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Relationship Between Polymorphism of Cystathionine beta Synthase Gene and Congenital Heart Disease in Chinese Nuclear Families[1]

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Objective To study the relationship between polymorphism of cystathionine beta synthase (CBS) gene and development of congenital heart disease (CHD). **Methods** One hundred and twenty-seven CHD case-parent triads were recruited from Liaoning Province as patient group, and 129 healthy subjects without family history of birth defect were simultaneously recruited as control group together with their biological parents. For all subjects the polymorphism of CBS gene G919A locus was examined by PCR-ARMS method. **Results** The frequencies of three genotypes (w/w, w/m, and m/m) in control group were 27.2%, 58.4%, and 14.4%, respectively, with no significant difference in gender. A significant difference in the allele frequency was found between CHD patients and controls, the wild allele frequency was 67.9% in patients and 55.7% in controls. CHD parents' genotype distribution was significantly different from that in controls. Further comparison of each type of CHD showed that genotype frequencies in several CHD subtypes were significantly different from those in their corresponding controls. The results of TDT analysis showed that no allele transmission disequilibrium existed in CHD nuclear families. **Conclusions** CBS gene G919A mutation is associated with the development of CHD, and the mutated allele may decrease the risk of CHD.

Key words: Congenital heart disease; Gene polymorphism; Cystathionine beta synthase; Case-control family study

INTRODUCTION

The term "congenital heart disease (CHD)" indicates that a structural problem (or defect) in a baby's heart is present at birth. It is the most common type of major birth defects and the leading cause of infant death. Currently, it is well recognized that both genetic and environmental factors play an important role in CHD etiology, but the specific causes of most CHD cases are still unknown.

Studies have shown that vitamins B_{12}, B_6, and folic acid are essential to the metabolism of homocysteine and supplementation of these vitamins, particularly folic acid, decreases levels of plasma homocysteine[1]. Animal experiments have shown that homocysteine impairs or disturbs the normal cardiovascular development in earlier stage, and then leads to the development of CHD in chicken fetus[2]. Epidemiological investigations have also implied that maternal hyperhomocysteinemia may be a risk factor for CHD[3], and folic acid can prevent the development of birth defects including cardiac defects[4].

The early work of our laboratory and other research groups has shown that gene variations of the key metabolic enzymes that can strongly influence the metabolic progress of folic acid and homocysteine, such as methylene-tetrahydrofolate reductase (MTHFR), methylene-tetrahydrofolate dehydrogenase (MTHFD), and methionine synthase (MS) are associated with the development of CHD[5-8]. Cystathionine beta synthase (CBS) is the first key enzyme of homocysteine transsulfuration pathway, and catalyzes the condensation of homocysteine with serine to form cystathionine, and ultimately, cysteine. CBS gene G919A change is a pyridoxine unresponsive mutation, and then the mild impairment of enzyme function affects homocysteine concentration[9-10]. Therefore we hypothesize that there may be an association between polymorphism

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of CBS and development of CHD. This work aimed to explore the relationship in a case-control family study.

MATERIALS AND METHODS

Subjects

One hundred and twenty-seven CHD case-parent triads were recruited from Liaoning Province by birth defect registration cards. The average age of these patients (59 males and 68 females) was 6.75 years (from less than 1 month to 27 years). All patients were diagnosed by specialists in pediatric cardiology. Among them, there were 27 patients with atrial septal defect (ASD), 55 with ventricular septal defect (VSD), 29 with patent ductus arteriosus (PDA), 31 with tetralogy of Fallot (TOF) and other types (pulmonary artery stenosis, Ebstein's anomaly, *et al.*) (We counted twice or more when patients had more than one type of defect).

One hundred and twenty-nine healthy subjects (from 9 months old to 33 years old, 73 males, and 56 females) without family history of birth defect were simultaneously recruited from the same geographic area as control group together with their biological parents.

After informed consent was obtained, 3-5 mL venous blood was drawn from all the subjects and their parents.

Genetic Analysis

Genomic DNA was extracted manually from blood clot using a standardized salting-out method. The CBS gene fragment containing G919A mutation locus was amplified by PCR method with the primers[11]: wild type sense: 5′-CTA CGA GGT GGA AGG GAT CG-3′; mutant type sense: 5′-CTA CGA GGT GGA AGG GAT CA-3′; and antisense: 5′-GCC TCC TCA TCG TTG CTC TT-3′. Reaction was carried out in a volume of 20 μL containing 1.2 units Taq DNA polymerase, 10 mmol/L Tris-HCl, 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 0.25 mmol/L deoxynucleoside triphosohates, 0.5 μmol/L primers, and 2 μL genomic DNA templates. After denaturation for 4 min at 94℃, temperature was cycled 35 times (94℃, 45 sec; 60℃, 75 sec; and 72℃, 120 sec), followed by extension at 72℃ for 7 min. Then the reaction mixtures were subjected to 1% agarose gel electrophoresis at 100 V. When the genotype was wild type, the 655 bp fragment was amplified only under the operation of wild type primer; and when the genotype was mutant type, the same size fragment was amplified only under the operation of mutant type primer; and both when heterozygous.

Statistical Analysis

Statistical analysis was performed using SPSS11.0 and Epi Info 6. Chi-square tests, transmission disequilibrium test (TDT), and haplotype-based haplotype relative risk (HHRR) analysis were used where appropriate. The odds ratio (OR) was calculated by Epi Info. $P<0.05$ was considered statistically significant.

RESULTS

Comparison of Genotype Distribution and Allele Frequency Between Patient Group and Control Group at CBS G919A Locus

In controls and their parents, the frequencies of three genotypes (w/w, w/m, and m/m) were 27.2%, 58.4%, and 14.4%, respectively, with no significant difference in gender (P=0.48, data not shown). Results of comparison of genotype distribution showed that there was a significant difference between CHD patients and controls, with OR 0.43 (95%CI: 0.24-0.78). However, when groups were divided into subgroups by gender, the significant difference disappeared in male subgroup (P=0.181), but not in female subgroup (P=0.010). Comparison of allele frequencies between patients and controls in female subgroup disclosed a significant difference, with wild allele frequency 72.6% in patients *vs* 52.2% in controls (Table 1).

In mother and father subgroups, significant differences were found in the genotype distribution between patients and controls. The allele frequency was also different between patient and control groups. In mother subgroup, the frequencies of mutant allele in patients and controls showed a significant difference (34.0% and 43.8%, respectively; P=0.041). While in father subgroup, the frequency of mutated allele in patients (28.8%) was also lower than that in controls (42.7%) (P = 0.002) (Table 2).

Comparison of CBS Genotype Distribution Between Different Types of CHD Patients and Controls at CBS G919A Locus

Regarding each type with or without other types of CHD as one subgroup, we compared the CBS genotype distribution in each subgroup with controls. The results of patients showed that PDA subgroup was significantly different from those of controls (P=0.027), while other subgroups showed no significant difference ($P>0.05$) (Table 3).

TABLE 1

Comparison of Genotype Distribution and Allele Frequency in CHD Patients With Controls at CBS G919A Locus

Group	Gender	Number	Genotype (*n*)			OR[a] (95% CI)	*P*-value	Allele Frequency (%)		*P*-value[b]
			w/w	w/m	m/m			w	m	
Patient	Male	42	17	18	7	0.57 (0.25-1.31)	0.181	61.9	38.1	0.593
	Female	53	25	27	1	0.32 (0.13-0.78)	0.010	72.6	27.4	0.003
	Total	95	42	45	8	0.43 (0.24-0.78)	0.005	67.9	32.1	0.012
Control	Male	61	17	37	7			58.2	41.8	
	Female	45	10	27	8			52.2	47.8	
	Total	106	27	64	15			55.7	44.3	

Note. [a]Put w/m and m/m together, and compared with corresponding control by chi-square test. [b]Compared with corresponding control.

TABLE 2

Comparison of Genotype Distribution and Allele Frequency in CHD Parents With Controls at CBS G919A Locus

Group	Parent	Number	Genotype (*n*)			OR[a] (95% CI)	*P*-value	Allele Frequency (%)		*P*-value[b]
			w/w	w/m	m/m			w	m	
Case	Mother	103	44	48	11	0.45 (0.25-0.81)	0.007	66.0	34.0	0.041
	Father	111	54	50	7	0.47 (0.27-0.82)	0.007	71.2	28.8	0.002
	Total	214	98	98	18	0.46 (0.31-0.69)	0.001	68.7	31.3	0.001
Control	Mother	104	26	65	13			56.2	43.8	
	Father	110	34	58	18			57.3	42.7	
	Total	214	60	123	31			56.8	43.2	

Note. [a]Put w/m and m/m together, and compared with corresponding control by Chi-square test. [b]Compared with corresponding control.

TABLE 3

Comparison of CBS Genotype Distribution Between Different Types of CHD Patients and Controls

Group	Number	Genotype (*n*)		OR[a] (95%CI)	Chi-squares	*P*-value
		w/w	w/m + m/m			
Atrial Septal Defect	19	6	13	0.74 (0.26-2.14)	0.31	0.578
Ventricular Septal Defect	41	17	24	0.48 (0.23-1.03)	3.61	0.058
Patent Ductus Arteriosus	25	12	13	0.37 (0.15-0.91)	4.91	0.027
Tetralogy of Fallot and Other Types	24	10	14	0.48 (0.19-1.20)	2.52	0.112
Control	106	27	79			

Note. [a]Compared with control.

The results of comparison of parents' genotype distribution in different subgroups with controls are listed in Table 4. Significant differences were seen between ASD mother, VSD mother, VSD father, TOF plus other types in mother subgroup and their corresponding controls, respectively. The *P*-values were all lower than 0.05. The *P*-value of TOF plus other types in father subgroup was below 0.05, but its 95% confidence interval contained 1.

TABLE 4

Comparison of CBS Genotype Distribution Between Different Types of CHD Patients and Control

Group	Parent	Number	Genotype (*n*)		OR[a] (95%CI)	Chi-squares	*P*-value
			w/w	w/m + m/m			
Atrial Septal Defect	Mother	21	11	10	0.30 (0.12-0.80)	6.29	0.012
	Father	23	8	15	0.84 (0.32-2.17)	0.13	0.716
Ventricular Septal Defect	Mother	47	21	26	0.41 (0.20-0.85)	5.85	0.016
	Father	51	27	24	0.40 (0.20-0.79)	7.19	0.007
Patent Ductus Arteriosus	Mother	23	5	18	1.20 (0.41-3.55)	0.11	0.742
	Father	26	11	15	0.61 (0.25-1.47)	1.23	0.267
Tetralogy of Fallot and Other Types	Mother	25	14	11	0.26 (0.11-0.65)	9.05	0.003
	Father	25	13	12	0.41 (0.17-1.00)	3.99	0.046
Control	Mother	104	26	78			
	Father	110	34	76			

Note. [a] Compared with corresponding control.

Analysis of Allele Transmission Disequilibrium in CHD Nuclear Families

Transmission disequilibrium of alleles was analyzed by transmission disequilibrium test (TDT) (Table 5) and haplotype-based haplotype relative risk (HHRR) calculation (Table 6). The results showed that there existed no allele transmission disequilibrium in CHD families (χ^2=0.012, $P>0.05$). HHRR calculation also showed that there was no significant difference between alleles transmitted and not transmitted from parents (χ^2=0.02, $P>0.05$).

TABLE 5

Analysis of TDT in CHD Nuclear Families

Alleles not Transmitted From Parents	Alleles Transmitted From Parents		Total
	w	m	
w	61	40	101
m	41	8	49
Total	102	48	150

Note. McNemar χ^2=0.012, *P*=0.913.

TABLE 6

Analysis of HHRR in CHD Nuclear Families

Alleles	Number of Alleles		Total
	w	m	
Transmitted From Parents	102	48	150
Not Transmitted From Parents	101	49	150
Total	203	97	300

Note. HHRR(χ^2) = 0.02, *P*=0.902.

DISCUSSION

Homocysteine is an intermediary metabolite of intracellular methionine. Some studies have shown that key enzyme gene variations in remethylation pathway are associated with CHD[5-8]. Up to now, there is no report about the relationship between polymorphism of CBS gene and CHD.

The main biochemical function of CBS is to catalyze the condensation of homocysteine with serine to produce cystathionine. The human CBS gene has been localized precisely in the region of band 21q22.3 of chromosome 21 and the entire gene was cloned and sequenced in 1998. Up to now, more than 130 different mutations have been identified in the human CBS gene, and the number is still increasing. Among them, G919A mutation is one of the most common mutations, but the mutation frequencies vary greatly. In a study examining the genetic influence on homocysteine level, Tsai *et al*.[12] have not found G919A mutation in 87 healthy controls and 376 coronary artery disease patients. In studies on relations between CBS G919A mutation and diseases (such as coronary heart disease and neural tube defects) in China, the mutation homozygous genotype frequency and the mutation

allele frequency ranges from 2.8%, 6.8%, to 33%, 47% in healthy control groups. In our study, in controls and their parents, the frequencies of three genotypes (w/w, w/m, and m/m) were 27.2%, 58.4%, and 14.4%, respectively, with no significant difference in gender. The significant difference in genotype distribution among different populations, is due to their different sources (ethics, territories, *etc*). In addition, whether the control group represents the whole population may be another reason. Further investigations are needed to study the polymorphism of CBS gene G919A locus in Chinese.

In this study, we tried to explore the relationship between CBS G919A mutation and CHD in Chinese nuclear families, and found some interesting findings.

When the relationship between CBS G919A mutation and CHD was analyzed, a significant difference was found between CHD patients and controls, with OR 0.43 (95%CI: 0.27-0.78). This difference was mainly caused by female samples, because when groups were divided into subgroups by gender, the significant difference disappeared in male subgroup but not in female subgroup, suggesting that the gene mutation acts as a protective factor for CHD. As to the genotype distribution of parents, mutant frequencies were higher in controls than in CHD parents, indicating that parents carrying the mutant allele may decrease the risk of giving birth to a CHD offspring.

Further comparison of each type of CHD gave more complex results. The genotype frequencies in several subgroups (PDA patient, ASD mother, VSD mother, VSD father, TOF, and other types mother subgroups) were significantly different from those in their corresponding controls, respectively, illuminating that the gene mutation may be a protective factor for corresponding type of CHD, and decreases the risk of CHD development in offsprings. It may be useful to know parents' and fetus' genotype for early screening and prevention of CHD. But the mechanism remains unknown. At the same time, we should remember that CHD is multifactorial in its etiology. Multiplicative effects of several loci and environmental factors are involved in the development of CHD. As to the overall risk of disease, the contribution of any one locus is likely to be relatively small.

The transmission disequilibrium test proposed by Spielman *et al*.[13] is a popular method to assess the association of disease with candidate genes. Through this research, the selection bias of different genetic background can be overcome without the necessity of finding corresponding controls[14]. Our study showed that there existed no allele transmission disequilibrium in CHD nuclear families, suggesting that the mutant allele is not transmitted from parents to children more proportionally than the wild allele. Since the sample size was not large enough, we could not analyze the transmission disequilibrium by each type of CHD, which requires more patients.

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