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A66G and C524T polymorphisms of the methionine synthase reductase gene are associated with congenital heart defects in the Chinese Han population

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ABSTRACT. Congenital heart defects (CHDs) are the most common birth defects; genes involved in homocysteine/folate metabolism may play important roles in CHDs. Methionine synthase reductase (*MTRR*) is one of the key regulatory enzymes involved in the metabolic pathway of homocysteine. We investigated whether two polymorphisms (A66G and C524T) of the *MTRR* gene are associated with CHDs. A total of 599 children with CHDs and 672 healthy children were included; the polymorphisms were detected by PCR and RFLP analysis. Significant differences in the distributions of A66G and C524T alleles were observed between CHD cases and controls, and slightly increased risks of CHD were associated with 66GG and 524CT genotypes (odds ratios = 1.545 and 1.419, respectively). The genotype frequencies of 524CT

in the VSD subgroup, 66GG and 524CT in the PDA subgroup were significantly different from those of controls. In addition, the combined 66AA/524CT, 66AG/524CT and 66GG/524CT in CHDs had odds ratios = 1.589, 1.422 and 1.934, respectively. Increased risks were also observed in 66AA/524CT and 66GG/524CT for ASD, 66AG/524CT for VSD, as well as 66GG/524CT for PDA. In conclusion, *MTRR* A66G and C524T polymorphisms are associated with increased risk of CHDs.

Key words: Congenital heart defect; Polymorphisms; Homocysteine; Methionine synthase reductase; Folic acid

INTRODUCTION

Congenital heart defects (CHDs), which include the defects of the heart and its major blood vessels, are the most common birth defects and the leading cause of death from birth defects. CHDs occur in approximately 1% of live births, with a much higher percentage in those aborted spontaneously or stillborn (Hoffman and Kaplan, 2002). Every year, approximately 140-160 thousand children in China are born with CHDs. CHDs are mainly the result of incomplete development of the heart during the first six weeks of pregnancy (Botto et al., 2004). However, the pathogenesis of CHDs is not fully understood. Population studies have suggested that CHDs are associated with a genetic syndrome (e.g., Down syndrome) or they can be of multifactorial origin, involving genetic and environmental factors (Tennstedt et al., 1999; Botto and Correa, 2003).

Several population-based studies and experiments with animal models have shown that periconceptional multivitamin/folic acid supplementation has a strong protective effect during early stages of embryo development, resulting in a significant reduction in the occurrence of developmental defects, including neural tube defects, CHDs, limb defects, and facial-clefting (Itikala et al, 2001; Botto et al., 2003, 2004; Czeizel et al., 2004). Women who take 4 mg folic acid daily from the time of their periconceptional examination until the end of the first trimester of pregnancy have a 40-80% reduced risk of having a baby with neural tube defects (Czeizel and Dudás, 1992; Berry et al., 1999; Huhta et al., 2006). Several large-scale population-based case-control studies have shown that at least one in four heart defects could be prevented by periconceptional use of multivitamin supplements (Botto et al., 1996, 2000; Czeizel, 1998). In addition, epidemiological evidence has revealed that elevated plasma homocysteine levels (hyperhomocysteinemia) confer an increased risk of congenital defects (Rosenquist et al., 1996; Verkleij-Hagoort et al., 2006). Low or inadequate intakes of folic acid are involved in the disruption of methionine metabolism, because methytetrahydrofolate, the primary form of folate in the circulation, acts as the carbon donor for homocysteine remethylation to yield methionine and tetrahydrofolate (Elmore et al., 2007). Therefore, genes that participate in homocysteine/folate metabolism are attractive candidates for investigation of the mechanisms underlying the protective role of folic acid in CHDs (Fredriksen et al., 2007).

Methionine synthase reductase (*MTRR*) is one of the key regulatory enzymes involved in the homocysteine metabolic pathway. During methionine synthase (*MTR*)-catalyzed remethylation, the methyl group of 5-methyltetrahydrofolate is transferred to homocysteine, and

in this cobalamin-dependent reaction, cofactor cob(I)alamin is oxidized to cob(II)alamin, which inactivates *MTR*. By catalyzing the reductive methylation of cob(II)alamin, *MTRR* restores *MTR* activity (Figure 1) (Olteanu and Banerjee, 2001; Silaste et al., 2001; Gellekink et al., 2005).

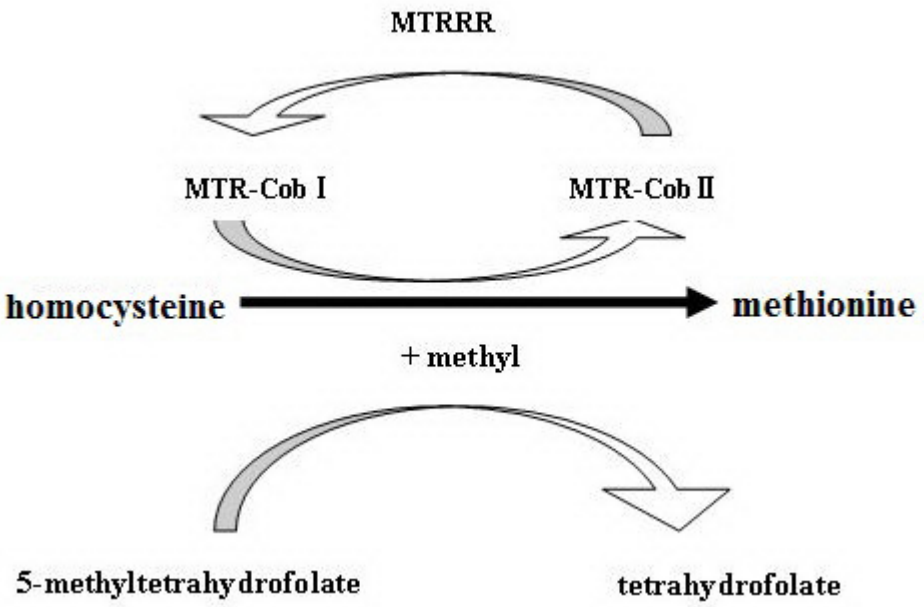

Figure 1. MTRR in the metabolism of homocysteine to methionine.

Since *MTRR* plays a crucial role in maintaining the activity state of *MTR*, nonsynonymous genetic variations within the *MTRR* gene may confer susceptible or protective effects against CHDs (Swanson et al., 2001; Elmore et al., 2007; Deng et al., 2008). The *MTRR* gene is located on chromosome 5 at 5p15.3-p15.2 (Leclerc et al., 1999). A common variant has been identified within the flavin mononucleotide-binding domain of the *MTRR* gene, the A66G polymorphism (rs1801394), which results in an isoleucine-to-methionine (I22M) substitution and appears to interact less effectively with methionine synthase (Olteanu and Banerjee, 2001). Another polymorphism C524T (rs1532268) leads to an amino acid change from serine to leucine (S175L). To our knowledge, few studies have been published concerning *MTRR* polymorphisms in relation to CHDs in the Chinese Han population. We investigated whether the two polymorphisms of the *MTRR* gene are associated with CHDs.

SUBJECTS AND METHODS

Patients and controls

A total 599 children (260 males and 339 females) with CHDs were included in this study from the Department of Pediatric Cardiology, West China Second University Hospital

in Chengdu, Sichuan Province, China. All patients were diagnosed based on echocardiography and cardiac catheterization (Table 1); 672 healthy subjects (317 males and 355 females) were recruited from the same hospital as the control group via health screening. Folate levels were not measured. Exclusion criteria were race other than Han, CHD in association with aneuploidies and as a part of genetic syndromes, maternal intake of potentially teratogenic pharmaceuticals as well as maternal exposure in an environment, which may cause abnormal embryo development (including smoking, alcohol, toxins, and radiation). All the participants were of the same ethnic origin and this study was approved by the Institutional Ethics Committee of West China Second University Hospital, Chengdu.

Table 1. Classification of the congenital heart disease groups.

Heart defect	Patients (N)
Atrial septal defect (ASD)	104
Ventricular septal defect (VSD)	282
Patent ductus arteriosus (PDA)	167
Pulmonary stenosis (PS)	46

Polymorphism detection

Peripheral blood samples were collected from cases and controls in 1-mL EDTA vacutainer tubes, and genomic DNA was extracted using standard salt fractionation and stored at -80°C. Information on the SNPs of the *MTRR* gene was derived from the SNP database (dbSNP) established by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/). Polymorphisms of the *MTRR* gene were detected by PCR and RFLP analysis (Table 2).

Table 2. Details of the PCR-RFLP method to determine each SNP of the *MTRR* gene.

SNP	Primers	PCR-RFLP method
rs1532268	5'-GTCAAGCAGAGGACAAGAG-3'	*Xho*I
(C524T)	5'-AGAGACTCCTGCAGATGTAC-3'	T allele: 309 bp
C/T		C allele: 62, 247 bp
rs1801394	5'-GCAAAGGCCATCGCAGAAGACAT-3'	*Ned*I
(A66G)	5'-AAACGGTAAACGGTAAAATCCACTGTAACGGC-3'	A allele: 25, 126 bp
A/G		G allele: 151 bp

The PCR was carried out in a volume of 25 μL, containing 100 pmol forward and reverse specific primers, 1.5 U Taq DNA polymerase, 200 μM dNTP, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 3 μL genomic DNA templates. PCR amplification conditions were as follows: after an initial denaturation step of 5 min at 95°C, 35 cycles (denaturation at 95°C for 30 s, annealing for 30 s at 59.3°C, and an extension step at 72°C for 30 s), followed by a final extension for 10 min at 72°C. Restriction enzyme digestion was performed, using specific *Xho*I and *Ned*I restriction enzymes (New England Biolabs, Beverly, MA, USA), and the digested PCR product was separated by electrophoresis on a 2.5-3% agarose gel. To assure accuracy of the PCR-RFLP results, each sample was analyzed twice. In order to verify

the genotypes by PCR-RFLP, the purified PCR products of random samples selected were sequenced directly (Figure 2) using the AB Sequence Detection System 7500 v1.4 software package (Applied Biosystems).

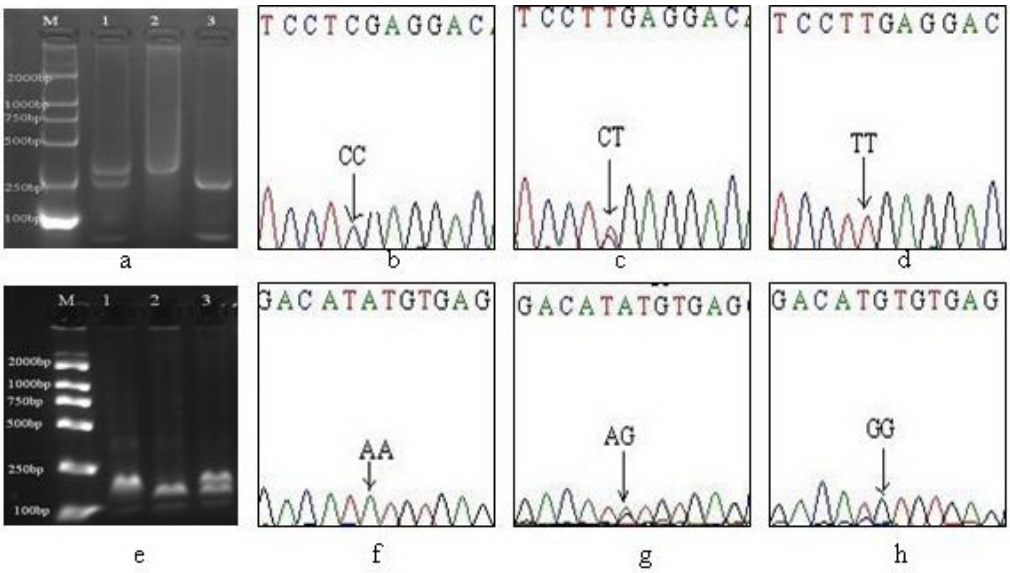

Figure 2. Determination of C524T and A66G genotypes of *MTRR* by gel electrophoresis after PCR-RFLP, verified by DNA sequencing tests. **a.** PCR amplifications of locus C524T digested by *Xho*I. *Lane M* = DNA marker; *lane 1* = CT; *lane 2* = TT; *lane 3* = CC. **b.** to **d.** DNA sequencing pictures of each genotype of C524T; SNP positions are indicated by arrows. **e.** PCR amplifications of locus A66G digested by *Ned*I. *Lane M* = DNA marker; *lane 1* = GG; *lane 2* = AA; *lane 3* = AG. **f.** to **h.** DNA sequencing pictures of each genotype of A66G; SNP positions are indicated by arrows.

Statistical analysis: case-control study

Genotype and allele frequency distributions of two SNPs in the *MTRR* gene were compared between CHD cases and controls, using the chi-square test. The odds ratios (OR) were calculated for relative risk along with the 95% confidence intervals (95%CI). All analyses were performed using SPSS for Windows, version 16.0. P values were two-tailed, and were considered to be significant at 0.05.

RESULTS

A66G and C524T allele frequencies

The distribution of the *MTRR* A66G and C524T alleles in CHD cases and controls was compatible with Hardy-Weinberg equilibrium (Table 3). A significant difference was observed between CHD cases and controls for A66G. The frequency of the 524T allele was significantly higher in CHD cases than in controls.

Table 3. A66G and C524T allele frequencies in congenital heart defect (CHD) cases and controls.

SNP	CHD cases	Controls	χ^2	P value	OR (95%CI)
A66G A	71.12 (852)	74.63 (1003)			Reference
G	28.88 (346)	25.37 (341)	3.956	0.047	1.194 (1.002-1.423)
C524T C	80.72 (967)	83.93 (1128)			Reference
T	19.28 (231)	16.07 (216)	4.505	0.034	1.248 (1.017-1.530)

Data are reported as percent with number in parentheses. OR = odds ratio; 95%CI = 95% confidence interval.

A66G and C524T genotype frequencies

Table 4 shows the distributions of *MTRR* genotypes. There were significant differences in the genotype frequencies of 66GG and 524CT for CHD cases compared with controls.

The prevalence of *MTRR* A66G and C524T combined genotypes in CHD cases and controls were also investigated (Table 4). When compared with controls, significant differences were observed in the combined genotype distributions of the three genotypes, 66AA/524CT, 66AG/524CT and 66GG/524CT (Table 4). None of the other comparisons were significant.

Table 4. Prevalence of the *MTRR* A66G and C524T polymorphisms in congenital heart defect (CHD) cases and controls.

Genotype	CHD cases	Controls	χ^2	P value	OR (95%CI)
A66G AA	51.58 (309)	55.80 (375)			Reference
AG	39.07 (234)	37.65 (253)	0.954	0.331	1.122 (0.889-1.417)
GG	9.35 (56)	6.55 (44)	4.109	0.043	1.545 (1.012-2.357)
C524T CC	63.94 (383)	70.83 (476)			Reference
CT	33.56 (201)	26.19 (176)	8.010	0.005	1.419 (1.113-1.810)
TT	2.50 (15)	2.98 (20)	0.041	0.840	0.932 (0.471-1.845)
66AA/524CC	36.23 (217)	43.45 (292)			Reference
66AA/524CT	14.19 (85)	10.71 (72)	6.411	0.011	1.589 (1.109-2.276)
66AA/524TT	1.17 (7)	1.64 (11)	0.100	0.752	0.856 (0.327-2.245)
66AG/524CC	22.87 (137)	23.36 (157)	1.189	0.275	1.174 (0.880-1.567)
66AG/524CT	15.52 (93)	13.10 (88)	4.130	0.042	1.422 (1.012-1.999)
66AG/524TT	0.67 (4)	1.19 (8)	0.415	0.519	0.673 (0.200-2.263)
66GG/524CC	4.84 (29)	4.02 (27)	1.719	0.190	1.445 (0.832-2.512)
66GG/524CT	3.84 (23)	2.38 (16)	3.930	0.047	1.934 (0.998-3.749)
66GG/524TT	0.67 (4)	0.15 (1)	2.821	0.093	5.382 (0.597-48.496)

Data are reported as percent with number in parentheses. OR = odds ratio; 95%CI = 95% confidence interval.

Subgroup analysis

The *MTRR* A66G and C524T genotype distributions of CHD subgroups were compared with controls (Table 5). The genotype frequencies of 524CT in the ventricular septal defect (VSD) subgroup, 66GG and 524CT in the patent ductus arteriosus (PDA) subgroup were significantly different from those of controls.

There were significant differences in the distributions of three combined genotypes (66AA/524CT, 66AG/524CT and 66GG/524CT) between CHDs and controls. To further identify the association between these three combined genotypes and CHD risk, we explored their distributions in the CHD subgroups. The frequencies of 66AA/524CT and 66GG/524CT genotypes in patients with atrial septal defect (ASD) were significant higher than controls. We also found that the frequency of 66AG/524CT genotype in patients with VSD and 66GG/524CT genotype in patients with PDA were significant higher than controls.

Table 5. Prevalence of the *MTRR* A66G and C524T polymorphisms in congenital heart disease subgroups.

Genotype	Control	ASD	VSD	PDA	PS
A66G AA	55.80 (375)	50.00 (52)	51.77 (146)	49.70 (83)	60.87 (28)
AG	37.65 (253)	39.42 (41)	41.49 (117)	35.92 (60)	34.78 (16)
GG	6.55 (44)	10.57 (11)	6.74 (19)	14.37 (24)*	4.35 (2)
C524T CC	70.83 (476)	62.50 (65)	63.83 (180)	61.68 (103)	76.09 (35)
CT	26.19 (176)	35.58 (37)	33.69 (95)*	35.33 (59)*	21.74 (10)
TT	2.98 (20)	1.92 (2)	2.48 (7)	2.99 (5)	2.17 (1)
66AA/524CC	43.45 (292)	32.69 (34)	36.88 (104)	34.73 (58)	45.65 (21)
66AA/524CT	10.71 (72)	16.35 (17)*	13.12 (37)	14.37 (24)	15.22 (7)
66AG/524CT	13.10 (88)	13.46 (14)	17.73 (50)*	16.17 (27)	4.35 (2)
66GG/524CT	2.38 (16)	5.77 (6)*	2.84 (8)	4.79 (8)*	2.17 (1)

Data are reported as percent with number in parentheses. *Compared with controls, $P < 0.05$. For subgroup abbreviations, see Table 1.

DISCUSSION

Single nucleotide polymorphisms are very helpful for finding genes that contribute to diseases (Brookes, 1999). Some SNP alleles are the actual DNA sequence variants that cause differences in gene function or regulation that directly contribute to disease processes (Brookes, 1999; Lai, 2001). Most SNP alleles, however, probably contribute little to disease. They are useful as genetic markers that can be used to find the functional SNPs because of associations between the marker SNPs and the functional SNPs.

Since *MTRR* is essential to maintain *MTR* function, it is a central regulatory enzyme in the metabolic pathway of homocysteine/folate. Malfunctioning of this enzyme may be associated with hyperhomocysteinemia. Hyperhomocysteinemia has been regarded as a modifiable risk factor for heart and vascular disease, because it is one of the causes of endothelial damage (Kapusta et al., 1999; Tierney et al., 2004; Verkleij-Hagoort et al., 2006).

In this case-control study, we looked for a possible association between the A66G and C524T polymorphisms of the *MTRR* gene and the development of CHDs. We calculated the ORs (see Tables 3, 4 and 6) and found that moderately higher risks of CHD were associated with 66GG and 524CT genotypes. We noted that even the 524T allele in CHD cases had a higher frequency than in controls; there were no significant differences in 524TT frequencies between the two groups. Possibly, the 524TT genotype was present at a low frequency in the population, and due to the limited sample size in our study, we did not detect differences between the two groups.

Considering the interaction of the two genotypes, we further explored the combined genotype frequencies of A66G and C524T of the *MTRR* gene. The combined 66AA/524CT, 66AG/524CT and 66GG/524CT gave significant ORs. The combined 66AA/524CT and 66AG/524CT genotypes of the *MTRR* gene may be risk factors for CHDs.

In order to find out whether the A66G and C524T polymorphisms of the *MTRR* gene have different effects on the most common types of CHD, we compared the frequencies in the four subgroups of CHD cases with controls. Many of the ORs were above 1, but only four of them reached significance (Tables 5 and 6). The patients with the combined genotypes 66AA/524CT and 66GG/524CT had higher risks of ASD (2.2- and 3.22-fold, respectively). In addition, the 66AG/524CT combined genotype was associated with a 1.6-fold higher risk of VSD, while the 66GG/524CT combined genotype was associated with a 2.5-fold increased risk of PDA, compared with controls.

Table 6. Calculated odds ratios (OR) with 95%CI (confidence intervals; in parentheses) of the *MTRR* polymorphisms in congenital heart disease subgroups.

Genotype	ASD	VSD	PDA	PS
A66G AA	Reference	Reference	Reference	Reference
AG	1.169 (0.753-1.813)	1.188 (0.888-1.589)	1.071 (0.741-1.549)	0.847 (0.449-1.598)
GG	1.803 (0.876-3.710)	1.109 (0.627-1.963)	2.464 (1.420-4.277)	0.609 (0.140-2.643)
C524T CC	Reference	Reference	Reference	Reference
CT	1.540 (0.992-2.388)	1.427 (1.055-1.932)	1.549 (1.077-2.229)	0.773 (0.375-1.593)
TT	0.732 (0.167-3.206)	0.926 (0.385-2.226)	1.155 (0.424-3.150)	0.680 (0.089-5.216)
66AA/524CC	Reference	Reference	Reference	Reference
66AA/524CT	2.208 (1.073-3.833)	1.443 (0.915-2.275)	1.678 (0.977-2.883)	1.352 (0.553-3.303)
66AG/524CT	1.366 (0.702-2.611)	1.595 (1.056-2.411)	1.545 (0.923-2.585)	0.316 (0.073-1.374)
66GG/524CT	3.221 (1.181-8.783)	1.404 (0.584-3.377)	2.517 (1.029-6.156)	0.869 (0.110-6.875)

For subgroup abbreviations, see Table 1.

Although some previous researchers reported that the two polymorphisms (A66G and C524T) of the *MTRR* gene are not associated with an increased risk of CHDs (van Beynum et al., 2006; Fredriksen et al., 2007; Verkleij-Hagoort et al., 2008; Shaw et al., 2009), we found a modest association between the A66G and C524T alleles of the *MTRR* gene and CHDs in the Chinese Han population. These differences between reports might be due to ethnic heterogeneity. However, as information concerning t-Hcy levels and the maternal genotypes of *MTRR* A66G and C524T polymorphisms is not available for all subjects, we could not further clarify the mechanism underlying the relationship between the *MTRR* A66G and C524T polymorphisms and CHD.

In conclusion, *MTRR* A66G and C524T polymorphisms are associated with increased risks of suffering CHDs. To further elucidate the relationship between *MTRR* A66G and C524T polymorphisms and CHDs, other risk factors, such as t-Hcy levels, enzyme activity, parental genotypes, and vitamin complex intakes should be investigated.

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