basis of this evidence, James *et al*.(1999) suggested the possibility that gene-nutrient interactions associated with abnormal folate metabolism and DNA hypomethylation might increase the risk of chromosome nondisjunction. In Brown *et al*.(1999)'s study, mothers of children with Down syndrome were

* Project (No. 2003ABA148) supported by the Science Foundation of Hubei Province, China

found to have mildly elevated plasma homocysteine levels and a 2.6-fold increased frequency of the 677C→T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene, compared with control mothers (95% *CI*, 1.2~5.8). The data from this study further suggested that polymorphisms in other genes in the folate pathway may increase the risk of having a child with Down syndrome (James *et al*., 1999; Rosenblatt, 1999).

MTHFR catalyzes the synthesis of 5-methyltetrahydrofolate, the methyl donor for the Vitamin B_{12}-dependent remethylation of homocysteine to methionine via the methionine synthase reaction. Methionine is the precursor for the synthesis of *S*-adenosylmethionine (SAM), the major cellular methyl donor for DNA, RNA, protein and phospholipid methylation. The reduction in enzyme activity associated with the 677C→T MTHFR polymorphism raises the dietary requirement for folic acid to maintain normal remethylation of homocysteine to methionine (Leclerc *et al*., 2003). Consequently, low folate status in individuals with the MTHFR polymorphism results in an increase in homocysteine and a decrease in methionine levels. Chronic elevation in intracellular homocysteine can lead to a decrease in the ratio of SAM to *S*-adenosylhomocysteine (SAH) that is associated with inhibition of the DNA methyltransferase and DNA hypomethylation (Pilsner *et al*., 2007). Methionine synthase reductase (MTRR) is a related flavoprotein that maintains the methionine synthase enzyme in an active state for the remethylation of homocysteine to methionine. The cloning of the cDNA for MTRR led to the identification of a polymorphism, 66A→G, that was recently associated with increased risk for spina bifida (Gué-ant-Rodriguez *et al*., 2003). Because of the importance of the methionine synthase reaction in maintaining normal folate metabolism and DNA methylation, we hypothesized that this polymorphism could be a second maternal genetic risk factor for Down syndrome.

The association between folate deficiency and DNA hypomethylation suggests that genetic and/or nutritional deficiencies that negatively affect folate metabolism may be mechanistically related to an increased risk of nondisjunction and Down syndrome. In the present study, we analyzed the association between the MTHFR 677C→T polymorphism and the maternal risk of Down syndrome in a yellow population, and we have evaluated, for the first time in a yellow population, the association between the MTRR 66A→G polymorphism and Down syndrome risk.

SUBJECTS AND METHODS

Study population

Blood samples from women who had a pregnancy affected by karyotypically confirmed full trisomy 21 (case mothers) were obtained from two study sites: the laboratory of prenatal eugenics diagnosis, and the laboratory of pediatric metabolic disease of Tongji hospital. For the purposes of our analyses, case and control mothers were limited to yellows, to reduce the ethnic variation between groups. Mothers of live-born infants with trisomy 21 were ascertained with the assistance of the laboratory of prenatal eugenics diagnosis, and details regarding the methods and sources have been described by Edmonds and Oakley (1981). Case mothers were identified at genetic laboratories that record all abnormal karyotypes of live births in the monitored counties. Recruitment of mothers participating in the study was done through Down syndrome support group newsletters and the Internet. Each case mother was asked to recruit a control mother who was approximately the same age, resided in the same geographic area (with a similar socioeconomic status), and had experienced no miscarriages or abnormal pregnancies. Informed consent was obtained from all participants.

Specimen collection and genotype analyses

Genomic DNA was isolated from lymphocytes in whole blood by use of standard chloroform/phenol extraction, and was stored at −20 °C until the genotype analysis was performed. DNA samples obtained from case and control mothers were analyzed at the laboratory of prenatal eugenics diagnosis for the MTHFR genotype and the MTRR genotype. For both polymorphisms, genotyping was done by PCR followed by appropriate restriction enzyme digestion and has been described in detail by Engel *et al*.(2006). Laboratory personnel who performed the genotyping were unaware of whether the samples came from case or control mothers.

Statistical analyses

Allele frequencies were calculated for each genotype, and the differences in allele frequencies between case and control mothers were determined using a Pearson χ^2 test. Expected genotype frequencies were calculated from the allele frequencies under the assumption of Hardy-Weinberg equilibrium. Odds ratios for both the heterozygous and homozygous mutant genotypes, as compared with the wild types, were calculated as a measure of the association between the MTHFR and MTRR genotypes and a Down syndrome-affected pregnancy. These odds ratios were used as an estimate of relative risk. The interaction between the two genotypes was evaluated by calculation of the odds ratio for the exposure genotype of either MTHFR or MTRR while the other was controlled, followed by calculation of the odds ratio, for the presence of both genotypes compared with the absence of both. For analysis of gene-gene interactions, the MTRR homozygous AA and the heterozygous AG genotypes were combined because neither genotype was associated with increased risk of neural tube defects or Down syndrome (van der Linden *et al*., 2006). For MTHFR, the heterozygous CT and homozygous TT genotypes were combined for the gene-gene interaction analysis, because both genotypes reduce MTHFR enzyme activity, and because both have been associated with an increased risk of neural tube defects (O'Leary *et al*., 2005) and Down syndrome (Acácio *et al*., 2005). Throughout, a two-tailed *P* value of 0.05 was interpreted as indicating a statistically significant difference. All statistical analyses were done with SAS software, version 9.

RESULTS

Allele frequencies

The distribution of the MTHFR genotypes in the control population was found to be in Hardy-Weinberg equilibrium. Table 1 indicates the MTHFR and MTRR allele frequencies for women with pregnancies affected by Down syndrome and control women, using the combined data from the two sources. The frequencies of the MTHFR 677T allele and the MTRR 66G allele were both significantly higher among case mothers than among control mothers. The MTHFR mutant allele frequency was 53.1% (68/128 alleles) among the case mothers and 27.8% (39/140 alleles) among the control mothers ($P<0.01$). The mean distribution of the T allele frequency in the combined control group was 0.28, which is in the range that recently was reported in a meta-analysis of several studies of North American whites (Botto and Yang, 2000). For the MTRR gene, the mutant allele frequency was 62.5% (80/128 alleles) among case mothers, compared with 41.5% (58/140 alleles) among the control mothers ($P<0.01$). These data confirm that, in the current study population, the mutant allele frequencies for both MTHFR and MTRR genes are significantly higher among case mothers than among control mothers.

Table 1 Allele frequencies of MTHFR 677C→T and MTRR 66A→G in women with Down syndrome-affected pregnancies (case mothers) and control mothers

Genotype	Allele	Case		Control		χ^2	*P*
		n	*f* (%)	*n*	*f* (%)		
MTHFR	C	60	46.9	101	72.2		
	T	68	53.1	39	27.8	17.8	<0.01
MTRR	A	48	37.5	82	58.5		
	G	80	62.5	58	41.5	11.9	<0.01

n: Number of alleles; *f*: Frequency

Maternal MTHFR genotype and risk of Down syndrome

The composite data in Table 2 show that the frequencies of the CC, CT and TT genotypes among all control mothers were 51.4% (36/70), 41.4% (29/70) and 7.2% (5/70), respectively. The corresponding frequencies among all case mothers were 21.8% (14/64), 50% (32/64) and 28.2% (18/64), respectively. The presence of the MTHFR 677C→T substitution in one or both alleles was associated with a 3.78-fold increase in the risk of having a child with Down syndrome (95% confidence interval (*CI*), 1.78~8.47). The odds ratio for the heterozygous CT genotype was 2.84 (95% *CI*, 1.28~6.29), whereas for the homozygous TT genotype, the odds ratio was 9.26 (95% *CI*, 2.88~29.74).

Maternal MTRR genotype and risk of Down syndrome

In Table 3, the distribution of MTRR genotypes in the control population was 34.3% (24/70) for the homozygous wild type, 48.6% (34/70) for the het-

erozygous genotype, and 17.1% (12/70) for the homozygous mutant genotype. The corresponding frequencies in the case mothers were 15.6% (10/64) for homozygous wild type, 43.8% (28/64) for heterozygous genotype, and 40.6% (26/64) for homozygous mutant genotype. Stratified by genotype, the data show that homozygosity for the MTRR 66A→G mutation was associated with a 2.81-fold increased risk of having a Down syndrome-affected pregnancy (95% *CI*, 1.06~7.39) compared with homozygous normal subjects. The heterozygous mutation, however, was not associated with a significant increase in risk (odds ratio, 1.98; 95% *CI*, 2.25~4.82).

Interaction between MTHFR genotype and MTRR genotype

To evaluate potential gene-gene interactions, both MTHFR and MTRR polymorphisms were evaluated in a two-by-four table, as suggested by Guéant-Rodriguez *et al*.(2003). Using this approach, it was possible to evaluate the risk associated with each genotype independently and, also to assess the combined risk when both polymorphisms are present. As is shown in Table 4, case mothers were more likely to be heterozygous or homozygous for the MTHFR mutant genotype and not have the MTRR mutant GG genotype (26/64) than were control mothers (odds ratio, 2.7; 95% *CI*, 1.091~ 6.685). Case mothers who were homozygous for the MTRR mutant polymorphism but were negative for the MTHFR mutant allele (8/64) were at a 2.7-fold increased risk (95% *CI*, 0.798~9.140). The presence of both MTHFR mutant alleles and the MTRR homozygous mutant allele (20/64) was associated with a 6-fold increased risk of having a child with Down syndrome (95% *CI*, 2.058~17.496). Although the presence of both polymorphisms consistently conferred a greater risk of Down syndrome than did the presence of either alone, the polymorphisms appear to act without a multiplicative gene-gene interaction.

Table 2 Association between maternal MTHFR genotype and Down syndrome-affected pregnancies (case mothers) (*n*=64) and control mothers (*n*=70)

Genotype	Number (percentage) of case mothers	Number (percentage) of control mothers	Odds ratio	95% *CI*	*P*
CC	14 (21.8%)	36 (51.4%)	1.00		
CT	32 (50.0%)	29 (41.4%)	2.84	1.28~6.29	>0.05
TT	18 (28.2%)	5 (7.2%)	9.26	2.88~29.74	<0.01
CT or TT	50 (78.2%)	34 (48.6%)	3.78	1.78~8.47	>0.05

Table 3 Association between maternal MTRR genotype and Down syndrome-affected pregnancies (case mothers) (*n*=64) and control mothers (*n*=70)

Genotype	Number (percentage) of case mothers	Number (percentage) of control mothers	Odds ratio	95% *CI*	*P*
AA	10 (15.6%)	24 (34.3%)	1.00		
AG	28 (43.8%)	34 (48.6%)	1.98	2.25~4.82	>0.05
GG	26 (40.6%)	12 (17.1%)	5.20	1.90~14.22	<0.025
AG or GG	54 (84.4%)	46 (65.7%)	2.81	1.06~7.39	>0.1

Table 4 Interaction between MTHFR and MTRR genotypes in women with Down syndrome-affected pregnancies (case mothers) (*n*=64) and control mothers (*n*=70)

MTHFR	MTRR	Number (percentage) of case mothers	Number (percentage) of control mothers	Odds ratio	95% *CI*	*P*
CC	AA or AG	10 (15.6%)	27 (38.6%)	1.0		
TT or CT	AA or AG	26 (40.6%)	26 (37.1%)	2.7	1.091~6.685	>0.05
CC	GG	8 (12.5%)	8 (11.4%)	2.7	0.798~9.140	>0.05
TT or CT	GG	20 (31.3%)	9 (12.9%)	6.0	2.058~17.496	<0.025

DISCUSSION

Maternal age is the only well-established risk factor for Down syndrome, and the associated risk increases exponentially at age 35 years (Hassold and Jacobs, 1984). It has been estimated that 15%~20% of all human conceptions are chromosomally abnormal because of errors in meiotic division; however, the majority of these errors are embryonic lethal and result in fetal loss (Hunt and Lemaire-Adkins, 1998). The high frequency of maternal nondisjunction is now thought to be due to the absence of a meiotic checkpoint in the oocyte, and this provides a plausible biological explanation for the predominance of maternal nondisjunction (McCaffrey *et al*., 2006). In the human female, primordial oocytes enter meiosis I during fetal development, undergo DNA replication and homologous recombination, and then remain arrested in prophase I (diplotene stage) for several decades until initiation of oocyte maturation and ovulation in the adult female (Hunt and Lemaire-Adkins, 1998). Recent preliminary studies have implicated the MTHFR 677C→T polymorphism (Acácio *et al*., 2005) and excessive smoking as maternal risk factors for Down syndrome. Interestingly, low folate status has been associated with each of these potential risk factors. A recent clinical study has more directly implicated folate deficiency as a risk factor for human aneuploidy. Lymphocytes from women consuming a controlled folate-deficient diet were found to have a significantly increased frequency of kinetochore positive micronuclei, which are surrogate markers for abnormal chromosome segregation (Warburton, 2005). Folate supplementation after the folate-depletion phase in this metabolic study was associated with a significant decrease in these centromeric fragments. Taken together, these studies support the possibility that multifactorial gene-environment interactions that compromise maternal folate status may promote meiotic nondisjunction and the risk of a Down syndrome conception. The results of the present study indicate that the simultaneous presence of the MTHFR 677T polymorphism and the homozygous MTRR 66G polymorphism conferred a 6-fold increase in the maternal risk of having a child with Down syndrome (95% *CI*, 2.058~17.496). In the combined populations, the MTHFR 677T substitution in one or both alleles was associated with a 3.78-fold increase in risk (95% *CI*, 1.78~8.47), and the presence of the homozygous 66G allele in MTRR was associated with a 5.2-fold increase in risk (95% *CI*, 1.90~14.22). A frequent challenge in conducting studies of gene-gene interactions is balancing the need for relatively large study populations against the need to minimize bias that may occur because of population admixture. To acquire sufficient statistical power, case mothers in the present study were drawn from two birth defect registries and a volunteer sample that included case mothers from 7 cities in middle China. Control mothers were limited to women who were documented to have no children with birth defects. To minimize ethnic bias, all participants were restricted to yellows. Thus, an inherent limitation of the current study is the potential bias of population admixture introduced by pooling control mothers from geographical areas different from those that provided the case mothers. The MTHFR 677T allele frequency in our control group was 0.28, which was within the range of frequencies reported in the meta-analysis. We had more difficulty in evaluating potential bias in the association between MTRR and Down syndrome, since there are no published data on allele frequencies or genotype distribution outside of our study sites. Thus, we compared the genotype distribution among our control mothers with that among fathers samples, who may be more ethnically similar to the case mothers than were a convenience sample of control mothers. The frequency of MTRR 66A→G homozygosity was 18.2% among the fathers and 17.0% among the control mothers. Taken together, these observations suggest that our combined control samples were representative of the MTHFR allele frequencies and MTRR homozygosity within middle China yellows. Nonetheless, the findings of the current study need to be confirmed in a large prospectively designed population-based case-control study. Because MTHFR and MTRR require folate and Vitamin B_{12}, respectively, to support the methionine synthase reaction, the metabolic impact of both polymorphisms is magnified by low levels of folate or Vitamin B_{12} (Gomez *et al*., 2006). Accordingly, risk estimates that stratify mutant genotypes by nutritional status result in more sensitive risk estimates than do those based on genotype alone. For example, stratification of the MTHFR 677C→T genotype by folate status or the MTRR 66A→G genotype by Vitamin B_{12} status has resulted in more sensitive risk estimates for neural tube defects (García-Casal *et al*., 2005). A limitation of the present study is that we did not have access to plasma samples for evaluation of folate, Vitamin B_{12} or homocysteine levels in case and con-

trol mothers. A compromise in the methionine synthase reaction caused by genetic and/or dietary factors could promote abnormal chromosome segregation by an indirect effect on oocyte DNA methylation patterns and higher-order chromatin structure. The secondary structure of pericentromeric heterochromatin, at repetitive satellite sequences, is involved in protein-DNA binding and in cohesion between sister chromatids (Neglia *et al*., 2003). The abundant methyl-binding protein, MeCP2, preferentially binds to pericentromeric DNA in a complex with histone deacetylase, resulting in local histone deacetylation and chromatin condensation (Valinluck *et al*., 2004). Recent evidence linking DNA methylation to histone deacetylation and chromatin condensation supports the possibility that DNA methylation patterns may be linked mechanistically to the epigenetic alterations in chromatin structure required for normal chromosome segregation. In non-replicating cells, such as oocytes, the loss of methyl groups at methylated CpG sites in DNA can occur during excision repair, by spontaneous deamination of 5-methyl cytosine to thymine, or during DNA strand exchange associated with recombination (Harvey and Newport, 2003). The failure of the DNA methyltransferase to remethylate the newly synthesized strands would result in permanent loss of DNA methylation patterns under conditions of localized folate methyl deficiency. The importance of stable pericentromeric DNA methylation for normal chromosome segregation has been underscored by several recent discoveries. For example, lymphocytes from individuals with the rare autosomal disorder ICF (immunodeficiency, centromeric instability, and facial anomalies) exhibit profound chromosomal abnormalities, including selective undermethylation of pericentromeric satellite DNA, chromosome decondensation, and complex multiradiate chromosomes (Lezhava *et al*., 2004). The genetic origin of this disorder was recently discovered to be a mutation in the de novo DNA methyltransferase 3B (*DNMT3B*) and supports a causal association between DNA hypomethylation, pericentromeric decondensation, and abnormal chromosome segregation (Geiman *et al*., 2004). Treatment of cultured cells with 5-azacytidine, a potent demethylating agent, similarly results in pericentromeric decondensation and profound chromatid mis-segregation in anaphase (Mosiolek *et al*., 2005). Chromosomal deletions, translocations and instability were recently reported in murine embryonic stem cells nullizygous for the major DNA methyltransferase (*Dnmt1*) gene (Ko *et al*., 2005). Aneuploidy and chromosomal instability are present in most human cancers, and chromosomal instability has recently been shown to be directly related to the extent of DNA hypomethylation (Kim *et al*., 2006). These data strongly suggest that stable methylation in pericentromeric DNA is an essential prerequisite for normal chromosome organization, stabilization and segregation. The association between folate deficiency and DNA hypomethylation lends support to the possibility that the increased frequency of the MTHFR and MTRR polymorphisms observed in the present study may be associated with chromosomal nondisjunction and Down syndrome. Further research is needed to evaluate the paradigm suggested by the present results: an association between maternal folate metabolism and Down syndrome. Additional studies of other candidate genes in the folate pathway, as well as a systematic study of interactions with other micronutrients involved in folate methyl metabolism in women with Down syndrome-affected pregnancies, may suggest opportunities to improve public health strategies for the primary prevention of Down syndrome.

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