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RESEARCH ARTICLE

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Investigation of MTHFR gene C677T polymorphism in cardiac syndrome X patients

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Background: Definition of Cardiac Syndrome X (CSX) refers to groups of patients with positive exercise stress test and normal epicardial coronary arteries on coronary angiography accompanied by chest pain. Although the etiology of CSX is not completely understood, there is a common consensus that its pathophysiology may be associated with endothelial dysfunction resulting in impaired coronary flow. Some polymorphisms observed on the MTHFR gene cause inactivation of the MTHFR enzyme, leading to hyperhomocysteinemia and homocysteinuria, which are prominent risk factors of cardiovascular and cerebrovascular diseases. It was aimed to explain the association of the endothelial dysfunction, which is thought to play a role in the pathophysiology of CSX, with C677T polymorphism on MTHFR gene based on genetic basis.

Methods: A total of 176 CSX patients and 196 healthy subjects with similar age and clinical features were compared in terms of C677T polymorphism of the MTHFR gene.

Results and Conclusion: There was no significant difference in terms of MTHFR gene C677T polymorphism between CSX patients and controls. When genotypic distribution was compared based on gender in both patients and controls, no significant difference was found between male and female subjects ($P>.05$). As fasting blood sugar and urea values were significantly higher, alanine aminotransferase and gamma-glutamyl transferase levels were significantly lower in the patients than the controls ($P<.05$). Described family story of the patients was significantly higher than the controls ($P<.05$). These suggest that homocysteine metabolism in CSX is not directly related to the endothelial dysfunction and thus the effect on the microvascular circulation.

KEYWORDS

C677T, cardiac syndrome X, microvascular dysfunction, MTHFR, polymorphism

1 | INTRODUCTION

Cardiac Syndrome X (CSX) indicates patients with chest pain, positive exercise stress test, and normal epicardial coronary arteries on coronary angiography.[1,2] Today CSX is used to describe patients with typical ischemic chest pain, ischemic electrocardiographic changes in the exercise stress test and angiographically normal coronary arteries.[3] Although the etiology of CSX is not completely understood, there is a common consensus that its pathophysiology may be associated with endothelial dysfunction resulting in impaired coronary flow.[4] The most basic pathophysiological mechanism thought to be present in patients with CSX is microvascular ischemia caused by endothelial dysfunction. Today, it is known that endothelium is a multifunctional organ that covers the vascular lumen and regulates blood flow using substances such as nitric oxide (NO).[5] Possible causes of coronary microvascular dysfunction are present in CSX patients. It has been reported that traditional cardiovascular risk factors (including hypertension and diabetes) contribute to coronary microvascular dysfunction, in particular to impaired endothelium-dependent

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vasodilatation.[3,6,7] A state of endothelial dysfunction is often prominent in patients with CSX. Decreased coronary blood flow against acetylcholine, atrial pacing, and other endothelium-mediated vasodilator stimuli trigger endothelial dysfunction. It is thought that impaired NO release and/or activation play an important role here.[8]

5,10-methylenetetrahydrofolate reductase enzyme (MTHFR) is a flavoprotein, which plays a role in folate metabolism and is a member of the MTHFR family.[9] A polymorphism occurring on the MTHFR gene causes a decrease in enzyme activity. As a result of the decrease in MTHFR activity, level of 5-methyl-tetrahydrofolate (5-methyl-THF) decreases and plasma homocysteine levels are increased due to the amount of 5,10-methylene-tetrahydrofolate (5,10-methylene-THF).[10]

Increase in plasma homocysteine levels is observed due to insufficient vitamin intake and inadequate vitamin cofactors in addition to genetic alterations of enzymes involved in homocysteine metabolism and malnutrition.[11,12] Homocysteine is an independent risk factor for atherosclerosis and venous thromboembolism.[13] Among the polymorphisms seen on the MTHFR gene, it has been suggested that C677T polymorphism, which occurs on the catalytic domain of the enzyme, is closely associated with vascular diseases.[14,15]

2 | MATERIALS AND METHODS

The study was approved by Ethical Committee of Cerrahpasa Medical Faculty with the approval number of 49484 and it was conducted in accordance with Helsinki Declaration of World Health Organization.

2.1 | Patient and control groups

A total of 176 consecutive CSX patients (mean age 52.6±11.1, 66.5% female) diagnosed in Bakirkoy Dr. Sadi Konuk Training and Research Hospital Cardiology Clinic based on ESC 2006 Stabilization Angiography criteria and a control group consisting of 196 consecutive asymptomatic healthy individuals (mean age 50.9±11.2, 56.1% female) with a 10-year cardiac event rate of <10% according to the Framingham risk score with similar age and gender characteristics were included in the study. Coronary artery disease, valvular heart disease, congestive heart failure, previous thromboembolic incident, presence of active infection and neoplasia were determined as exclusion criteria. Besides, diabetes mellitus and metabolic syndrome were not excluded in our study. All the patients were hospitalized in Bakirkoy Dr. Sadi Konuk Training and Research Hospital Cardiology Clinic between October 2015 and August 2016. Besides, written informed consent was taken from each individual participating in the study.

2.2 | The protocol

2.2.1 | DNA isolation and real-time polymerase chain reaction

Venous blood samples were drawn from the antecubital veins of the individuals in the patient and control groups after 12 hours of overnight fasting, centrifuged at 1500 *g* for 30 minutes, and the sera were separated and stored at −80°C. Genomic DNA samples obtained from blood samples drawn from the same groups were stored at −20°C until use.

Genomic DNA isolation from blood samples taken into sterile K3-EDTA tubes from individuals in the patient and control groups was done according to the standard protocol in the kit (Invitrogen PureLink Genomic DNA Kit, catalogue number: K1820-02; Thermo Fisher Scientific, Waltham, MA, USA).

Real-time polymerase chain reaction analysis of the isolated DNA samples was performed according to the standard protocol stated in the TaqMan® SNP Genotyping Assay (rs1801133).

After the mixture consisting of 5 μL of Master Mix, 0.25 μL of assay and 3.75 μL of distilled water for each well, was placed in a 96-well plate in a way that there is 9 μL in each well, 1 μL DNA samples were added and real-time PCR was performed with the (Applied Biosystem 7500® Instrument; Thermo Fisher Scientific). In the genotyping process, C and T alleles were defined using VIC/FAM dyes in the given target sequence of GAAAAGCTGCGTGATGATGAAATCG[G/A]CTCCCGCAGACACCT TCTCCTTCAA. As C allele was determined with the VIC dye, FAM dye indicated the presence of T allele.

2.3 | Statistical analysis

Mean, standard deviation, median, lowest, highest values were used in the descriptive statistics of the data. The distribution of the variables was checked by the Kolmogorov-Smirnov test. Independent sample *t*-test and Mann-Whitney *U* test were used in the analysis of the quantitative data. Statistical analyses were performed using the SPSS 22.0® program (IBM Corp., Armonk, NY, USA).

3 | RESULTS

Based on real-time PCR analysis, the distribution of the genotypes seen as a result of C677T polymorphism on the MTHFR gene was determined as 47.7% CC, 45.5% CT, 6.8% TT in patients with CSX, and in the control group, they were determined as 55.6% CC, 36.2% CT and 8.2% TT (Table 1). There was no significant difference in the incidence of MTHFR gene C677T polymorphism between the patient and control groups (*P*=.194).

When MTHFR C677T polymorphism was compared in patients and control subjects based on gender, genotypic distribution in males and females in the patient group did not show a significant difference

TABLE 1 Table of genotypic distribution between individuals in the patient and control groups

		Patient group		Control group		
		n	%	n	%	*P*
Genotype	CC	84	47.7	109	55.6	.194
	CT	80	45.5	71	36.2	
	TT	12	6.8	16	8.2	

Chi-square test.

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TABLE 2 Table of genotypic distribution in individuals between the patient and control groups for males and females

		Female		Male		
		n	%	n	%	*P*
Patient group						
Genotype	CC	52	44.4	32	54.2	.286
	CT	55	47.0	25	42.4	
	TT	10	8.5	2	3.4	
Control group						
Genotype	CC	63	57.3	46	53.5	.293
	CT	41	37.3	30	34.9	
	TT	6	5.5	10	11.6	

Chi-square test.

TABLE 3 The table of allelic distribution between the patient and control group

		Patient group		Control group		
		n	%	n	%	*P*
Allele	C	248	70.45	289	73.72	.326
	T	104	29.54	103	26.27	

(*P*=.286). Likewise, genotypic distribution in males and females in the control group did not show a significant difference (*P*=.293; Table 2).

The allelic distribution of MTHFR C677T polymorphism was 70.45% for C allele and 29.54% for T allele in CSX patients, whereas C allele was found to be 73.72% and T allele 26.27% in the control group. There was no significant difference between the patient and control groups in terms of the distribution of MTHFR gene C and T alleles (*P*=.326; Table 3).

In the patient group, FBS and urea values were significantly higher than the control group (*P*<.05). Besides, ALT and GGT levels were significantly lower in the patient group compared to the control group (*P*<.05). AST, creatinine, HDL, HGB, LDL, MPV, PLT, TG, total cholesterol, uric acid values showed no significant difference between the patient and control groups (Table 4).

Family story (FS) described as the rate of diabetes mellitus (DM), hypertension (HT), ARB/ACE inhibitor usage and development of cardiovascular disease, myocardial infarctions or sudden death in relatives in the first degree (mother, father, sibling) before the age of 55 in males and 65 in females, was found to be significantly higher in the patient group (*P*<.05) compared to the control group. Rate of smoking and the rates of beta blocker, calcium channel blocker and statin use did not differ significantly (*P*>.05) between the patient and control groups (Table 5).

4 | DISCUSSION

Cardiac Syndrome X (CSX) is a syndrome defined as benign characterized by angina, normal coronary arteries and ST segment depression. However, today it is considered as a syndrome, which increases the risk of cardiovascular events and an important cause of morbidity. Failure to clearly elucidate the pathogenesis makes diagnosis and consequently treatment difficult.[16]

Today, with more successful identification and investigation of CSX, endothelial dysfunction is thought to play a role in microvascular dysfunction, and therefore studies in the microvascular level have begun.[17] Pharmacological treatments and lifestyle changes have offered successful but limited treatment approaches for CSX. In addition, a clear treatment approach has not yet been specified in the treatment guidelines.[18]

In a study conducted among 42 consecutive women with CSX, it was determined that 14 (33%) subjects were homozygous for MTHFR C677T polymorphism, whereas 16 (16%) of 100 individuals in the control group were homozygous.[19]

In the analysis using logistic regression model for dependent variables in group distribution, it was concluded that homozygosity for C677T polymorphism was an important predictor of CSX (OR=2.62; 95% CI: 1.14-6.05). In our study, 12 of the 176 individuals in the patient group (6.8%) were homozygous for the MTHFR C677T polymorphism, whereas 16 of the 196 individuals in the control group (8.2%) were homozygous. In the patient group, it was determined that 10 (8.5%) female and 2 (3.4%) male subjects were homozygous for MTHFR C677T polymorphism. Based on the results of another study, it was suggested that particularly in CSX patients with MTHFR polymorphism, microvascular dysfunction may be defined as "physiologically-frozen" arterial syndrome accompanied by both endothelium-dependent and endothelium-independent dysfunction.[19] Also, Arroyo-Espliguero et al.[20] have reported increased arterial stiffness in CSX patients.

MTHFR is a key enzyme involved in folate metabolism. Some polymorphisms on the MTHFR gene cause inactivation of the MTHFR enzyme, leading to hyperhomocysteinemia and homocysteinuria, which are important risk factor for cardiovascular and cerebrovascular diseases.[18] In various studies, the relationship between MTHFR C677T region polymorphism and cardiac effects was found to be associated with target organ damage.[21,22] In other studies, no association was detected between homozygous MTHFR C677T polymorphism and increased risk of cardiovascular disease.[23,24] This result was confirmed by a recent large-scale epidemiological study. In this study, despite low serum folate levels and high homocysteine levels, the MTHFR 677TT genotype was associated with a low risk of cardiovascular disease.[25]

In a meta-analysis study involving 23 study outcomes, MTHFR C677T polymorphism was studied in 5869 patients with cardiovascular disease and a control group composed of 6644 patients. While the prevalence of the TT genotype ranged between 5.4% and 16.0% among different groups in the control group; in the patient group, it ranged between 6.5% and 29.7% among different groups. In all studies, it has been observed that the distributions of different genotypes and allele frequencies were almost the same in patient and control groups. The percentage of TT genotype in the patients was 11.9%, whereas, in the control group it was reported as 11.7%. As another result of the same meta-analysis, it has been indicated that although individuals with TT genotype have an average 25% higher homocysteine levels

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TABLE 4 Demographic data of the patients and the control group

	Patient group		Control group		
	Mean±SD	Med (Min-Max)	Mean±SD	Med (Min-Max)	*P*
FBS	104.1±26.8	99.0 (72.0-266.0)	92.2±15.7	90.0 (71.0-196.0)	***.000****
ALT	20.1±13.0	18.0 (7.0-152.0)	23.9±14.9	20.0 (8.0-91.0)	***.007****
AST	20.7±7.0	20.0 (6.0-74.0)	21.9±9.3	19.0 (11.0-103.0)	.494
Creatinine	0.9±1.1	0.7 (0.4-9.2)	0.7±0.5	0.7 (0.4-7.0)	.446
GGT	25.4±18.2	21.0 (9.0-156.0)	26.1±12.6	23.0 (7.0-74.0)	***.040****
HDL	48.5±14.2	47.0 (24.0-129.0)	49.8±29.5	44.0 (23.0-383.0)	.186
HGB	13.0±1.6	13.2 (5.5-17.3)	13.2±1.5	13.3 (7.3-16.6)	.345
LDL	123.7±42.7	121.0 (14.0-254.0)	125.5±32.6	120.0 (44.0-224.0)	.502
MPV	8.7±1.2	8.6 (6.3-14.2)	8.5±0.9	8.5 (6.6-12.6)	.077
PLT	252.7±55.1	250.5 (39.0-398.0)	261.4±58.2	250.0 (131.0-477.0)	.405
TG	144.9±78.7	130.0 (41.0-603.0)	131.8±65.0	125.0 (17.0-452.0)	.218
Total Cholesterol	201.2±44.6	199.5 (101.0-343.0)	201.9±37.3	204.0 (52.0-318.0)	.867
Urea	33.7±13.4	32.0 (3.9-132.0)	28.6±7.8	29.0 (12.0-53.0)	***.000****
Uric acid	4.9±2.2	4.8 (2.2-28.0)	5.0±1.4	4.9 (1.0-8.0)	.181

FBS, Fasting Blood Sugar; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; GGT, Gamma-glutamyl transferase; HDL, High density lipoprotein; HGB, Hemoglobin; LDL, Low density lipoprotein; MPV, Mean Platelet Volume; PLT, Platelet count; TG, Triglyceride.
Mann-Whitney *U* test/Independent sample *t*-test.
*$P<.05$ for significant differences between patient and control groups in FBS, ALT, GGT and urea levels.

than individuals with normal wild-type CC genotype, they did not generally have an increased risk of cardiovascular disease or specifically coronary heart diseases. Also, the fact that polymorphism of MTHFR, which is commonly associated with small homocysteine elevations in individuals with cardiovascular disease, is not associated with increased cardiovascular risk was proven by this meta-analysis. In the given meta-analysis, which argues that increased levels of homocysteine in patients with cardiovascular disease are epiphenomena; this has been addressed as a consequence of the effects of well-understood standard risk factors for vascular diseases and renal function, and is not indicated as a direct risk factor.[26] In our study, parallel to these findings, TT genotype was detected as 8.2% in the control group compared to 6.8% of TT genotype in the patient group.

In this study, we evaluated 176 CSX patients (mean age 52.6±11.1, 66.5% women) and a control group consisting of 196 healthy asymptomatic individuals with similar age and sex characteristics (mean age 50±11.2, 56%, 1 female) in terms of MTHFR gene C677T polymorphism. In parallel with the study suggesting that CSX is relatively more common in females, the proportion of female patients in our study group was found to be significantly higher than the control group ($P<.05$).

Also, in terms of genotypic and allelic distributions obtained as a result of genotyping analysis in our study, no significant difference ($P>.05$) was determined between males and females in the patient and control group.

Although many clinical studies have been conducted on MTHFR variants, there are many studies with conflicting results due to the inadequacy in understanding the poly-genetic, epigenetic and environmental

TABLE 5 Demographic data of the patients and the control group

		Patient group		Control group		
		n	%	n	%	*P*
Smoking	No	121	68.8	148	75.5	.146
	Yes	55	31.3	48	24.5	
DM	No	139	79.0	185	94.4	***.000****
	Yes	35	19.9	11	5.6	
HT	No	122	69.3	162	82.7	***.003****
	Yes	54	30.7	34	17.3	
ARB/ACE inhibitor	No	139	79.0	171	87.2	***.033****
	Yes	37	21.0	25	12.8	
FS	No	105	59.7	159	81.1	***.000****
	Yes	71	40.3	37	18.9	
Beta blocker	No	146	83.0	173	88.3	.143
	Yes	30	17.0	23	11.7	
Calcium channel blocker	No	149	84.7	177	90.3	.099
	Yes	27	15.3	19	9.7	
Statin	No	131	74.4	157	80.1	.192
	Yes	45	25.6	39	19.9	

DM, Diabetes Mellitus; HT, Hypertension; ARB/ACE Inhibitor, Angiotensin II Receptor Blocker/Angiotensin-Converting Enzyme Inhibitor; FS, Family story.
Chi-square test.
*$P<.05$ for significant differences between patient and control groups in DM, HT, ARB/ACE inhibitor and FS values.

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factors, which may interact with multifactorial structures of disease and syndromes in addition to the MTHFR polymorphisms.[18,19,26]

However, the fact that the statistical results of our study did not show any significant difference between patients and control groups in terms of polymorphism, indicating that homocysteine metabolism in CSX does not have a direct effect on endothelial dysfunction and thus microvascular circulation. MTHFR enzyme activity and that activity-related homocysteine levels may not be involved in development of microvascular dysfunction in CSX pathogenesis. The reason is that rather than thromboembolism, microvascular dysfunction in consequence of endothelial dysfunction seems to play an important role in the pathogenesis of the disease.[27,28]

Although it is a known fact that the enzyme MTHFR is directly involved in folate and homocysteine metabolism, yet there are many aspects of genetic regulation which are unknown and need to be elucidated. To understand the regulation of the regulatory region of the MTHFR gene and its genome better, multinational studies with a larger number of patients with MTHFR gene involvement are needed. Besides, another important limitation is that only genetic polymorphism of MTHFR was determined in our study. In addition to that, plasma homocysteine levels and MTHFR ezyme activity are also needed to be measured for better elucidation of the relation between homocysteine metabolism and CSX. To our knowledge, this is the first study investigating the relation of MTHFR with CSX on a genetic basis.

In conclusion, although we did not find a statistically significant relationship between MTHFR C677T polymorphism and CSX in this study, we believe that it is necessary to plan and conduct new studies by increasing the number of individuals in the patient and control groups to clarify this relationship with more detailed and further analyses.

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