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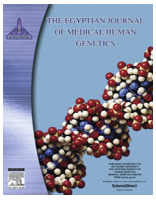

Original article

Common variant of 5,10-methylenetetrahydrofolate reductase may increase risk of coronary artery disease in the Iranian population

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ABSTRACT

Background: Coronary artery disease (CAD) is the most prevalent form of cardiovascular disease that is caused by the formation of plaque in the arteries walls. Both genetic and environmental factors play an important role in the development of CAD.
Aim: The aim of this study was to determine the association of 5,10-methylenetetrahydrofolate reductase (*MTHFR*) C677T gene polymorphism with CAD in an Iranian population.
Subjects and methods: In this case/control study, sequence specific primer-polymerase chain reaction (SSP-PCR) method was used for genotyping of 310 patients with CAD and 367 healthy controls.
Results: Frequency of C/T genotype was significantly higher in the patients group than the control group *(P = .03, OR: 1.6, 95% CI: 1.04–2.47).* Based on the assumption that T is a risk allele, dominant model compares C/C genotypes to C/T + T/T genotypes. A significant association was observed in *MTHFR* C677T when the effect of the polymorphism was considered under a dominant genetic model *(OR = 1.59; 95% CI = 1.03–2.46; P = .02).* Evaluating genotype frequencies in 4 different ethnic groups (Fars, Turkmen, Sistani, and others) demonstrated significant statistical association of C/T genotype in Fars sub-groups *(OR = 1.8; 95% CI = 1.11–3.06; P = .01)* but this association is not observed in other populations. Significant association of C/T *(P = .01, OR: 2.21, 95% CI: 1.15–4.4)* genotype was found in women, but this association was not observed in men.
Conclusion: The results of this study showed that C/T genotype in *MTHFR* C677T position is a causative factor, especially in women, and might be associated with susceptibility to CAD in the Iranian population.

1. Introduction

Coronary artery disease (CAD) is caused by atherosclerotic plaque build-up in the wall of coronary arteries and with decreased blood flow [1]. CAD is the most common type of cardiovascular disease and the most important cause of death globally [2].

Previous studies have shown that both genetic and environmental factors affect the incidence of this disease [3]. Factors such as age, smoking, high blood pressure, family history, diabetes, lack of exercise, obesity, stress, high blood cholesterol, poor diet and excessive alcohol increase the risk of developing CAD [4,5]. Genetic factors play a major role in CAD progression in younger people [6].

5,10-Methylenetetrahydrofolate reductase (*MTHFR*) gene is located on the short (p) arm of chromosome 1 at position 36.3 (1p36.3). *MTHFR* reduces 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is the carbon donor for remethylation of homocysteine to methionine [7].

Wilcken et al. reported that CAD patients have higher concentrations of homocysteine than healthy individuals [8]. In subsequent studies, Hyperhomocysteinaemia has been recognized as a risk factor for coronary heart disease [9]. Previous investigations reported reduced *MTHFR* activity with a thermo-unstable enzyme in patients with CAD [10].

Goyette et al. found nine polymorphisms in the *MTHFR* gene via single-strand conformation polymorphism and direct sequencing of PCR fragments [11]. Using the same procedures, Frosst et al. reported that C to T substitution at nucleotide 677 leads to alanine to valine amino acid change at the 222nd position of *MTHFR* protein (Ala222Val). In most populations, the C677T polymorphism (rs1801133) in *MTHFR* is an even more common polymorphism with an allele frequency of 30–40% [7,12].

Some studies reported an association between C677T polymorphism and risk of cardiovascular disease, while others did not find such association [13].

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This study aimed to determine and compare the prevalence of *MTHFR* (C677T) polymorphism in patients with CAD and healthy individuals to provide baseline epidemiological data for future clinical investigations of CAD and other diseases in Iran.

Iran is the 17th most populous nation in the world and has central location in Eurasia and Western Asia [14]. Different ethnicities live in Iran including the Persians (the largest groups) Turkic groups (include the Turkmen and the Qashqai peoples) Kurds, Lurs, Sistanis, Balochis, Arabs and others. Because of the ethnic diversity, evaluating genetic variation in Iran is valuable and it may be used in future studies.

2. Subjects and methods

2.1. Patients

In this case-control study, the frequency of *MTHFR* C677T gene polymorphism was evaluated in 310 patients with CAD and 367 healthy controls. The mean age of subjects in the case and control group was 59.25 ± 10.54 and 51.94 ± 10.88 years respectively. The percentages of men and female was 60.32% and 39.68% in the cases and 35.62% and 64.38% in the controls respectively. Control subjects were matched with patients by age, ethnicity, and geographical area.

CAD patients were diagnosed by angiography test and approved by a cardiologist, from March 2013 to March 2014, at Kowsar Heart Center in Kordkuy, Iran. Inclusion criteria for patients group was coronary stenosis of 70% and more in at least one major coronary artery. Patients with coronary stenosis below 70% were excluded from the study. None of the patients had major autoimmune or hematologic disease.

Individuals with normal angiography at rest, without symptoms of myocardial ischemia during exercise were selected as controls.

All of subjects (including case and control) were divided into four main ethnic groups of Fars, Turkmen, Sistani and others.

In agreement with the Helsinki Declaration, all participants signed the informed consent and the Ethics Committee of Golestan University of Medical Sciences approved this study. The sample size was determined by the Quanto software V-1.2 with a statistical power of 80%.

2.2. DNA extraction and genotyping

Five ml peripheral whole blood was used for extracting genomic DNA by a standard protocol [15].

C677T gene polymorphism of *MTHFR* was detected based on the specific sequence primer polymerase chain reaction (SSP-PCR) method. Human growth hormone (*HGH*) gene was used as an internal control for false-negative reactions. The polymorphism was identified by the sequence-specific forward primers 5-GAGAAGGTGTCTGCGGGAGC-3′ and 5-GAGAAGGTGTCTGCGGGAGT-3′ in combination with the consensus reverse primer 5-CTCAATCACGTCCTTGATCTC-3′ [16]. The following control primer pairs that amplify a conserved region of the HGH were used: HGH-F: 5′-GCCTTCCCAACCATTCCCTTA-3′ and HGH-R: 5′-TCACGGATTTCTGTTGTGTTTC-3′.

SSP-PCR amplification was done using a 15 ml reaction mixture containing one ml of genomic DNA, 0.9 ml of 25 mm MgCl2 (Qiagen, USA), 1.5 ml of each 10X reaction buffer, 1.5 ml of 10 mM dNTP, 2.2 ml of sucrose 60%, 0.5 VL-for-specific primers (10 pM), 0.5 ml of 10 pM HGH primers, and 0.2 of Taq polymerase (Qiagen, USA).

PCR was carried out by a thermal cycler (Techne, UK), using the following program: 1 min at 95 °C followed by 10 cycles of 15 s at 94 °C, 15 s at 65 °C, 40 s at 72 °C, followed by 20 cycles of 10 s at 94 °C, 50 s at 59 °C and 40 s at 72 °C, with 5 min at 72 °C as final extension.

The PCR products were electrophoresed on 1.5% agarose gel (Merck, Germany) stained with ethidium bromide. Photographs were taken using a gel documentation system (UVITEC, UK) and DNA bands were visualized under the UV radiation.

2.3. Statistical analysis

Statistical analysis was done using Statistical Package for the Social Sciences (SPSS, version 17.0, IBM, USA) and the mean of parametric variables was calculated. Allele and genotype frequencies were calculated and compared between groups using non-parametric tests, followed by exact Fisher's analysis by STATA V-8 (California, USA). The risk associated with genotypes/alleles was calculated as the odds ratio (OR) with 95% confidence interval (CI). P-value less than .05 were considered as statistically significant.

3. Results

The allelic and genotypic distributions of the *MTHFR* gene C677T in healthy controls and patients with CAD under co-dominant, dominant and recessive models are shown in Table 1.

Frequency distributions of genotypes C/C, C/T and T/T in the control subjects were about 20.4%, 72.8% and 6.8%, respectively.

Table 1
Frequency of the *MTHFR* C677T allele and genotypes among patients (N = 310) and controls (N = 367) under Co-dominant, Dominant and Recessive model.

Alleles and Genotype	Controls	CAD patients	OR (95% CI)*	P-value
C/C	75 (20.4 %)	43 (13.9 %)	1 (–)**	–
C/T	267 (72.8%)	244 (78.7 %)	1.6 (1.04–2.47)	**.03**
T/T	25 (6.8%)	23 (7.4 %)	1.6 (0.77–3.34)	NS* (.2)
C	417 (57%)	330 (53%)	1 (–)	–
T	317 (43%)	290 (47%)	1.16 (0.93–1.44)	NS (.2)
Model of Inheritance				
Dominant				
C/C	75 (20.4 %)	43 (13.9 %)	1 (–)	–
C/T + T/T	292 (79.6%)	267 (86.1%)	1.59 (1.03–2.46)	**.02**
Recessive				
C/T + C/C	342 (93.2%)	287 (92.6%)	1 (–)	–
T/T	25 (6.8%)	23 (7.4 %)	1.1 (0.58–2.06)	NS (.8)
Co-dominant				
C/C + T/T	100 (27.2 %)	66 (21.3)	0.72 (0.5–1.0)	NS (.07)
C/T	267 (72.8%)	244 (78.7 %)	1 (1)	–

*OR: Odds Ratio, CI: Confidence Interval, NS: Not Significant.
**The frequency of CC genotype was more in the control group and was selected as reference group.

The frequency of these genotypes in the patients with CAD was about 13.9%, 78.7%, 7.4% respectively.

The frequency of C/T genotype was significantly higher in the case group compared to the controls *(P = .03, OR: 1.6, 95% CI: 1.04–2.47)*. Frequencies of C/C and T/T genotypes and C and T alleles were different between the controls and patients group but they were not statistically significant.

Based on the assumption that T is a risk allele, dominant model compares C/C genotypes to C/T + T/T genotypes. A significant association was observed in *MTHFR* C677T when the effect of the polymorphism was evaluated under a dominant genetic model *(OR = 1.59; 95% CI = 1.03–2.46; P = .02)*.

Genotype frequencies in four different ethnic groups represented significant statistical association in Fars sub-groups but this association was not observed in other populations.

The C/T genotype frequency was significantly increased in Fars patients compared to Fars controls *(OR = 1.8; 95% CI = 1.11–3.06; P = .01)*. However, there was no significant association between T/T genotype and CAD in the Fars sub-groups (Table 2).

The frequency of C/T *(P = .01, OR: 2.21, 95% CI: 1.15–4.4)* genotype was significantly different among female subjects, while no significant difference was found among male subjects (Table 3).

Frequencies of genotypes in CAD group according to a number of affected arteries showed that genotype C/T was significantly associated with the three vessel disease type (p = .05) (Table 4).

4. Discussion

Cardio- and cerebrovascular diseases are multifactorial, as their etiopathogenesis is determined by genetic and environmental factors and by gene-gene and gene-environment interactions [17]. Several studies have investigated the association of several genes polymorphisms and CAD [18–21].

Although data have concluded conflicting results, several studies have been carried out to test the hypothesis that genetic polymorphisms in the *MTHFR* gene might be associated with CAD risk.

Morita et al. examined the distribution of the *MTHFR* C677T genotypes (Ala222Val) in 362 Japanese male patients with CAD

Table 2
Frequency of the *MTHFR* C677T genotypes within four different ethnic groups.

Model	Genotype	Total	Controls	CAD patients	OR (95% CI)	P-value
Fars	C/C	90 (18.9%)	57 (23.2%)	33(14.4%)	1 (–)	–
	C/T	351 (73.9%)	170 (69.1%)	181 (79.0%)	1.8 (1.11–3.06)	**.01**
	T/T	34 (7.2%)	19 (7.7%)	15(6.6%)	1.36 (0.56–3.27)	NS (.5)
	Total	475 (100.0%)	246 (100.0%)	229 (100.0%)		
Turkmen	C/C	16 (16.5%)	10 (17.9%)	6 (14.6%)	1 (–)	–
	C/T	73 (75.3%)	44 (78.6%)	29 (70.7%)	1.1 (0.31–4.1)	NS (1)
	T/T	8 (8.2%)	2 (3.6%)	6 (14.6%)	5 (0.58–62)	NS (.2)
	Total	97 (100.0%)	56 (100.0%)	41 (100.0%)		
Sistani	C/C	9 (12.2%)	6 (13.3%)	3 (10.3%)	1 (–)	–
	C/T	63 (85.1%)	37 (82.2%)	26 (89.7%)	1.4 (0.26–9.4)	NS (.7)
	T/T	2 (2.7%)	2 (4.4%)	0 (0.0%)	0 (0–5.7)	NS (1)
	Total	74 (100.0%)	45 (100.0%)	29 (100.0%)		
Other	C/C	2 (7.7%)	1 (5.9%)	1 (11.1%)	2.3 (0.02–196)	NS (.5)
	C/T	20 (76.9%)	14 (82.4%)	6 (66.7%)	1 (–)	–
	T/T	4 (15.4%)	2 (11.8%)	2 (22.2%)	2.3 (0.13–38)	NS (.5)
	Total	26 (100.0%)	17 (100.0%)	9 (100.0%)		

*OR: Odds Ratio, CI: Confidence Interval, NS: Not Significant.
**The frequency of CC genotype was more in the control group and was selected as reference group.

Table 3
Frequency of the *MTHFR* C677T genotypes within SEX.

Gender	Genotype	Controls	CAD patients	Total	OR (95% CI)	P-value
Men	C/C	23 (17.7%)	28 (15.0%)	51 (16.1%)	1 (–)	–
	C/T	99 (76.2%)	138 (73.8%)	237 (74.8%)	1.1 (0.59–2.2)	NS (.7)
	T/T	8 (6.2%)	21 (11.2%)	29 (9.1%)	2.2 (0.7–6.7)	NS (.15)
	Total	130 (100.0%)	187 (100.0%)	317 (100.0%)		
Women	C/C	52 (22.1%)	15 (12.2%)	67 (18.7%)	1 (–)	–
	C/T	166 (70.6%)	106 (86.2%)	272 (76.0%)	2.21 (1.15–4.4)	**.01**
	T/T	17 (7.2%)	2 (1.6%)	19 (5.3%)	0.4 (0.04–2.1)	NS (.3)
	Total	235 (100.0%)	123 (100.0%)	358 (100.0%)		

*OR: Odds Ratio, CI: Confidence Interval, NS: Not Significant.
**The frequency of CC genotype was more in the control group and was selected as reference group.

Table 4
Genotyping investigation on patients suffering from one-, two- or three-vessel-stenosis.

Genotype	Controls, n(%)	1 VD, n(%)	P-value	2VD, n(%)	P-value	3VD, n(%)	P-value
C/C	75 (20.4%)	19 (16.7%)	–	10 (12.3%)	–	14 (12.2%)	–
C/T	267 (72.8%)	89 (78.1%)	NS (.4)	63 (77.8%)	NS (.15)	92 (80.0%)	**.05**
T/T	25 (6.8%)	6 (5.3%)	NS (1)	8 (9.9%)	NS (.15)	9 (7.8%)	NS (.19)

VD: vessel disease; N.S: Not Significant.

and found that the frequency of Val/Val genotype was significantly higher in the patients compared to controls [22].

Study of Alam et al. on 100 patients with CAD and 100 controls from North India, has shown the positive association of *MTHFR* (C677T) gene polymorphism and risk of CAD [23].

Tripathi et al. examined 329 patients with CAD and 331 healthy individuals from the same region and found that the frequency of T allele was significantly higher in patients [24].

Sarecka-Hujar et al. genotyped the *MTHFR* C677T polymorphism in angiographically documented patients with premature CAD and showed that presence of *MTHFR* TT genotype, smoking or elevated levels of low-density lipoprotein cholesterol affect the risk of premature CAD [25].

Kolling et al. demonstrated that *MTHFR* gene C677T polymorphism is not associated with CAD [26].

Meta-analyses studies on the association of *MTHFR* genotype with risk of coronary heart disease showed a significant heterogeneity between the results obtained in different regions [27,28]. However, a meta-analysis of *MTHFR* case-control studies failed to find a correlation between risk of coronary heart disease and T/T versus C/C homozygotes for the *MTHFR* C677T polymorphism [9].

The present study evaluated the association of *MTHFR* gene polymorphism with susceptibility to CAD in 310 Iranian patients with CAD and 367 healthy matched controls.

In Iran, Aleyasin et al. evaluated C677T *MTHFR* polymorphism in 100 CAD cases and 100 healthy controls and concluded that patients had a significantly higher frequency of T allele [29]. Nasiri et al. genotyped 54 patients with a history of MI (Myocardial Infarction) and 54 control subjects in Fars province of Iran and found a strong positive correlation between the T/T genotype and risk of myocardial infarction [30].

Saffari et al. reported no significant difference between patients with multi-vessel CAD and subjects with less than 30% stenosis of the main arteries in southern Iran [31].

Our results showed that distribution of the C/T genotype was significantly different between the CAD patients and healthy controls. Other genotypes showed that there was a mild difference between the frequency of C/T and T/T genotypes and C and T allele in CAD cases and healthy controls but they were not statistically significant.

In the present study, genotype frequencies in the Fars ethnic group were similar to *MTHFR* genotype diversity in the total study population. However, this association was not observed in other ethnic groups and more studies should be carried out in larger population to further validate the results.

Significant differences in the frequency of C/T genotype in females indicates that the *MTHFR* gene may act as a major risk factor in women. It has been suggested that environmental factors could be considered as risk factors in men. One study in south of Iran showed that number of male CAD patients was higher than women and concluded that sex hormones (Estrogen and progesterone) may play protective role in women [32].

The present study found genotype C/T is significantly associated with the three vessel disease type but showed almost similar frequencies for the C/C and T/T genotypes regarding to stenosis of one, two or three vessels. Unlike some findings of previous studies that showed the lack of association of genotype with the disease [24,33–35]. Nevertheless, Morita et al. reported a significant association and significantly higher frequency of the mutant genotype in patients with triple-vessel disease [22]. Mousavi et al. in Iran showed significant association of mutant genotype with the two and three vessel disease type [19].

The most important limitation of this study was the ethnic differences of populations. The allelic and genotype frequency can be significantly different in various populations. The sample size is second limitation. It is important to perform study with a larger statistical population to clarify the role of polymorphism in the population.

5. Conclusion

Results of this study reported that the frequency of C/T genotype in *MTHFR* C677T position is significantly different between the patients with CAD and healthy individuals, which might be a causative factor and genetic risk factor for cardiovascular disease in the Iranian population.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Contributors

Majid Shahbazi initiated the research program. Marzieh Attar and Seyedpayam Shirangi genotyped the patient and control subjects. Marzieh Attar, Farnoosh Shateri and Majid Shahbazi accumulated and banked all of the DNA samples. Majid Shahbazi carried out the statistical analyses. Majid Shahbazi supervised the project. Marzieh Attar, Seyedpayam Shirangi and Majid Shahbazi wrote the paper.

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