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Impact of *MTHFR* Gene Polymorphisms C677T and A1298C on Congenital Atrial Septal Defect Risk in an Iranian Cohort

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Abstract

Background: Congenital heart defects (CHD) are recognized as the most common heart abnormalities amongst newborns and children, and atrial septal defect (ASD) is recognized as one of the most frequent forms of CHD. Prior studies indicated that the methylenetetrahydrofolate reductase (*MTHFR*) gene contributes to the etiology of CHD. Therefore, we designed a case-control study to assess the possible role of the *MTHFR* gene, specifically the C677T (rs1801133) and A1298C (rs1801131) polymorphisms within the Iranian ASD population sample.

Method: A total of 166 subjects (81 children diagnosed with ASD and 85 control participants) were enrolled in this research. Samples genotyped for *MTHFR* rs1801133 and rs1801131 polymorphisms using the PCR-RFLP and ARMS-PCR approaches.

Results: Our results indicated that rs1801131 variant reduced the risk of ASD in codominant (OR [95%CI]: 0.41[0.21-0.83], P=0.012), dominant (OR[95%CI]: 0.48 [0.25-0.93], p=0.028) and overdominant (OR[95%CI]: 0.44 [0.23-0.81], P=0.009) models. Moreover, rs1801133 variant increased the risk of ASD in codominant (OR[95%CI]: 2.68[1.39-5.16], P = 0.003), dominant (OR [95% CI]: 2.72 [1.43–5.14], P = 0.002), overdominant (OR [95% CI]: 2.50 [1.31–4.78], P = 0.005), and allelic (OR [95% CI]: 2.16 [1.27–3.69], P = 0.004) models.

Conclusion: Our findings suggest that *MTHFR* rs1801133 and rs1801131 variants may potentially affect the onset of ASD.

Keywords: Atrial Septal Defect, Congenital Heart Defects, Folate Metabolism, Genetic Polymorphism, Methylenetetrahydrofolate Reductase.

Introduction

Congenital heart diseases or defects (CHD) are the most common heart malformations among newborns and children (1). This condition occurs due to inadequate heart development during the first six weeks of pregnancy (2). Even though in recent years, the ability to diagnose and treat CHDs has advanced significantly, the disorder still imposes a significant burden in many countries. Compared to other childhood disorders, the treatment of CHDs remains more costly and complex (3, 4).

Nowadays, it is commonly acknowledged that CHD is a multifactorial disorder that includes environmental and genetic components (5, 6). Based on some epidemiological findings, one-third of individuals with CHD are affected by genetic factors (7). In addition, the interaction

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between genes and environment is involved in the etiology of CHD, highlighting the complexity of the disorder (8, 9). Atrial septal defect (ASD), ventricular septal defect (VSD), and atrioventricular septal defects (AVSD) are three main forms of CHD (10). ASD, as one of the more prevalent forms of CHD, has a prevalence of around 100 per 100,000 live births (11) and is responsible for 10-15% of CHD cases worldwide (12-14).

Numerous investigations have shown that maternal periconceptional intake of multivitamins or folic acid may decrease the risk of CHD in offspring of females with mutations in genes involved in folate metabolism (15). Nevertheless, research is still ongoing on the mechanism of this effect. It has been shown that homocysteine concentration is affected by folate and vitamin B12 (2). Previous research has demonstrated that the methylenetetrahydrofolate reductase *(MTHFR)* gene (NM_005957.5) contributes to CHD etiology (16). It acts as a major r regulatory protein in the metabolism of folate (17). The conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate is catalyzed by this enzyme, which is essential for the remethylation process of homocysteine (Hcy) to methionine (18). The *MTHFR* gene contributes to folate metabolism, and mutations in this gene prevent the removal of Hcy, an independent risk factor for CHD occurrence (19, 20).

The prior studies indicated that two well-studied SNPs in the *MTHFR* gene, C677T (rs1801133, Ala222Val) and A1298C (rs1801131), are correlated with reduced enzyme activity and elevated plasma homocysteine levels (21). Numerous studies have assessed the role of *MTHFR* gene polymorphisms in various disorders, such as childhood acute lymphoblastic leukemia (22), Alzheimer disease (23), breast cancer (24), pregnancy complications (25), and ischemic stroke (26). In addition, several studies investigated the possible effect of *MTHFR* gene polymorphisms on CHD in different ethnicities (1, 27-33); however, the outcomes have been inconsistent. Therefore, we conducted the current research for the first time among ASD patients from an Iranian population sample.

Materials and Methods

In current case-control research, 81 children diagnosed with ASD and 85 healthy individuals were recruited in southeast Iran. Both groups were paired based on age and gender. The methodology and enrollment process has been previously elucidated (22).

The Local Ethics Committee of Zahedan University of Medical Sciences authorized the research protocol (IR.ZAUMS.REC.1402.360), and parents of both patients and controls provided informed consent. In our study, we included all children under the age of 18 with a confirmed diagnosis of ASD who were referred to the pediatric cardiology clinics at Ali Asghar and Ali Ibn Abi Talib Hospitals in Zahedan, or who were admitted to these facilities. Exclusion criteria included individuals with other cardiac abnormalities, such as tetralogy of Fallot, complex septal defects, single ventricle, or a history of exposure to teratogenic substances. As a control group, we enrolled healthy children attending the Ali Asghar Clinic for routine annual examinations, provided they had no known underlying diseases.

We employed the salting-out approach to extract DNA from peripheral whole blood (34). The primers were synthesized by SinaClon, Iran. To genotype of MTHFR C677T polymorphism, the PCR-RFLP approach was utilized. Each PCR reaction comprised 1 µl of DNA, 1 µl of both forward and reverse primers, 10 µl of Premix (2X Prime Taq, Genet Bio, South Korea), and 7 µl of water. The PCR products were treated with restriction enzyme *HinfI* (Thermo Scientific, USA). Digestion of the T allele resulted in 222-bp and 167-bp fragments, whereas the undigested C allele produced a 389-bp fragment. Genotyping of the *MTHFR* A1298C variant was performed by using the ARMS-PCR approach. The A or C allele generated a 317-bp fragment, with the internal control producing a 574-bp fragment.

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The primers for amplifying both loci were reported in our previous publication (22).

Statistical Analysis

An independent sample t-test and $\chi 2$ test were employed for data analysis using SPSS software (version 20). The correlation between the *MTHFR* C677T (rs1801133) and A1298C (rs1801131) variants and ASD risk was examined through logistic regression analyses to calculate the OR and 95% CI. We also used SNPStats software for haplotype analysis. A p-value below 0.05 was considered statistical significance.

Results

The research population consisted of eighty-one ASD cases (36 males, 45 females; age: 6.01 ± 3.98 years) and eighty-five healthy children (43 males, 42 females; age: 5.74 ± 2.44 years). There were no statistically significant differences between the groups regarding gender or age (p = 0.428 and p = 0.588, respectively).

Table 1 summarizes the genotype and allelic distribution of *MTHFR* gene variants. The results showed that the rs1801131 (A1298C) variant was associated with a decreased risk of ASD in codominant (OR[95%CI]: 0.41[0.21-0.83], p=0.012, AC vs AA), and dominant (OR[95%CI]: 0.48[0.25-0.93], p=0.028, AC+CC vs. AA) and overdominant (OR[95%CI]: 0.44[0.23-0.81], P=0.009, AC vs. AA+CC).

Table 1. Genotypic and allelic frequencies of *MTHFR* gene polymorphisms in childhood ASD and control subjects.

Polymorphisms	ASD n (%)	Control n (%)	OR (95%CI)	P-value
rs1801131				
Codominant				
AA	33(40.7)	21(24.7)	1	-
AC	35(43.3)	54(63.5)	0.41(0.21- 0.83)	**0.012**
CC	13(16.0)	10(11.8)	0.83(0.31- 2.23)	0.707
Dominant				
AA	33(40.7)	21(24.7)	1	-
AC+CC	48(59.3)	64(75.3)	0.48(0.25- 0.93)	**0.028**
Recessive				
AA+AC	68(84.0)	75(88.2)	1	-
CC	13(16.0)	10(11.8)	1.43(0.59- 3.48)	0.425
Overdominant				
AA+CC	46(56.7)	31(36.5)	1	-
AC	35(43.3)	54(63.5)	0.44(0.23- 0.81)	**0.009**
Alleles				
A	101(62.3)	96(56.5)	1	-
C	61(37.7)	74(43.5)	0.78(0.505- 1.22)	0.276
rs1801133				
Codominant				
CC	38 (46.9)	60 (70.6)	1	-
CT	39 (48.2)	23 (27.1)	2.68(1.39- 5.16)	**0.003**
TT	4 (4.9)	2 (2.3)	3.16(0.55- 18.09)	0.197
Dominant				
CC	38 (46.9)	60 (70.6)	1	-
CT+TT	43 (53.1)	25 (29.4)	2.72(1.43- 5.14)	**0.002**
Recessive				
CC+CT	77 (95.1)	83 (97.7)	1	-
TT	4 (4.9)	2 (2.3)	2.16(0.38- 12.11)	0.373
Overdominant				
CC+TT	42(51.8)	62(72.9)	1	-
CT	39 (48.2)	23 (27.1)	2.50(1.31- 4.78)	**0.005**
Alleles				
C	115(71.0)	143 (84.1)	1	-
T	47 (29.0)	27 (15.9)	2.16(1.27- 3.69)	**0.004**

OR: Odds Ratio, CI: Confidence Interval, Significant p-values are shown in bold ($p < 0.05$).

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Conversely, the rs1801133 variant (C677T) was found to increase the risk of ASD across all assessed genetic models. Specifically, in the codominant (OR[95%CI]: 2.68[1.39-5.16], p=0.003, CT vs. CC), dominant (OR[95%CI]: 2.72[1.43-5.14], p=0.002, CT+TT vs. CC), overdominant (OR[95%CI]: 2.50[1.31-4.78], P=0.005, CT vs. CC+TT) and allele (OR [95%CI]: 2.16 [1.27- 3.69], p=0.004, T vs. C) models.

Furthermore, we also examined the interaction of *MTHFR* polymorphisms on ASD risk (Table 2). Compared to the reference *MTHFR* rs1801131AA/ rs1801133 CC, the AA/CT genotypes were associated with an elevated risk of ASD (OR[95%CI]: 5.56[1.50-20.53], p=0.007). Table 3 presents the results of haplotype analysis. The results revealed that TA haplotypes were associated with an increased risk of ASD compared to CA haplotypes (rs1801133C/ rs1801131A), (OR [95%CI]: 3.64 [1.25-10.63], P= 0.02).

Table 2. Interaction of *MTHFR* rs1801131and rs1801133 polymorphisms on ASD risk.

rs1801131	rs1801133	ASD n (%)	Control n (%)	OR (95%CI)	P-value
AA	CC	13(16.1)	17(20.0)	1.00	-
AA	CT	17(21.0)	4(4.7)	5.56(1.50- 20.53)	**0.007**
AA	TT	3(3.7)	0(0.0)	-	-
AC	CC	18(22.2)	35(41.1)	0.67(0.27- 1.69)	0.396
AC	CT	16(19.8)	17(20.0)	1.23(0.46- 3.32)	0.682
AC	TT	1(1.2)	2(2.4)	0.65(0.05- 8.02)	0.738
CC	CC	7(8.6)	8(9.4)	1.14(0.33- 3.97)	0.832
CC	CT	6(7.4)	2(2.4)	3.92(0.68- 22.71)	0.111
CC	TT	0(0.0)	0(0.0)	-	-

OR: Odds Ratio, CI: Confidence Interval, Significant p-values are shown in bold ($p < 0.05$).

Table 3. Haplotype frequencies of *MTHFR* polymorphisms in ASD risk.

Haplotype $_{rs1801131, rs1801133}$	ASD n (%)	Control n (%)	OR (95%CI)	p-value
AC	0.4081	0.4917	1.00	-
CC	0.3018	0.3494	1.00(0.53- 1.90)	0.99
AT	0.2153	0.0731	3.64(1.25- 10.63)	**0.02**
CT	0.0748	0.0859	1.12(0.37- 3.41)	0.84

OR: Odds Ratio, CI: Confidence Interval, Significant p-values are shown in bold ($p < 0.05$).

Discussion

This study demonstrates that the MTHFR gene polymorphisms rs1801133 and rs1801131 are significantly associated with congenital atrial septal defect (ASD) susceptibility in an Iranian cohort. Specifically, the rs1801133 variant was associated with an increased risk of ASD, whereas the rs1801131 variant was found to be associated with a decreased risk. These findings are consistent with previous studies indicating the importance of MTHFR polymorphisms in congenital heart defects (CHD).

It is well-established that the genetic and environmental aspects are potential contributing factors for CHD. However, numerous genetic investigations have indicated that genetic factors are one of the key causes of CHD susceptibility. Twin studies revealed that monozygotic twins had a 63% higher risk of CHD compared with dizygotic twins. In addition, whole exome sequencing research showed an 8.5%

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diagnostic rate for pathogenic genetic variations in fetuses exhibiting structural cardiac anomalies and a 15.4% ratio in those with multisystemic anomalies. Genetic factors are also identified as the main agents of pathogenesis in ASD, the most common congenital heart defect, in both familial and sporadic patients. Thus, finding the involved genes and related mutations in the onset of ASD is essential for disease prevention and prenatal diagnosis (35).

Various metabolic pathways regulate the neural tube formation and cardiac development throughout the embryogenesis period. Among them, the folate-homocysteine metabolic pathway has a significant role in controlling the level of folate and homocysteine, two important factors significantly contributing to the development of CHD (2). Therefore, the genes modulating the folate-homocysteine metabolic pathway, as well as mutations within their sequences, are likely crucial risk factors for CHD and ASD disorders. For this reason, in the current study, we selected two common exonic single nucleotide polymorphisms (SNPs) (rs1801133 and rs1801131) of the *MTHFR* gene to evaluate the correlation of *MTHFR* gene variations with the risk of ASD.

The rs1801133 variant is located in the fourth exon of *MTHFR* at the 677th nucleotide position (C667T). This mutation leads to the amino acid substitution of alanine to valine (Ala222Val). It results in a reduction in MTHFR activity and consequent metabolic impairment of Hcy, which slightly elevate plasma Hcy levels slightly (36). Furthermore, the rs1801133 missense variant, localizing at 1298 position (A1298C), resulting in glutamine to alanine change (Glu429Ala), which significantly decreases the activity of MTHFR enzyme (37). Our results demonstrated that rs1801133 increased the ASD risk in four genetic models (allelic, codominant, dominant, and overdominant). In comparison, rs1801131 was associated with a decreased risk of ASD in three studied models (codominant, dominant, and overdominant).

Our finding was consistent with the meta-analysis report regarding the association between rs1801133 SNP and CHD by Liu et al (2020). They detected a significant link between this polymorphism and CHD occurrence under the recessive model (OR[95% CI]: 1.35[1.06-1.71]) for the entire population, including 15 eligible studies. Subgroup analyses by ethnicity showed similar correlations in Asians but not for Caucasians (38). Similar data were obtained from the next meta-analysis (2022) by pooling the results of 26 eligible studies. A statistically significant association between rs1801133 and CHD was identified under all applied genetic models. This correlation was also seen in Asian populations but not in others (2). However, Ali et al. (2024) reported a lack of a significant association between rs1801133 and congenital septal defects in Pakistan, similar to our data, they observed that heterozygous mutant (CT) was the more prevalent genotype in CHD patients in comparison with controls (39). In another study by Shivkar et al. (2022), indicative hyperhomocysteinemia in carriers of T allele for rs1801133 genotype was discovered in young coronary artery disease (CAD) patients. The authors proposed that the link between hyperhomocysteinemia and CAD depended on typical cardiovascular risk factors. Thus, the risk of hyperhomocysteinemia and young CAD could be assessed through *MTHFR* polymorphism analysis, followed by measuring serum levels of homocysteine, folate, and vitamin B12 (40). Moreover, the maternal rs1801133 variant was detected as a CHD risk factor for their offspring, possibly attributable to hyperhomocysteinemia resulting from abnormal metabolism of Hcy (41).

There is limited data on the influential role of rs1801131 on CHD risk. Sun et al. (2021) found that rs1801131 was significantly associated with the risk of CHD in the homozygote comparisons in the Chinese population (42); their result was in concordance with our finding. Furthermore,

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Zidan et al. reported the correlation of rs1801131 CC genotype as well as a higher level of homocysteine with a raised risk of CHD in the Egyptian population. They also observed that the frequencies of rs1801131 AC and CC genotypes and C allele were remarkably raised among mothers with children affected by CHD (43). Moreover, the meta-analysis of 18 studies revealed a significant correlation between the rs1801131 and myocardial infarction risk (44). Song et al., nonetheless, found no relationship between rs1801131 and coronary artery disease development in Qingdao, China (45).

In conclusion, our data revealed the potential role of *MTHFR* rs1801133 and rs1801131 polymorphisms on ASD susceptibility. More genetic association studies in larger groups, various ethnicities, and functional studies are suggested to strengthen data validity.

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Ethical Approval
The Local Ethics Committee of Zahedan University of Medical Sciences authorized the research protocol (IR.ZAUMS.REC.1402.360). The written consent was obtained prior the research from all participants.

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Competing Interest
The authors declare that they have no competing interests

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