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Analysis of *MTR* and *MTRR* Gene Polymorphisms in Chinese Patients With Ventricular Septal Defect

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Background: Congenital heart defects (CHDs) are the most common birth defects and ventricular septal defects (VSDs) are one of the most common types of CHDs. Genes involved in homocysteine/folate metabolism may play important roles in CHDs. Methionine synthase and methionine synthase reductase (MTRR) are key regulatory enzymes involved in the metabolic pathway of homocysteine.

Methods: We investigated whether a polymorphism (A2756G) of the methionine synthase and 2 polymorphisms (A66G and C524T) of the MTRR gene are associated with VSDs. A total of 183 children with VSDs and 201 healthy children were studied.

Results: The polymorphisms were detected by polymerase chain reaction amplification and sequencing of the amplified product. Significant differences in the distributions of the A66G and C524T alleles were observed between VSD cases and controls, and a slightly increased risk of VSDs was associated with either of the 66AG, 524CT, and 524TT genotypes [odds ratios (OR) = 1.796, 1.909, and 2.088, respectively]. The genotype frequency of 66AG in VSDs patients was significantly different from those of controls (ORs = 3.147). In addition, the combined 66AG/524CT and 66GG/524TT in VSDs had ORs 2.937 and 5.344, respectively.

Conclusions: MTRR A66G and C524T polymorphisms are associated with increased risk of VSDs.

Key Words: ventricular septal defects, polymorphisms, methionine synthase, methionine synthase reductase, folic acid

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Congenital heart defects (CHDs) are among the most prevalent and serious birth defects, occurring in ~8 to 11 of every 1000 live births.[1–3] Ventricular septal defects (VSDs) is one of the most common types of CHDs. However, the pathogenesis of VSDs is not fully understood. Epidemiological studies have shown that folate supplements can significantly reduce the risk of cardiovascular disease,[4] neural tube defects, and cancer.[5,6] The mechanisms underlying these reduced risks have not been fully elucidated, although it has been speculated that folic acid restores some normal developmental functions.

Several population-based studies and experiments in animal models have shown that periconceptional multivitamin/folic acid supplementation has a substantial 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 clefts.[7–10] Numerous large-scale population-based case-control studies have shown that at least 1 in 4 heart defects could be prevented by periconceptional use of multivitamins.[11,12] In addition, homocysteine (Hcy), an intermediary in the synthesis of cysteine from methionine, is a nonessential sulfur-containing amino acid. Both environmental and genetic factors affect plasma Hcy levels. Low consumption of folate, vitamin B12 and B6, smoking and obesity are considered as environmental factors, whereas the genetic factors include several variants of enzymes involved in the Hcy metabolic pathway. Accordingly, genes that participate in Hcy/folate metabolism are attractive candidates for investigation of the mechanisms underlying the protective role of folic acid in CHDs.[13]

Methionine synthase reductase (MTRR) is one of the key regulatory enzymes involved in the Hcy metabolic pathway. During the methionine synthase (MTR)-catalyzed remethylation, the methyl group of 5-methyltetrahydrofolate is transferred to Hcy, and in such cobalamin-dependent reaction, the cofactor cob(I)alamin is oxidized to cob(II)alamin, which inactivates MTR. By catalyzing the reductive methylation of cob(II)alamin, MTRR restores the activity of MTR (Fig. 1).[14–16]

Since MTRR plays a crucial role in maintaining the activity level of MTR, nonsynonymous genetic variations within the MTRR gene may confer susceptibility to or protective effects against CHDs.[17–19]

To our knowledge, few studies have been published about MTR and MTRR polymorphism connected to

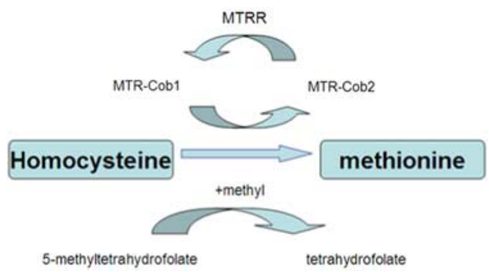

FIGURE 1. Methionine synthase reductase (MTRR) participates in the metabolism of homocysteine to methionine. full color online

folate influence on fetuses at risk for selected VSDs. Using population-based data, we investigated a single nucleotide polymorphism (SNP) in the MTR gene and 2 SNPs in the MTRR gene in the complex folate pathway as possible risk factors for VSDs.

METHODS

A total of 183 children with VSD admitted to Beijing Children's Hospital Heart Centre from September 2006 to December 2011 were recruited for this study. The diagnosis and classification of the VSDs was performed by a pediatric cardiologist based on the clinical and echocardiography findings with the surgical notes. In addition, 201 healthy control subjects with no history of congenital heart disease were recruited for our study.

Ethical approval was given by the Institutional Research Ethics Committee of Beijing Children's Hospital, Beijing, China. Informed consents were obtained from their parents. Similarly, consent forms were obtained from the parents of all the control subjects enrolled in this study.

Detection of Polymorphisms in MTR and MTRR Genes

Genomic DNA was isolated from peripheral blood leukocytes using standard salt fractionation and stored at -80°C. Primers (Table 1) were designed to amplify the MTR (A2756G) and MTRR (A66G, C524T) genes by polymerase chain reaction in patients and controls.[20,21] After purification using the AxyPrepDNA Gel Extraction kit (Axygen), the polymerase chain reaction products were sequenced (Fig. 2). We used an ABI3730XL sequence analyzer for DNA sequencing by Sangers method, which is based on the use of dideoxynucleotides (ddNTP's) in addition to the normal nucleotides (NTP's) found in DNA.[22] After sequences were obtained, we compared and analyzed them through https://www.ncbi.nlm.nih.gov/snp. In addition, identical results were obtained when genotyping was performed for 15% of the samples in 2 separate occasions.

Statistical Analysis: Case-Control Study

Genotype and allele frequency distributions of 2 SNPs in the MTR and MTRR genes were compared between the VSD cases and controls, using the χ^2 test. The odds ratios (ORs) were calculated for the relative risk along with the 95% confidence intervals. All analyses were performed using SPSS for Windows, version 16.0 (IBM). *P*-values are 2-tailed, and are considered as significant at 0.05.

RESULTS

MTR (A2756G) and MTRR (A66G and C524T) Allele Frequencies

The distribution of the MTR (A2756G) and MTRR (A66G and C524T) alleles in the VSD cases and controls was compatible with Hardy-Weinberg equilibrium (Table 2). Significant differences were observed between the CHD cases and controls for the A66G allele, but not for C524T and A2756G.

A66G and C524T Polymorphisms of the MTRR Gene

For the MTRR A66G polymorphism, the percentages of the A66A, A66G, and G66G genotypes in the VSD cases are shown in Table 2. Actually, there were 2 significant differences between the cases and controls ($P < 0.05$). In addition, the derived allele frequencies of the A and G alleles in the VSD cases and controls are shown in Table 2. There was a significant difference between the allele frequency in the cases and controls ($P < 0.05$).

For the MTRR C524T polymorphism, the genotype and its frequency differed significantly between the cases and controls ($P < 0.05$) (Tables 3, 4). The allele frequencies (Table 2) also showed no significance difference between the cases and controls ($P < 0.05$).

A2756G Polymorphisms of the MTR Gene

For the MTR A2756G polymorphism, the genotype and its frequency differed significantly between the cases

TABLE 1. Polymerase Chain Reaction Primer Sequences

Gene	SNP	Primers	Product (bp)
MTR	A2756G(rs1805087)	F: 5′-TGTTCCCAGCTGTTAGATGAAAATC-3′ R: 5′-GATCCAAAGCCTTTTACACTCCTC-3′	211
MTRR	A66G(rs1801394)	F: 5′-GCAAAGGCCATCGCAGAAGACAT-3′ R:5′-AAACGGTAAACGGTAAAATCCACTGTAACGGC-3′	151
	C524T(rs1532268)	F: 5′-GTCAAGCAGAGGACAAGAG-3′ R: 5′-AGAGACTCCTGCAGATGTAC-3′	309

MTR indicates methionine synthase; MTRR, methionine synthase reductase; SNP, single nucleotide polymorphism.

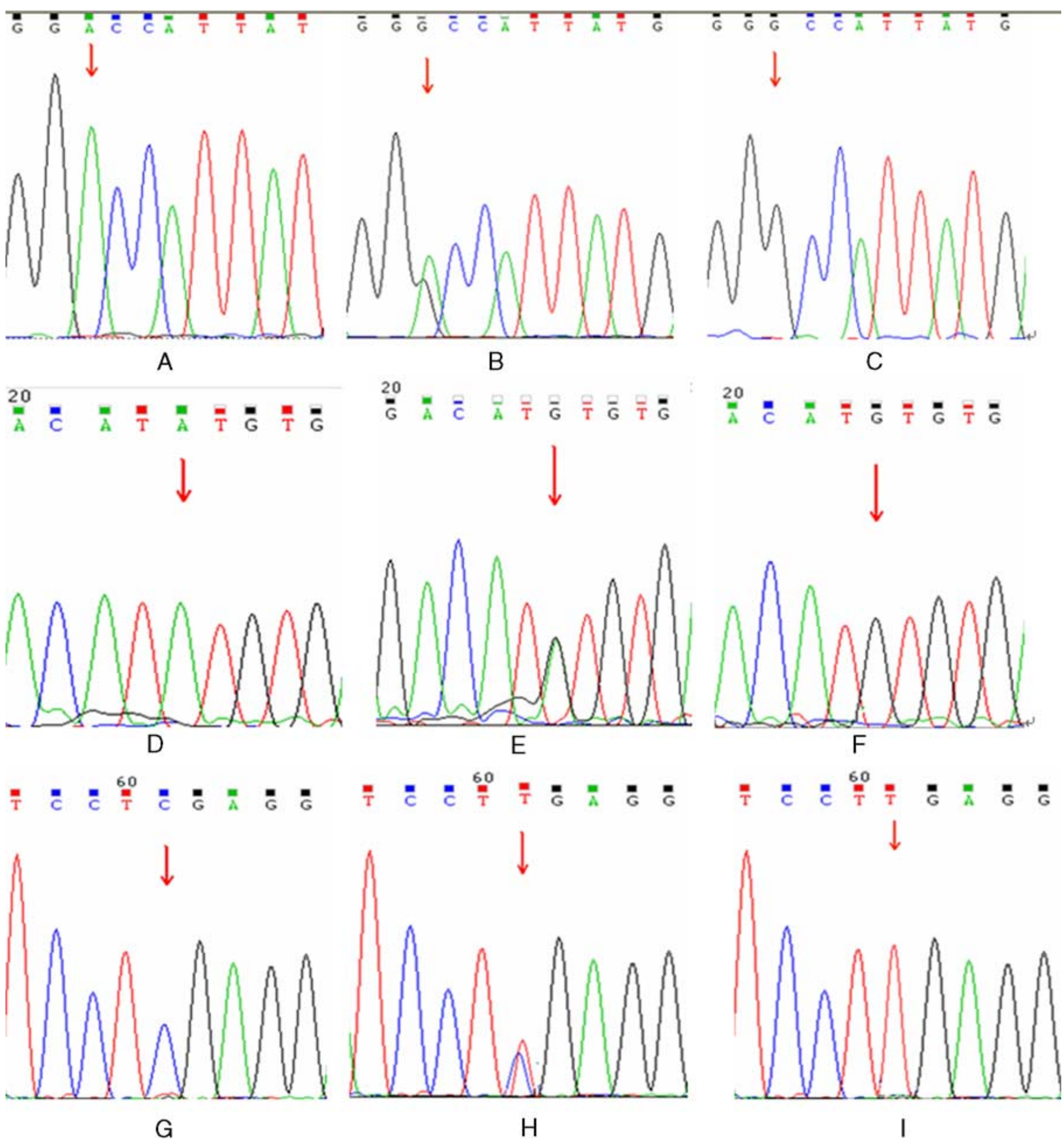

FIGURE 2. Determination of the genotypes of MTR and MTRR by DNA sequencing analysis (A–C). DNA sequencing pictures of each genotype of A2756G (D–F). DNA sequencing pictures of each genotype of A66G (G–I). DNA sequencing pictures of each genotype of C524T. SNP positions are indicated by arrows. MTR indicates methionine synthase; MTRR, methionine synthase reductase; SNP, single nucleotide polymorphism.

TABLE 2. MTR and MTRR Allele Frequencies in VSD Cases and Controls

Gene	SNP	VSD Cases [n (%)]	Controls [n (%)]	χ^2	*P*	OR (95% CI)
MTR	A2756G A	256 (66)	270 (67.2)	0.687	0.407	1.138
	G	132 (34)	132 (32.8)			(0.8382-1.544)
MTRR	A66G A	233 (63.6)	299 (74.4)	10.377	0.01*	0.603
	G	133 (36.4)	103 (25.6)			(0.443-0.823)
	C524T C	238 (63.3)	290 (72.1)	2.612	0.106	0.785
	T	138 (36.7)	112 (27.9)			(0.585-1.053)

*P-value < 0.05.

CI indicates confidence interval; MTR, methionine synthase; MTRR, methionine synthase reductase; OR, odds ratio; SNP, single nucleotide polymorphism; VSD, ventricular septal defect.

TABLE 3. Genotypic and Allelic Distribution of MTRR Gene Polymorphisms

SNP	Genotypes and Alleles	Case (%)	Control (%)
A66G genotypes	AA	68 (37.2)	107 (53.2)
	AG	97 (53)	85 (42.3)
	GG	18 (9.8)	9 (4.5)
	χ^2	*P*	OR (95% CI)
AG vs. AA	7.483	0.006*	1.796 (1.179-2.736)
GG vs. AA	7.399	0.007*	3.147 (1.337-7.407)
GG vs. AG	1.698	0.193	1.753 (0.748-4.107)
	Genotypes and alleles	Case (%)	Control (%)
C524T Genotypes	CC	66 (36.1)	105 (52.2)
	CT	96 (52.5)	80 (39.8))
	TT	21 (11.4)	16 (8)
	χ^2	*P*	OR (95% CI)
CT vs. CC	8.864	0.003*	1.909 (1.245-2.928)
TT vs. CC	4.123	0.042*	2.088 (1.017-4.288)
TT vs. CT	0.06	0.806	1.904 (0.535-2.236)

*P-value <0.05.
CI indicates confidence interval; MTRR, methionine synthase reductase; OR, odds ratio; SNP, single nucleotide polymorphism.

and controls ($P<0.05$; Table 2). However, the allele frequencies, listed in Tables 3, 4, showed no significant difference between the cases and controls ($P>0.05$).

DISCUSSION

SNPs are very helpful for identifying genes that contribute to disease pathogenesis.[23] Some SNP alleles are the actual DNA sequence variants that cause alterations in gene function or regulation which directly contribute to the disease processes.[23,24] Most SNP alleles, however, probably contribute little to the pathogenesis of disease. They are helpful as genetic markers that can be used to find the functional SNPs because of associations between the marker SNPs and the actual functional SNPs.

MTR is critical for Hcy metabolism, and MTRR is required to maintain MTR in its active state. Frequent polymorphisms in the genes encoding each of these enzymes are associated with changes that alter the primary structure of each protein, and both have been extensively analyzed regarding metabolites and disease association. Malfunctioning of these enzymes may be associated with hyperhomocysteinemia. Hyperhomocysteinemia has been regarded as a modifiable risk factor for heart and vascular disease, because it underlies endothelial damage.[25–27]

TABLE 4. Genotypic and Allelic Distribution of MTR Gene Polymorphisms

SNP	Genotypes and Alleles	Case (%)	Control (%)
A2756G Genotypes	AA	87 (47.5)	96 (47.8)
	AG	82 (44.8)	78 (38.8)
	GG	14 (7.7)	27 (13.4)
	χ^2	*P*	OR (95% CI)
AG vs. AA	0.47	0.493	1.16 (0.759-1.774)
GG vs. AA	2.427	0.806	0.527 (0.282-1.161)
GG vs. AG	3.827	0.05	0.493 (0.241-1.009)

CI indicates confidence interval; MTR, methionine synthase; OR, odds ratio; SNP, single nucleotide polymorphism.

In this case-control study, we looked for a possible association of the MTR (A2756G) and MTRR (A66G, C524T) gene polymorphisms with the development of VSDs. We calculated the ORs (Table 2) and found moderately higher risks of VSDs associated with the 66GG and 524CT genotypes of the MTRR gene. We noted that the 66G allele had a higher frequency in the VSD cases than in the controls. However, the frequency of the 524T and 2756G alleles did not differ between the 2 groups.

Our analyses revealed no evidence that the MTR A2756G variant may influence the risk of the VSDs. This finding is consistent with the results of 2 case-control studies, which also showed no evidence of an association between VSDs and MTR A2756G.[28,29] Interestingly, maternal genotypes that include the MTR 2756G allele have been associated with increased offspring risk of spina bifida and cleft lip with or without cleft palate.[30,31]

We also calculated the ORs (Tables 3, 4) and found that moderately higher risks of VSDs were associated with the MTRR genotypes (66GG, 66AG, 524TT, and 524CT), but not with the MTR genotype (A2576G). We also noted a higher frequency of the MTRR 66G allele in the VSD cases than in the controls. Actually, the difference in the 66GG frequency between the 2 groups was significant. However, as for other alleles, due to the limited sample size in our study, we did not detect differences between the 2 groups. The A66G genotype frequency was consistent with those predicted by the Hardy-Weinberg distribution, but the C524T genotype was not.

Considering the interaction of the 2 genotypes, we further explored the combined frequencies of the MTRR A66G and C524T genotypes. The combined 66AG/524CT and 66GG/524TT had significant ORs. Thus, the combined 66AG/524CT and 66GG/524TT genotypes of the MTRR gene may represent risk factors for VSDs. The

TABLE 5. Prevalence of Combined Genotype Frequencies of A66G and C524T of the MTRR Gene in VSD Cases and Controls

Genotype	VSD Cases (%)	Controls (%)	χ^2	*P*	OR (95% CI)
66AA/524CC	32	57		Reference	
66AA/524CT	28	39	0.55	0.458	1.279 (0.667-2.451)
66AA/524TT	8	11	0.254	0.614	1.295 (0.473-3.551
66AG/524CC	29	45	0.18	0.671	1.148 (0.607-2.17)
66AG/524CT	61	37	12.895	0*	2.937 (1.619-5.325)
66AG/524TT	7	3	4.364	0.37	4.156 (1.005-17.197)
66GG/524CC	5	3	2.192	0.193	2.969 (0.665-13.244)
66GG/524CT	7	4	3.153	0.076	3.117 (0.847-11.467)
66GG/524TT	6	2	4.696	0.03*	5.344 (1.018-28.044)

*P-value <0.05.
CI indicates confidence interval; MTRR, methionine synthase reductase; OR, odds ratio; VSD, ventricular septal defect.

ORs were significant, 2.937-fold and 5.344-fold, respectively (Table 5). This finding is somewhat consistent with the result of a previous study.[21] However, previously reported studies indicate that the 2 polymorphisms (A66G and C524T) of the MTRR gene are not associated with an increased risk of CHDs.[13,32–34] The differences between these reports and our study might be attributed to ethnic heterogeneity. However, as information on the t-Hcy levels and the frequencies of the maternal genotypes of MTRR A66G and C524T polymorphisms are not available for all subjects, we could not further elucidate the mechanism underlying the relationship between the MTRR A66G and C524T polymorphisms and VSDs.

In summary, the MTRR A66G and C524T polymorphisms are associated with increased risks of developing VSDs. Other risk factors, such as t-Hcy levels, enzyme activity, parental genotypes, and vitamin complex intakes should also be investigated to further elucidate the relationship between MTRR A66G and C524T polymorphisms and VSDs.

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