Impact of A1298C Polymorphism of Methylenetetrahydrofolate Reductase Gene on Risk of Congenital Heart Disorders in Iranian Population

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Received: 07 Sep. 2017; Accepted: 19 Dec. 2017

Abstract- Congenital heart disorders (CHDs) encompass inborn defects of the heart compartments. Polymorphisms of methylenetetrahydrofolate reductase **(**MTHFR) gene have continuously been reported as a modulating factor for CHDs. In a case-control study, the association of A1298C polymorphism of MTHFR gene with ventricular septal defect (VSD) and Tetralogy of Fallot (TOF), two common forms of CHDs, in the children of southeastern Iran was investigated. The presence of A1298C polymorphism was investigated using tetra-ARMS-PCR method, and statistical processes were carried out applying SPSS software (V:19). Males in the control group formed 40% and 60% (including 46.74% in VSD and 13.26% in in TOF). Although the association between gender and CHDs was not statistically significant, males had a higher risk of VSD (OR=1.7, 95% CI; 0.9-3.1, *P*=0.08). Overall frequency of AA, AC ,and CC genotypes of A1298C polymorphism were 55%, 43%, and 2% in CHDs patients respectively; while respective ratios were 50%, 48% and 2% in the controls (*P*>0.05). Patients with VSD were more commonly identified with AC+CC genotypes (52.8%) compared to TOF cases (23%, *P*=0.01). In stratified regression analysis, heterozygote genotype showed a significant protection against TOF (OR=0.3, 95% CI; 0.1-0.8, *P*=0.01). Frequency of variant allele was obtained 31.58% in CHD patients and 33.8% in the controls (*P*>0.05). Moreover, frequency of variant (C) allele was 35.2% in VSD compared to 18.8% in TOF (*P*>0.05). A1298C polymorphism of MTHFR gene seems to exert a significant protective effect against TOF.

Acta Med Iran 2018;56(6):386-391.

Keywords: Congenital heart disorders; Methylentetrahydrofolate reductase; Polymorphism; Tetralogy of Fallot; Ventricular septal defect

Introduction

Congenital heart disorders (CHDs) are types of malformations emerging in one or more heart structures with the incidence of 4-50 in 1000 live births (1). These are the most common birth defects accounted as the leading causes of premature death across the world (2). Almost 40-50% of neonates with CHDs are diagnosed within the first week of life, while the remainder will be detected during the first month or first year of their lives (2). The incidence of ventricular septal defect (VSD), as one of the commonest CHDs, is estimated between 30% -35% (1), while tetralogy of Fallot (TOF), the most common cyanotic CHDs, occurs with the prevalence of 10% in all CHDs (3).

Several potential genetic and environmental risk factors could contribute to CHDs; however, the chief etiology is unknown in most cases (2). Pathophysiological aspects of CHDs are supposed to be the result of interaction between multisystem genes and environmental determinants (4). Mutations in some of the specific transcription factors acting during the development of cardiac tissue such as GATA transcription factor-4 (GATA-4), T-box transcription

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factor-5 (TBX-5), and NK2 transcription factor-locus 5 (NKX2-5) have been suggested as potential triggering events for CHDs (5); nevertheless, conclusions are inconsistent in this regard.

Methylenetetrahydrofolate reductase (MTHFR) is an enzyme encoded by a gene located at ch1p36.3. The enzyme catalyzes reduction of 5,10-MTHF to 5-MTHF, which severs as the initial source of methyl group in diverse metabolic pathways; most importantly in DNA methylation process (6). Multiple functional polymorphisms have been detected within this gene. A1298C polymorphism is one of the most studied variations. This polymorphism has been mapped within exon 7 of MTHFR gene, and corresponds to the regulatory domain of the enzyme causing reduced catalytic activity of MTHFR (6).

Association of A1298C polymorphism and various inborn heart abnormalities are inconclusive (7). Particularly, higher occurrence of heart dysfunctions have been described in individuals with A1298C polymorphism (8). On the other hand, some studies have indicated a significant protective role for A1298C polymorphism against CHDs (9). It is noteworthy that the relationship between these two is less straightforward in the studies with heterogeneous patient populations having different subtypes of CHDs (10,11). Nevertheless, there are infrequent studies on the role of A1298C polymorphisms in isolated VSD or TOF patients. In the present study, the role of this polymorphism in Iranian children affected with VSD and TOF in the south-east province, Sistan and Balouchistan was assessed.

Materials and Methods

Sample population

Children diagnosed with VSD and TOF were recruited from "Cardiac Care center of Imam Ali Hospital in Zahedan city" in 2014-2015.Their Parents were requested to sign the respective consent form before the study. A history of CHDs throughout the family was checked based on a questionnaire from both parents, and the cases with familial syndromes were excluded. The case group included VSD (76 subjects) and TOF (24 subjects) as a subgroup of CHDs. Diagnosis was made based on echocardiography, cardiac catheterization, and surgery information. The controls (100 randomized healthy individuals) were homogenized regarding age, gender, and ethnicity; and they were selected from the same hospital. The controls had no precedent and current cardiac abnormalities as confirmed by echocardiography. Our study was approved by the ethical committee of research deputy of Zahedan University of Medical Sciences.

Polymorphism genotyping

Standard proteinase k method was applied to extract genomic DNA from 2 ml EDTA venous blood samples (12). We used tetra-ARMS (amplification refractory mutation system) PCR method to determine the presence of A1298C polymorphism utilizing two pairs of inner and outer primers (Table 1).

PCR reaction

Each 20 µl PCR mixture contained 100 ng genomic DNA, 1.5 µl of each inner primers and 0.9 µl of each outer primers. Taq DNA polymerase master mix (Pishgam, Iran) (10 µl) along with 5 µl of distilled water were included in the reaction tube. PCR was performed in Eppendorf thermal cycler (Eppendorf, Germany) with temperature profile of initial denaturation at 95˚ C for 5 min followed by 30 cycles of denaturation at 95˚ C; annealing at 60˚ C and extension at 72˚ C (each of 60s). A final extension phase at 72˚ C was allowed for 5 min to maximize the amplification process. PCR products were then stained with Ethidium bromide and visualized by electrophoresis on 2% agarose gel under ultraviolet emission.

Statistical analysis

Chi-square test was used to assess significant difference of alleles and genotypes distributions. Odd ratios were exploited as degree of association between genotypes and risk of VSD or TOF. The obtained data from both the case and the control groups in association with the results of A1298C genotyping was analyzed by means of SPSS software (V: 19).

Table 1. Sequences of primers used to amplify alleles of A1298C polymorphism of MTHFR gene

Primer	Orientation	Primer sequence(5→3)	Product; Size (bp)
Outer Primers	Forward	GAA-GTT-TGC-ATG-CTT-GTG-GTT	Outer; 460
	Reverse	TCT-CCC-AAC-TTA-CCC-TTC-TCC	A allele; 271
Inner Primers	Forward	AGG-AGC-TGA-CCA-GTG-AGG-C	C allele; 230
	Reverse	AAC-GAA-GAC-TTC-AAA-GAC-ACC-TT	

Results

Out of 100 CHD patients studied, 74 cases had VSD, while 26 suffered TOF. In the control and CHD groups males formed 49% and 60%, respectively. There was no significant relationship between gender and CHDs (*P*=0.07). Mean age was 3.7±3.4 and 4.8±3.9 years in the cases and the controls, respectively (*P*=0.03).

Homozygote wild-type (AA) genotype of A1298C polymorphism was the most frequent combination in both the control (50%) and CHD groups (55%). The frequency of heterozygote genotype was 48% in the controls and 43% in the CHDs patients; while variant allele homozygote genotype frequency was 2% in both groups. Furthermore, penetrance of mutant allele (C) was 33.8% and 31.58% in the controls and CHD patients, respectively. Distribution of genotypes and alleles was not statistically different in patients and the controls. Stratified sampling showed that CHD and TOF patients were dominantly (77%) represented with wild type homozygote (AA) genotype (*P*=0.05). This was significantly different from genotype distribution in VSD patients, who were represented with higher frequency of either AC or CC genotypes (52.8%, *P*=0.01, Table 2).

Table 2. Frequencies of genotypes and alleles of A1298C polymorphism of MTHFR gene in healthy controls and children with CHDs

Polymorphism		Control (n=100)	CHDs (n=100)	*P*	Stratified CHDs		
					VSD (n=74) N (%)	TOF (n=26) N (%)	*P*
Genotype	AA	50	55		35(47.2)	20(77)	
	AC	48	43	0.7	37(50)	6(23)	0.05
	CC	2	2		2(2.8)	0(0)	
	AC+CC	50	45	0.2	39(52.8)	6(23)	0.01
Allele	A	66.2	68.5	0.3	64.8	81.2	0.1
	C	33.8	31.6		35.2	18.8	

Regarding risk association analysis, it was found that the males had ORs of 1.5, 1.7 and 1.2 for CHDs, VSD and TOF; respectively. Regression analysis also showed a significant protective role for heterozygote (AC) genotype of A1298C polymorphism against TOF (OR=0.3, 95% CI; 0.1-0.8, *P*=0.01). Table 3 summarizes risk association analysis of selected parameters for CHDs, VSD, and TOF.

Discussion

In the current study, A1298C polymorphism of MTHFR gene was assessed in 100 CHD patients including 76 VSD and 24 TOF cases. A significant lower risk of TOF was observed in children with mutant heterozygote (AC) than compared to the cases with AA genotypes (OR=0.3, 95% CI=0.1-0.8, *P*=0.01). Nevertheless, risk assessment of mutant homozygote (CC) genotype was hindered; as no cases with this combination were identified in TOF patients. Although, no studies on the role of A1298C polymorphism in isolated TOF were detected, some reports have mentioned the polymorphism as a protective factor against the development of CHDs (9,13). In an investigation of polymorphisms of 12 genes involved in folate metabolism by Wang et al (14), ORs for AC and CC genotypes of A1298C polymorphism were 0.9 and 0.7 for CHDs, which are fairly close to our result for CHDs (OR=0.8 and 0.9, table 3). Inevitably, bioavailability of folate derivatives is required for cell growth and differentiation during cardiac biogenesis. In justifying the observed protective effect against CHDs of a polymorphism associated with potential reduced level of folate, one can address multifactorial etiological approach (participation of both genetic and environmental factors) of CHDs development. Maternal factors including folate taking has been proposed as a potential mediator. It's been noted that mothers with a CHD affected newborns had lower intake of folate supplements in comparison to those who conceived a healthy neonate (15,16). It's also been mentioned that sufficient nutrient bioavailabilityof folate can lessen negative effects of A1298C polymorphism in reducing folate levels (17,18). Reportedly, environmental mediators such as incontinence during pregnancy or certain maternal disorders during child-bearing period may increase the risk of CHD in embryos (19). Besides, environmental and occupational situations exposing pregnant women to toxic agents were higher in mothers with CHDs than otherwise (20).

Table 3. Analysis of odd ratios and confidence intervals for CHDs, VSD and TOF regarding gender along with genotypes and allele of A1298C polymorphism of MTHFR gene

Parameter		Logistic Regression analysis		
		OR	95% CI	*P*
CHD[a]	Sex (male)	1.5	0.8-2.7	0.1
	Genotype (AC)	0.8	0.4-1.4	0.4
	Genotype (CC)	0.9	0.1-6.6	0.9
	Allele (C)	0.9	0.5-1.4	0.6
VSD	Sex (male)	1.7	0.9-3.1	0.08
	Genotype (AC)	1.1	0.5-2	0.7
	Genotype (CC)	1.4	0.1-10.6	0.7
	Allele (C)	1	0.6-1.7	0.8
TOF	Sex (male)	1.2	0.5-2.8	0.6
	Genotype (AC)	0.3	0.1-0.8	0.01
	Allele (C)	0.4	0.1-1.1	0.1

[a] CHD; Congenital heart disease, VSD: Ventricular septal defect, TOF: Tetralogy of Fallot

In addition, residing of genetic polymorphisms of folate related genes in mothers has also been studied as risk factors for CHDs in their siblings (21). In a meta-analysis conducted on folate related genes polymorphisms in CHDs, it was revealed that accounting for the polymorphisms status in mothers may be essential in determining the risk of CHDs (7).

From molecular point of view, an observed protective role may be related to a more balanced and error-free DNA synthesis in the presence of the polymorphism. It is proposed that fewer developmental errors are amenable by a more balanced rate of synthesis and methylation of DNA through slightly decreased MTHFR activity (9,22). This notion is also supported by the observation of higher risk of CHDs in folate supplemented mothers bearing wild type allele of A1298C polymorphism (9). Regardless, more studies are required to clarify the effects of such potential interactive contributors.

In contrast to the studies supporting a protective effect, there are also studies indicating increased risk of CHDs in the presence of A1298C polymorphism. In the present study, heterozygote and mutant homozygote genotypes were characterized with relatively increased risk of VSD (OR=1.1 and 1.4 respectively, $P>0.05$). Statistically non-significant ORs observed here may be due to either small population of our study (74 VSD patients) or genetic heterogeneity of the population.In two recent comprehensive meta-analyses, overall OR related to A128C polymorphism was 1.3 for CHDs (6,23). In addition, CC and AC genotypes of A1298C polymorphism were significantly associated with the risk of CHDs in Egyptian (15,21,24) and Turkish (25) patients. In parallel, C allele of A1298C polymorphism was described to be associate with CHDs in Caucasian children (7). But no significant relationship was found between A1298C polymorphism and occurrence of CHDs in other studies (13,17,26,27). Regarding these conflicting results, more population based studies are requisite in order to reveal the etiological role of A1298C polymorphism in CHDs pathogenesis.

In the present study, an overall higher occurrence of CHDs in males (60%) was observed. Although the association was not statistically significant, it was close to significance threshold ($P=0.07$). This is in accordance with the result of Goldmuntz *et al*., who reported the dominancy of males (60%) in a population of CHDs patients (10). Likewise, our results demonstrated higher ORs for the males regarding VSD and TOF categories (1.7 and 1.2, $P=0.08$ and 0.6 respectively). Lower risk of CHDs in females compared to males may be related to hormonal difference between the two genders (28); however this is an uncertain hypothesis, and needs more clarification.

Our findings suggest a protective role for A1298C polymorphism against development of TOF. Association of this polymorphism with VSD was not significant; however, higher risk of VSD was observed in individuals with A1298C polymorphism. These results may imply differential effects of MTHFR gene in pathogenesis of different CHDs subtypes. However, the role of other genetic determinants and influence of related environmental factors should also be taken into account.

Acknowledgements

The authors would like to thank the patients' families for their kind cooperation in the study. They are also obliged to the research deputy of Zahedan University of Medical Sciences for their undertaking the research.

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