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Polymorphism of Methionine Synthase Gene in Nuclear Families of Congenital Heart Disease[1]

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Objective To investigate the relation of methionine synthase (MS) gene variation with congenital heart disease (CHD) phenotype. **Methods** One hundred and ninety three CHD patients (94 males and 99 females) and their biological parents (nuclear families) in Liaoning Province were selected as the case group, and another 104 normal persons (60 males and 44 females) and their parents without family history of birth defects as the control group. For all subjects the polymorphism of MS gene A2756G locus was examined by PCR-RFLP method. **Results** In offspring of the control group the frequencies of MS genotype (+/－) and allele (+) were 10.7% and 5.3%, without existence of homozygote. The MS genotype distribution and allele frequencies of CHD patients and their mothers were not significantly different from the control ($P > 0.05$). The frequency of allele (+) in case fathers (5.0 %) was apparently lower than that in the control (9.1%, P=0.060), and the odds ratio (OR) was 0.53 (95% CI: 0.25-1.09). There was no difference in parents' genotype combination between the two groups, and in genotype distribution among different types of CHD. Analysis of genetic transmission indicated that mutation allele (+) existed transmission disequilibrium in CHD nuclear families. The percentage of allele (+) transmitted from parents was lower than that allele (−) with OR 0.26 (95% CI: 0.11-0.60). **Conclusion** MS gene variation in parents is associated with occurrence of CHD in offspring, and mutation allele (+) in parents may be related with the decrease of CHD risk in offspring.

Key words: Methionine synthase; Gene polymorphism; Congenital heart disease; Nuclear family

INTRODUCTION

Congenital heart disease (CHD), the developmental malformation of embryo or fetus heart in uterus, is one of the most common pediatric diseases and the major cause leading to abortion and infant death. To date the etiology and mechanism of CHD remain unclear, and many studies interpreted that CHD was the result of interaction between genetic and environmental factors. In recent years the relation between homocysteine (HCY) and birth defects has become a focal point. Basic studies and epidemiological investigations have

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shown that hyperhomocysteinemia was an important risk factor of birth defects, especially of neural tube defects that are related closely to heart in development. Animal experiments indicated that HCY could lead to cardiovascular developmental malformation of chicken fetus[1,2]. Also genetic epidemiological studies showed HCY gene mutation of metabolic key enzyme, methylenetetrahydro-folate reductase (MTHFR), was the risk factor of CHD in infant[3]. The above results gave a clue that hyperhomocysteinemia might be related to occurrence of CHD[4]. HCY is the metabolic middle product of methionine, a kind of essential sulfur-bearing amino acid in nearly all tissues. One of the main metabolic paths of HCY is remethyled to methionine, which is catalyzed by methionine synthase (MS). So MS gene defects might lead to change of enzyme activity that influences HCY remethylation, and results in hyperhomocysteinemia and occurrence of CHD. But now there is not any report about the relation between MS gene variations and CHD.

Nuclear family consists of a child and his/her biological parents. In this kind of research design the alleles of offspring are thought as "case" and the non-inherited alleles of parents as "intra-control", to seek for the genetic biomarkers related to diseases. By designing the selection bias of genetic race difference could be overcome without looking for the same background control. In this study the relation between MS genotype and CHD phenotype was probed by group-matched case-control design, and MS allele transmission disequilibrium was investigated by intra-control design based on CHD nuclear families.

METERIALS AND METHODS

Subjects

By birth defects registration cards in Liaoning Province, 193 CHD patients (0-31 years old, 94 males and 99 females) and their biological parents were selected as the case group. The types of CHD were diagnosed by expert doctors, including 85 (44.0%) ventricular septal defect patients, 28 (14.5%) arterial duct patents, 19 (9.8%) tetralogy of Fallot, 16 (8.3%) arterial septal defects, 32 (16.6%) other types (pulmonary artery stenosis, Ebstein's anomaly and so on) and 13 (6.7%) combined types of CHD.

Another 104 normal persons (0-33 years old, 60 males and 44 females) and their parents were selected in same district as the control group, with age and sex matched and without birth defects family history.

Blood Sample Collection

Three-five mL fasting venous blood was collected from all subjects, and then the serum and blood clotting were separated.

MS Genotyping

Genome DNA was extracted from blood clotting by salting-out method. The 189 bp fragment of MS gene A2756G locus was amplified by PCR with a pair of primers[5]: 5′-CAT GGA AGA ATA TGA AGA TAT TAG AC-3 and 5′-GAA CTA GAA GAC AGA AAT TCT CTA-3′. PCR was performed in a total volume of 10 μL containing 2.0 mmol • L^{-1} $MgCl_2$, 0.5 μmol • L^{-1} primers, 0.25 mmol • L^{-1} dNTPs, 1.2 U Taq DNA polymerase and DNA template 2 μL. Reaction was conducted as an initial melting at 94℃ for 4 min followed by 30 cycles consisting of melting at 94℃ for 30 s, annealing at 55℃ for 30 s, and extension at 72℃ for 45 s. A final extension was lasted for 7 min at 72℃ to end the procedure. The

189 bp amplification fragment was digested by 6 U HaeⅢ restriction endonuclease for 4 hours at 37℃ and subsequent electrophoresis in a 12% polyacrylamide gel. If the enzyme site existed, the PCR product would be digested into fragments of 159 and 30 bp in the presence of (+) allele, and if not, there was only 189 bp in the presence of (-) allele.

Statistics

Data analysis was carried out using SPSS and Epi info software. Standard summary statistics, chi-square tests and calculation of odds ratio were used where appropriate. Statistical significance was interpreted as $P<0.05$.

RESULTS

Comparison of Genotype Distribution and Allele Frequencies at MS Gene A2756G Locus in Case Group With Control

In the individuals (offsprings in the control group), the frequencies of heterozygote (+/-) and mutation allele (+) were 10.7% and 5.3% respectively, with no difference of sex (P=0.263). And no homozygote (+/+) was detected. The comparison of genotype distribution and allele frequencies showed no significant difference between CHD patients and the control (P>0.05). The odds ratio (OR) of genotype (+/-) was 0.84 (95% confidence interval, 95% CI: 0.35-2.01) (Table 1).

TABLE 1

Comparison of Genotype Distribution in CHD Patients With Control at MS A2756G Locus

Group	Sex	*n*	Genotype *n* (%)		OR(95%CI)	*P*-value [a]	Allele Frequency (%)[b]	
			-/-	+/-			-	+
Case	Male	90	82(91.1)	8(8.9)	0.62(0.20-1.97)	0.368	95.6	4.4
	Female	96	87(90.6)	9(9.4)	1.41(0.33-6.99)	0.616	95.3	4.7
	Total	186	169(90.9)	17(9.1)	0.84(0.35-2.01)	0.672	95.4	4.6
Control	Male	59	51(86.4)	8(13.6)			93.2	6.8
	Female	44	41(93.2)	3(6.8)			96.6	3.4
	Total	103	92(89.3)	11(10.7)			94.7	5.3

Note. [a] Comparison of genotype frequency with control group by chi-square test, [b] Compared with control group [OR＝0.85(0.37-1.98) , P=0.680].

The analysis of mothers' genotype distribution and alleles frequencies showed no significant difference between the two groups (P>0.05). The percentage of carriers with allele (+), including (+/-) and (+/+) genotypes, was lower in CHD fathers than that in the control (10.0% and 17.2% respectively, P＝0.084), with OR 0.54 (95% CI: 0.25-1.16). And the mutation allele (+) frequency of CHD fathers was also decreased, compared with that of the control (5.0% and 9.1%, P=0.060) with OR 0.53 (95% CI: 0.25-1.09).

TABLE 2

Comparison of Genotype Distribution and Allele Frequencies in CHD Parents With Control at MS A2756G Locus

Group	Parent	*n*	Genotype *n* (%)			OR (95%CI)	*P*-value [a]	Allele Frequency (%)[b]	
			-/-	+/-	+/+			-	+
Case	Mother	184	160(87.0)	24(13.0)	0(0.0)	1.11(0.50-2.49)	0.778	24(6.5)	344(93.5)
	Father	180	162(90.0)	18(10.0)	0(0.0)	0.54(0.25-1.16)	0.084	18(5.0)	342(95.0)
	Total	364	322(88.5)	42(11.5)	0(0.0)	0.77(0.45-1.32)	0.311	42(5.8)	686(94.2)
Control	Mother	101	89(88.1)	12(11.9)	0(0.0)			12(5.9)	190(94.1)
	Father	99	82(82.8)	16(16.2)	1(1.0)			18(9.1)	180(90.9)
	Total	200	171(85.5)	28(14.0)	1(0.5)			30(7.5)	370(92.5)

Note. [a]Comparison of genotype frequency with control group by chi-square test, [b]Compared with control group by Chi-square test, Mother: OR＝1.10(0.52-2.40), *P*＝0.785, Father: OR＝0.53 (0.25-1.09), *P*＝0.060, Total: OR＝0.76(0.45-1.26) , *P*＝0.255.

Comparison of MS Genotype Distribution in Different Types of CHD With Control

Regarding one type of CHD with or without other types of CHD as one group, the comparison of MS genotype distribution in different types of CHD patients with the control showed no significant difference (Table 3). There was no difference among all the groups *(P* =0.481).

TABLE 3

Comparison of Genotype Distribution Among Different Types of CHD Patients With Control Group at MS A2756G Locus

Group	*n*	Genotype *n* (%)		OR(95％CI)	*P*-value [a]
		-/-	+/-		
Ventricular Septal Defect	91	80(87.9)	11(12.1)	1.15(0.44-3.04)	0.758
Atrial Septal Defect	21	19(90.5)	2(9.5)	0.88(0.00-4.78)	0.875
Patent Ductus Arteriosus	34	2(94.1)	2(5.9)	0.52(0.08-2.60)	0.327
Tetralogy of Fallot	20	20(100.0)	0(0.0)	0.00(0.00-2.02)	0.126
Other Types	9	9(100.0)	0(0.0)	0.00(0.00-4.94)	0.302
Control	103	92(89.3)	11(10.7)		

Note. [a]Compared with control group by Chi-square test.

The comparison of parents' genotype distribution in different types of CHD with the control showed no significant difference (Table 4). There was no difference among all the groups for mother (*P*=0.882) and father (*P*=0.515).

TABLE 4

Comparison of Genotype Distribution in Parents Among Different Types of CHD With Control at MS-A2756G Locus

Group	Parent	*n*	Genotype *n* (%)		OR (95%CI)	*P*-value [a]
			-/-	+/- and +/+		
Ventricular Septal Defect	Mother	90	79(87.8)	11(12.2)	1.03(0.40,2.68)	0.943
	Father	86	77(89.5)	9(10.5)	0.56(0.22,1.44)	0.192
Atrial Septal Defect	Mother	19	18(94.7)	1(5.3)	0.41(0.01,3.15)	0.396
	Father	21	19(90.5)	2(9.5)	0.51(0.05,2.46)	0.521
Patent Ductus Arteriosus	Mother	32	29(90.6)	3(9.4)	0.77(0.13,3.13)	0.697
	Father	36	31(86.1)	5(13.9)	0.78(0.21,2.45)	0.649
Tetralogy of Fallot	Mother	20	17(85.0)	3(15.0)	1.31(0.21,5.60)	0.713
	Father	19	18(94.7)	1(5.3)	0.27(0.01,1.96)	0.299
Control	Mother	101	89(88.1)	12(11.9)		
	Father	99	82(82.8)	17(17.2)		

Note. [a] Compared with control group by Chi-square test.

Comparison of Parents' Genotype Combination in Case Group With Control

There was no significant difference in genotype combination between parents of the case group and of the control (Table 5). Further comparison of families at least one parent carrying allele (+) with both parents carrying genotype (-/-) showed no difference between the two groups ($\chi^2=1.060$, $P=0.303$), and OR was 0.74(95% CI: 0.40-1.37).

TABLE 5

Comparison of Genotype Combination of CHD Parents With Control

Group	*n*	Genotype Combination of Parents [a] *n* (%)		
		-/- and -/-	-/- and (+/- or +/+)	+/- and (+/- or +/+)
Case	172	135(78.5)	33(19.2)	4(2.3)
Control	96	70(72.9)	26(27.1)	0(0.0)

Note. [a] Compared between two groups by Chi-square ($P=0.121$).

Analysis of Alleles Transmission Disequillibrium in CHD Nuclear Families

Transmission disequillibrium of alleles was analyzed by transmitted disequilibrium test (TDT) (Table 6) and haplotype-based haplotype relative risk (HHRR) (Table 7) calculation. In 1:1 matched case-control design, the pair of alleles of CHD patients transmitted from parents was regarded as case, and the other pair of alleles without transmission from parents as control. The results showed that mutation allele (+) was transmitted disproportionally in CHD nuclear families, which meant allele (-) was transmitted from parent to fetus more

proportionally, and increased the CHD risk of fetus. So it indicated that allele (+) might be related to decrease of CHD risk.

TABLE 6

Analysis of TDT in CHD Nuclear Families

Allele Without Transmission From Parents	Allele Number of CHD Patients [a]		Total
	+	-	
+	1	28	29
-	7	280	287
Total	8	308	316

Note. [a] TDT(χ^2) =10.028, P<0.05, OR=0.29.

TABLE 7

Analysis of HHRR in CHD Nuclear Families

Transmission of Allele	Allele Number [a]		Total
	+	–	
Transmitted From Parents	8	308	316
Not Transmitted From Parents	29	287	316

Note. [a] HHRR(χ^2)=12.66, P=0.000, OR=0.26 (95% CI:0.11-0.60).

DISCUSSION

The main biochemical function of MS is to catalyze the remethylation of HCY to methionine, so it is the key enzyme of HCY metabolic paths. MS gene is located at chromosome 1q43, and more than ten mutation loci of MS gene have been reported. The A2756G locus mutation (codon D919G) is common in western population leading to the appearance of endonuclease HaeIII, and might change the serum HCY level. The study showed in the normal control population the frequencies of heterozygote (+/-) and mutation allele (+) at MS gene A2756G locus were 10.7% and 4.6% separately, without any homozygote detected. It was reported that in Caucasian the frequencies of genotype (+/-) and (+/+) were 30% and 4%[6], and in Japanese the frequency of allele (+) was 17%[7]. Other studies reported in Chinese the heterozygote and homozygote frequencies were 17% and 1%, and allele (+) was 9.5%[5]. The results showed that the mutation frequency of the studied population was much lower than that of Caucasian and Japanese, even lower than that of other Chinese population reported. This indicated that there might be ethnic and territorial difference of genetic variation. Therefore, further investigations are needed on the polymorphism of MS gene A2756G locus in Chinese.

The analysis of relation of MS gene A2756G locus variation with CHD showed that there was no difference in the genotype distribution and alleles frequencies between the case and control groups (P>0.05), and the OR of heterozygote was 0.84 (95% CI: 0.35-2.01). Further comparison of different sex and CHD types showed no significant difference between the case and control groups, with OR 0-0.15 (P>0.05). The genetic variations of parents could transmit the mutation gene to offspring, and change the intrauterine environment and influence the development of fetus. So it is very important for early

screening, prevention and treatment to know the relation of parents' genotype with phenotype in offspring. In the study the MS gene variation of CHD parents was investigated and the results showed that the frequencies of genotypes and alleles in CHD mothers were not apparently different from the control, and the frequency of allele (+) in CHD fathers was lower than that in the control (5.0% vs 9.1%, *P*=0.060), with OR 0.53 (95% CI: 0.25-1.09). The results indicated that the parents (especially the father) carrying the mutation allele (+) might decrease the CHD risk of offspring.

Up to now there has been no report about the relations of MS gene polymorphism with CHD. Some of the studies focused on the association of MS gene variation with neural tube defects[8], and the results were always contradictory and could not clarify the association[9]. As mentioned above, MS is the key enzyme in metabolic path of HCY. The MS gene 2756A→G variation might lead to the deletion mutation of codon 919D→G, which made asparagic acid replaced by aminoacetic acid. Because the amino acid coded by codon 919 is located in the enzyme activity area, the gene locus mutation might increase or decrease the MS activity by changing the secondary structure of protein. This also could influence the HCY level *in vivo* and further disturb the development of multiple organs and systems, such as cardiovascular and neural systems in fetus[6]. Some studies showed that the HCY level was decreased progressively among (-/-), (+/-) and (+/+) genotypes[6,10], but others showed increasing tendency or no significant association between A2756G locus mutation and HCY [11,12]. In the study the result showed that the parent with mutation allele (+) might decrease the CHD risk of offspring, indicating that the MS gene variation could increase the enzyme activity and decrease HCY level and related birth defects risk. So in the next study the HCY level would be detected to certify the relation of A2756G locus variation with HCY, which is the bridge to investigate the association of MS gene mutation with CHD.

The case-parent control study was a matched study in fact, in which the two alleles of patients were as "case" and another two alleles of parents not transmitted to fetus as "control". The greatest advantage of the design was that it could overcome the selection bias of the control without selecting the same genetic background group with the case[13]. The study showed that there existed genetic disequilibrium of mutation allele (+) in CHD nuclear families, suggesting the percentage of allele (-) transmitted from parents was higher than that of allele (+). The results indicated that the MS mutation allele (+) might decrease the CHD risk of fetus with OR 0.26 (95% CI: 0.11-0.60). So it is concluded that the MS gene A2756G locus variation in parents is associated with the occurrence of CHD in offspring, and mutation allele (+) may decrease the CHD risk.

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