The Association of the *MTHFR* c.1625A>C Genetic Variant with the Risk of Congenital Heart Diseases in the Chinese

Yuting Wang,¹ Lei Sun,¹ Weina Du,² Shuang Song,¹ Shuo Wang,¹ Weiju Jiang,¹ Tianchu Huang,³ and Hui Li¹

The purpose of this study is to investigate the association of methylenetetrahydrofolate reductase (*MTHFR*) gene polymorphisms with the risk of congenital heart diseases (CHD). The genotypes of the *MTHFR* genetic variant were determined by the polymerase chain reaction–restriction fragment length polymorphism and DNA sequencing methods. Our data suggested that the allelic and genotypic frequencies of CHD patients were significantly different from non-CHD controls. The *MTHFR* c.1625A>C genetic variant was significantly associated with the increased risk of CHD (CC vs. AA: odds ratio [OR]=2.29, 95% confidence interval [CI] 1.15–4.53, $p=0.016$; C vs. A: OR=1.47, 95% CI 1.11–1.96, $p=0.008$). Results from this study indicate that the *MTHFR* c.1625A>C genetic variant influences the risk of CHD in the studied population.

Introduction

CONGENITAL HEART DISEASE (CHD) is one of the most common birth defects, occurring in ~4 to 10 per 1000 live births. It is a major cause of childhood morbidity and mortality worldwide (Hoffman and Kaplan, 2002; Marelli *et al.*, 2007; Gong *et al.*, 2012; Mamasoula *et al.*, 2013; Wang *et al.*, 2013; Wang *et al.*, 2014; Zhang *et al.*, 2014). It is generally accepted that genetic factors are considered to play important influences on the treatment effectiveness of CHD and the prognosis of patients. However, the exact etiology of CHD still remains poorly understood. Recently, several studies have indicated that the methylenetetrahydrofolate reductase (*MTHFR*) gene is an important candidate gene for influencing the prognosis of patients and the risk of CHD (Chambers *et al.*, 2000; Junker *et al.*, 2001; Klerk *et al.*, 2002; Lee *et al.*, 2005; Pereira *et al.*, 2005; Hobbs *et al.*, 2006; Zhu *et al.*, 2006; van Beynum *et al.*, 2007; Brandalize *et al.*, 2009; Garcia-Fragoso *et al.*, 2010; Nie *et al.*, 2011; Gong *et al.*, 2012; Sanchez-Urbina *et al.*, 2012; Yin *et al.*, 2012; Balderrabano-Saucedo *et al.*, 2013; Mamasoula *et al.*, 2013; Mehlig *et al.*, 2013; Wang *et al.*, 2013; Zidan *et al.*, 2013; Wang *et al.*, 2014; Zhang *et al.*, 2014). *MTHFR* genetic variants, such as C677T and A1298C, have been reported and identified with respect to their genetic influences on the risk of CHD in different populations (Chambers *et al.*, 2000; Junker *et al.*, 2001; Klerk *et al.*, 2002; Nie *et al.*, 2011; Wang *et al.*, 2013; Zidan *et al.*, 2013). However, to date, no similar studies were reported about the association of the *MTHFR*

c.1625A>C genetic variant with the risk of CHD. Thus, this study aimed to evaluate the influence of *MTHFR* c.1625A>C genetic variation on the risk of CHD.

Materials and Methods

Subjects

In the present study, we enrolled 507 subjects from the Shengjing Hospital of China Medical University between June 2009 and November 2013. All subjects were genetically unrelated Han Chinese and lived in Shenyang, China. CHD patients were diagnosed and confirmed by doctors. The non-CHD controls were free from CHD and present or past history of diseases. The non-CHD controls were frequency-matched to CHD patients by sex and age. The protocol of this study was approved by the Ethics Committee of the Shengjing Hospital of China Medical University (Shenyang, China). Informed written consent forms were obtained from all participants.

Genotyping of the *MTHFR* gene

Genomic DNA was isolated from peripheral venous blood using the Axygen DNA Isolation Kit (Axygen, CA). According to the human *MTHFR* gene reference sequences (DNA sequences, GenBank ID: NG_013351.1; mRNA sequences, GenBank ID: NM_005957.4), the polymerase chain reaction (PCR) primers were designed by the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA).

¹Department of Obstetrics and Gynecology, Shengjing Hospital of China Medical University, Shenyang, People's Republic of China.

²Maternal and Child Health Hospital of Dalian, Dalian, People's Republic of China.

³Experimental Research Center, Shengjing Hospital of China Medical University, Shenyang, People's Republic of China.

TABLE 1. THE CREATED RESTRICTION SITE-POLYMERASE CHAIN REACTION ANALYSIS FOR THE *MTHFR* c.1625A>C GENETIC VARIANT

Primer sequences	Annealing temperature (°C)	Amplification fragment (bp)	Region	Restriction enzyme	Genotype (bp)
Forward primer: 5'-CGGGTTAATTACCACCTTGTCG-3'	61.9	217	Exon10	TaqI	AA: 187, 20 AC: 217, 187, 20 CC: 217
Reverse primer: 5'-CACTTACACTGGCTGGTGGTCAC-3'					

Underlined nucleotides mark nucleotide mismatches enabling the use of the selected restriction enzymes for discriminating sequence variations.

MTHFR, methylenetetrahydrofolate reductase.

Table 1 shows the detailed information of the primer sequences, amplification region, annealing temperature, and fragment size. The PCR amplification reaction was carried out in a total volume of 20 μ L PCR solution, including 50 ng template DNA, 1 $\times$ buffer (100 mmol Tris-HCl, pH 8.3; 500 mmol KCl), 0.25 μ mol primers, 2.0 mmol MgCl₂, 0.25 mmol dNTPs (Bioteke Corporation, Beijing, China), and 0.5 U Taq DNA polymerase (Promega, Madison, WI). PCR cycling was performed using an initial denaturation at 94°C for 5 min, followed by 32 cycles at 94°C for 30 s, at 61.9°C for 30 s, at 72°C for 30 s, and a final extension at 72°C for 5 min. The created restriction site-PCR (CRS-PCR) method with one of the primers containing a nucleotide mismatch, which enables the use of restriction enzymes for discriminating sequence variations and DNA sequencing methods were utilized to investigate the genotypes of the *MTHFR* c.1625A>C genetic variant. Following the manufacturer's instructions, the PCR-amplified products (10 μ L) were digested with 5 U TaqI restriction enzyme (MBI Fermentas, St. Leon-Rot, Germany) (Table 2) at 37°C for 10 h. The digested products were electrophoresed and separated on a 2.5% agarose gel and observed for different genotypes under ultraviolet light.

Statistical analyses

The chi-squared (χ^2) test was utilized to estimate the Hardy-Weinberg equilibrium (HWE) and the differences in the genotype and allele distributions between CHD patients and non-CHD controls. The association of the *MTHFR* c.1625A>C genetic variant with the risk of CHD was evaluated by computing the odds ratios (ORs) with their 95% confidence intervals (CIs) using unconditional logistic regression analysis. The statistically significant level was settled at p -value < 0.05. All statistical analyses were analyzed by the Statistical Package for Social Sciences software (SPSS, Windows version release 15.0; SPSS, Inc., Chicago, IL).

Results

General characteristics of subjects

A total of 507 unrelated individuals, including 252 CHD patients (Male: 154, Female: 98; mean $\pm$ standard deviation [SD] age: 2.59 ± 1.43) and 255 sex- and age-matched non-CHD controls (Male: 165, Female: 90; mean $\pm$ SD age: 2.77 ± 1.35), were recruited in this case-control study. The CHD patients and non-CHD controls were frequency-matched by sex and age, as indicated by χ^2 -tests (for sex, $\chi^2 = 0.7020$, $p = 0.4021$; for age, $\chi^2 = 0.3192$, $p = 0.5721$, respectively).

Frequencies and genotyping of the *MTHFR* genetic variant

Through the CRS-PCR and DNA sequencing methods, a genetic variant (c.1625A>C) of the *MTHFR* gene was detected in this study. Our sequence analyses indicate that this genetic polymorphism is a nonsynonymous mutation that causes an A to C mutations in exon10 of the *MTHFR* gene. This mutation alters asparagine (Asn) to threonine (Thr) (p.Asn542Thr, the reference sequences GenBank IDs: NG_013351.1, NM_005957.4, and NP_005948.3). The TaqI restriction enzyme has been utilized to digest the PCR-amplified products, which were divided into three genotypes, AA (187 and 20 bp), AC (217, 187, and 20 bp), and CC (217 bp, Table 1). The allelic and genotypic frequencies in the studied populations are summarized in Table 2. The allelic frequencies of CHD patients (A, 71.43%; C, 28.57%) were not consistent with those of non-CHD controls (A, 78.63%; C, 21.37%; $\chi^2 = 7.0156$, $p = 0.0081$, Table 2). The genotypic frequencies of CHD patients (AA, 53.57%; AC, 35.72%; CC, 10.71%) were significantly different from those in non-CHD controls (AA, 62.75%; AC, 31.76%; CC, 5.49%; $\chi^2 = 6.6968$, $p = 0.0351$, Table 2). The genotype and allele

TABLE 2. THE FREQUENCIES OF ALLELIC AND GENOTYPIC *MTHFR* c.1625A>C GENETIC VARIANTS

Groups	Genotypic frequencies (%)			Allelic frequencies (%)		χ^2	p
	AA	AC	CC	A	C		
Cases ($n = 252$)	135 (53.57)	90 (35.72)	27 (10.71)	360 (71.43)	144 (28.57)	3.9375	0.1396
Controls ($n = 255$)	160 (62.75)	81 (31.76)	14 (5.49)	401 (78.63)	109 (21.37)	0.7682	0.6811
Total ($n = 507$)	295 (58.19)	171 (33.73)	41 (8.09)	761 (75.05)	253 (24.95)	5.0100	0.0817
	$\chi^2 = 6.6968$, $p = 0.0351$			$\chi^2 = 7.0156$, $p = 0.0081$			

TABLE 3. THE ASSOCIATION OF THE *MTHFR* c.1625A>C GENETIC VARIANT WITH THE RISK OF CONGENITAL HEART DISEASES

Comparisons	Test of association		
	OR (95% CI)	χ^2 -value	p-Value
Homozygote comparison (CC vs. AA)	2.29 (1.15–4.53)	5.80	0.016
Heterozygote comparison (AC vs. AA)	1.32 (0.90–1.92)	2.04	0.153
Dominant model (CC/AC vs. AA)	1.46 (1.02–2.08)	4.38	0.036
Recessive model (CC vs. AC/AA)	2.07 (1.06–4.04)	4.64	0.031
Allele contrast (C vs. A)	1.47 (1.11–1.96)	7.01	0.008

The χ^2 tests were used for statistical analyses.
CI, confidence interval; OR, odds ratio.

distributions of this genetic variant between CHD patients and non-CHD controls were in accordance with the HWE (all *p*-values >0.05, Table 2).

Association of the *MTHFR* genetic variant with the risk of CHD

The association of the *MTHFR* c.1625A>C genetic variant with the risk of CHD is shown in Table 3. Our data suggested that the *MTHFR* c.1625A>C genetic variant was statistically associated with the increased risk of CHD in a homozygote comparison (CC vs. AA: OR=2.29, 95% CI 1.15–4.53, $\chi^2=5.80$, *p*=0.016), dominant model (CC/AC vs. AA: OR=1.46, 95% CI 1.02–2.08, $\chi^2=4.38$, *p*=0.036), recessive model (CC vs. AC/AA: OR=2.07, 95% CI 1.06–4.04, $\chi^2=4.64$, *p*=0.031), and allele contrast (C vs. A: OR=1.47, 95% CI 1.11–1.96, $\chi^2=7.01$, *p*=0.008, Table 3).

Discussion

In recent years, a large number of epidemiologic studies have suggested that CHD is a huge medical problem and presents a significant economic burden to the families and society around the world (Junker *et al.*, 2001; Hoffman and Kaplan, 2002; Nie *et al.*, 2011; Gong *et al.*, 2012; Sanchez-Urbina *et al.*, 2012; Mamasoula *et al.*, 2013; Wang *et al.*, 2013). It is caused by complex interactions between environmental and genetic factors; furthermore, growing evidence indicated that genetic factors play key roles in the development of CHD. Previous studies reported that the *MTHFR* gene is one of the most important candidate genes for influencing the susceptibility to CHD. In the present study, we firstly investigated the distribution of the *MTHFR* c.1625A>C genetic variant by CRS-PCR, and evaluated the influence of the *MTHFR* c.1625A>C genetic variant on the risk of CHD in a Chinese Han population through association analyses on the basis of analysis of 252 CHD patients and 255 sex- and age-matched non-CHD controls. Our findings suggested that there were significant differences for the allelic and genotypic frequencies between the CHD patients and non-CHD controls (Table 2). Compared with allele-A and genotype-AA, the allele-C and genotype-CC were significantly associated with increased susceptibility to CHD (All *p*-values <0.05, Table 3). Allele-C and genotype-CC may be

significantly associated with the increased risk of CHD. The *MTHFR* c.1625A>C genetic variant lead to the Asn to Thr amino acid replacement and might impact the expression and function of the *MTHFR* protein, thus influencing the risk of CHD. It also might be linked to other nonsynonymous polymorphisms, such as C677T and A1298C in the *MTHFR* gene, which have been proven to associate with the risk of CHD (Chambers *et al.*, 2000; Junker *et al.*, 2001; Klerk *et al.*, 2002; Nie *et al.*, 2011; Wang *et al.*, 2013; Zidan *et al.*, 2013). The findings from this study could provide more evidence to explain the biological function and role of the *MTHFR* gene in the development of CHD.

In conclusion, to our knowledge, we are the first to investigate the potential association of the *MTHFR* c.1625A>C genetic variant with the risk of CHD. Results from this case-control study suggest that the *MTHFR* c.1625A>C genetic variant is significantly associated with the increased risk of CHD in the Chinese Han population, and could be a useful molecular biomarker for evaluating the susceptibility to CHD. Further studies with larger, different ethnic populations are needed to confirm these conclusions and to reveal the underline molecular mechanisms.

Author Disclosure Statement

The authors have no conflicts of interest.

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Address correspondence to:

Hui Li, PhD

Department of Obstetrics and Gynecology
Shengjing Hospital of China Medical University
No. 36, Sanhao Street
Heping District
Shenyang 110004
Liaoning Province
People's Republic of China

E-mail: huili_12@sina.com