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ORIGINAL ARTICLE / ÖZGÜN ARAŞTIRMA

MTHFR and MMP-9 Genetic Variants in Coronary Artery Disease

Koroner Arter Hastalığında MTHFR ve MMP-9 Genetik Variantları

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ABSTRACT

Objective: Coronary artery disease (CAD) is a multifactorial disease that influenced by both genetic and environmental factors. Single nucleotide polymorphisms (SNPs) in the candidate genes produce susceptibility to such multifactorial diseases. Therefore, investigations of SNPs, in the genes that may play role in etiopathogenesis of CAD, become crucial. In the present study we investigated the both independent and synergistically effects of matrix metalloproteinase (MMP) -1562 C/T and methylenetetrahydrofolate reductase (MTHFR) 677 C/T polymorphisms on the CAD occurrence.

Methods: In total 217 individuals, 109 coronary artery disease patients and 108 healthy controls were examined. We determined the genotypes for MMP-9 -1562 C/T and MTHFR 677 C/T polymorphisms by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP).

Results: We found no statistically significant differences between genotypes and allelic frequencies of both MMP-9 -1562 C/T and MTHFR 677 C/T polymorphisms and CAD (p>0.05). No TT homozygous genotype was found in any of groups for MMP9 -1562 C/T polymorphism. However, while C allele and CC genotype was found to be highly, TT genotype was found to be very rare for both polymorphisms in Southeastern Anatolia.

Conclusion: We have found no associations between MMP9 -1562 C/T and MTHFR 677 C/T polymorphisms and coronary artery disease. However, TT genotype was determined to be very rare in Southeast Anatolia.

Key words: Methylenetetrahydrofolate reductase (MTHFR) -677, Matrix metalloproteinase 9 (MMP9) -1562, polymorphism, coronary artery disease

ÖZET

Amaç: Koroner arter hastalığı (KAH), hem genetik hem de çevresel faktörlerden etkilenen çok faktörlü bir hastalıktır. Aday genlerdeki tek nükleotid polimorfizmleri (SNP) bu tür multifaktöryel hastalıklara yatkınlığa neden olurlar. Bu yüzden, KAH etiyopatogenezinde rol oynayan genlerde SNPlerin araştırılması, önemli hale gelir. Bu çalışmada, KAH oluşumu üzerinde, matriks metalloproteinaz-9 (MMP9) -1562 C/T ve metilentetrahidrofolat redüktaz (MTHFR) 677 C/T polimorfizmlerinin bağımsız ve sinerjistik etkileri araştırıldı.

Yöntemler: 109 koroner arter hastası ve 108 sağlıklı kontrol olmak üzere toplam 217 birey incelendi. MTHFR 677 C/T ve MMP-9 -1562 C/T polimorfizmleri için genotipler polimeraz zincir reaksiyonu (PCR)- restriksiyon fragmanı uzunluk polimorfizmi (RFLP) ile belirlendi.

Bulgular: KAH ile MMP-9 -1562 C/T ve MTHFR - 677 C/T polimorfizmlerinin genotipleri ve alel frekansları arasında istatistiksel olarak anlamlı bir farklılık olmadığı bulundu. (p> 0.05) MMP9 -1562 C/T polimorfizmi için TT homozigot genotipi hiç bir grupta bulunmadı. Bununla birlikte, Güneydoğu Anadolu Bölgesinde C aleli ve CC genotipi her iki polimorfizm için hakim iken, TT genotipi ise çok nadir olarak bulundu.

Sonuç: MTHFR 677 C/T ve MMP9 -1562 C/T polimorfizmleri ile koroner arter hastalığı arasında ilişki bulunmadı. Ancak Güneydoğu Anadolu bölgesinde TT genotipinin çok nadir olduğu belirlendi.

Anahtar kelimeler: Metilentetrahidrofolat redüktaz MTHFR 677 C/T, matriks metalloproteinaz-9 (MMP9) -1562 C/T, polimorfizm, koroner arter hastalığı

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INTRODUCTION

Coronary artery disease (CAD) is a complex genetic disease influenced by both genetic and environmental factors [1]. Although CAD mortality decreases due to primarily preventive measures and successful treatments, it remains as the most frequent cause of death even in developed countries [2]. Atherosclerosis is major cause of CAD characterized by plaque formation, alteration and progression to unstable plaques prone to rupture and complete occlusion [3,4].

The matrix metalloproteinases (MMPs) are a family of zinc dependent enzymes with proteolytic activity against connective tissue proteins such as collagens, proteoglycans and elastin [5]. MMPs are suspected to play an important role in the pathogenesis of cardiovascular diseases (CVD) including atherosclerosis and restenosis, because of their major significance in vascular remodeling [6,7].

The MMP-9 (gelatinase B or 92-kDa type IV collagenase) is a member of the MMP family and highly expressed in the vulnerable regions of the atherosclerotic plaques. It has been shown that elevated blood levels of MMP-9 lead to plaque rupture and induce development and progression of atherosclerotic lesions [1,6,7,8,9]. MMP-9 gene contains 13 exons and 7.7 kb of DNA [5]. The MMP-9 -1562 C/T promoter gene polymorphism (rs3918242) affects the expression and activity of MMP-9, influences the susceptibility, development and progression of atherosclerosis and related to presence and severity of CAD [10-12].

Methylene tetrahydrofolatereductase (MTHFR) gene is located on chromosome 1p36.3, composed of 11 exon and produced 2,2 kb long cDNA [5,13]. It encodes 656 amino acid consisting MTHFR enzyme that have been associated with CAD ethiopathogenesis. MTHFR enzyme involves in homocysteine (hcy) metabolism [13-17]. MTHFR gene 677 C/T polymorphism (rs1801133) is a common single nucleotide polymorphism (SNP) replaced alanine to valine at 226th position of MTHFR enzyme at folate binding site of catalytic domain [18]. It leads to reduced MTHFR enzyme activity and as consequence increases blood homocysteine levels by reducing of active folate production and remethylation to methionine [5,14,18-21]. In case hyperhomocysteinemia auto-oxidation of homocysteine produce free reactive oxygen species such as superoxide, hydrogen peroxide and hydroxyl radicals, and promote tissue injury and atherosclerosis caused from oxidative stress followed by lipid peroxidation, endothelial cytotoxicity, foam cell formation and smooth muscle cell proliferation [14,20].

In the present study we investigated the effects of MMP9 -1562 C/T and MTHFR 677 C/T polymorphisms on CAD both independently and synergistically in Turkish population from Southeast Anatolia region in Turkey.

METHODS

This study screened 298 consecutive patients with a suspected diagnosis of coronary artery disease and 108 healthy controls. Informed consent was obtained from each individual in accordance to study protocol approved by ethics committee of the Adıyaman University. The patients who applied to the Cardiology Department of Adıyaman 82nd Year State Hospital underwent elective coronary angiography for evaluation of cardiac symptoms, abnormalities in electrocardiograms (ECGs), or positive stress tests. 109 patients with stable CAD (presence of $\geq$ 60% coronary occlusion on cardiac catheterization) who were hospitalized for cardiac catheterization were eligible. Patients with history of acute coronary syndrome in the last one month, chronic cardiac failure, valvular heart disease, congenital heart disease, cardiomyopathy, chronic kidney disease, hepatic dysfunction, respiratory illness, prior stroke, and active infection were excluded from the study.

All patients underwent selective coronary angiography under local anesthesia through the femoral artery using the Judkins technique. The definition of the coronary tree segments is based on the classification proposed by the AHA and modified for the Arterial Revascularization Therapies Study (ARTS) I and II. A lesion is defined as significant when it causes a 60% reduction in luminal diameter by visual assessment.

In order to ensure genetic homogeneity, individuals from only a single region were included in this study.

Genomic DNA was extracted from blood samples (10 mL) using the salt method. DNA fragment amplification was performed by polymerase chain reaction (PCR).

For detection of MMP9 -1562 C/T polymorphism a 435 bp region in promoter was amplified by PCR using forward: 5'-GCCTGGCACATAGTAGGCCC-3' and reverse: 5'-TTCCTAGCCAGCCGGCATC-3' primer pair. Amplification was carried out for 35 cycles at 95°C for 45 sec, 60°C for 1 min, 72°C for 1.5 min, with a final extension period of 7 min at 72°C. Amplified PCR products were digested with 1 unit of the restriction enzyme SphI (New England Biolabs, Ontario, Canada) at 37°C overnight. Digested products were separated on 2% agarose gel and visualized with CCD camera and evaluated with Labsworks Software (BioRad). SphI digestion of MMP9 -1562 C/T PCR products produced CC, CT and TT genotypes with 435 bp, 435bp/ 247bp/188 bp and 247bp /188 bp fragments, respectively [12].

Genotypes of the MTHFR gene 677 C/T polymorphism were determined by PCR using forward: 5'-TGAAGGAGAAGGTGTCTGCGGGA-3' and reverse: 5'-AGGACGGTGCG GTGAGAGTG- 3' primer pair. Amplification was carried out for 35 cycles at 95°C for 45 sec, 61°C for 1 min, 72°C for 1.5 min, with a final extension period of 7 min at 72°C [15]. Amplified 198 bp PCR fragments were digested with 5 unit of the restriction enzyme HinfI (New England Biolabs, Ontario, Canada) at 37°C overnight. Digested products were separated on 2% agarose gel and visualized with CCD camera and evaluated with Labsworks Software (BioRad). HinfI digestion of MTHFR 677 C/T PCR products produced CC, CT and TT genotypes with 198 bp, 198 bp /175 bp /23 bp, and 175 bp /23 bp fragments, respectively.

Blood samples were centrifuged and separated serum samples were used immediately for analyzing C reactive protein (CRP), high-density lipoprotein (HDL), low-density lipoprotein (LDL), total cholesterol, and triglyceride. Serum CRP, HDL, LDL, total cholesterol and triglyceride levels were measured with commercial kit (Roche Diagnostics, Mannheim, Germany) using auto analyzer (Roche/ Hitachi Cobas c Systems, Basel, Switzerland).

Statistical analysis

Statistical analysis was performed by running a packaged program of IBM SPSS Statistics 20 software.

Comparison of the categorical variables (such as: gender, genotype, allele, haplotype…) between groups were performed with Pearson Chi-Square Test, Yates' Chi-Square Test, Fisher's Exact Test, One Proportion Exact p Value and Chi-Square Goodness of Fit Test analyses. On the other hand, continuous variables were compared between groups with Mann-Whitney U Test for the non-normal variables and Student's t Test for the normally distributed variables. We compared parameter values between the two groups by means of two independent sample t test and Mann Whitney-U test. Furthermore, we used Shapiro-Wilk Test for the normality. The p values less than 0.05 accepted as statistically significant.

RESULTS

Study populations were included 109 coronary artery disease patients and 108 healthy controls. Main characteristics of the patients and controls are listed in Table 1. Among risk factors for cardiovascular disease: Diabetes mellitus (DM), myocardial infarction (MI), total cholesterol, high-density lipoprotein (HDL) and C-reactive protein (CRP) were found to be different between the patient and control groups ($p<0.05$).

There were no differences in age, gender, stroke history, body mass index (BMI), and other cardiovascular risk factors such as LDL, triglyceride, systolic and diastolic blood pressure between the groups ($p>0.05$) (Table 1).

The genotype and allele frequencies of the MMP9 -1562 C/T polymorphism in CAD patients and controls are summarized in table 2. The TT genotype was not found both in patients and controls. We found genotype frequencies of MMP9 -1562 C/T polymorphism as 76.8% CC and 23.2% CT for controls and 76.1% CC and 23.9% CT for patients. We found -1562 T allele frequencies as 11.6% for controls and 11.9% for patients. While C allele frequencies were found as 88.4% for controls and 88.1% for the patients. There were no statistically

significant differences between control and patient groups in terms of numbers and percentages of genotypes and alleles of MMP-9 -1562 C/T (p=0.222, p=0.581 respectively).

Table 1. Comparison of baseline characteristics of coronary artery disease patients and healthy controls (Mean ± standard deviation)

Risk factors	CAD (n=109)	Controls (n=108)	p
Age (years)	59.05 ±12.84	58.25 ± 11.82	0.633**
Gender (female/male)	41 / 66	40 / 68	0.889***
BMI (kg/m^2)	27.18 ± 3.69	26.76 ± 3.89	0.708*
Systolic BP (mmHg)	123.56 ± 20.88	124.52 ± 11.94	0.273*
Diastolic BP (mmHg)	76.13 ± 12.09	78.34 ± 10.17	0.091*
Total cholesterol, mg/dL	187.37± 53.68	195.20 ± 34.40	0.018*
HDL cholesterol, mg/dL	39.82 ± 9.57	45.96 ± 13.86	0.003*
LDL cholesterol, mg/dL	112.71 ± 47.90	115.16 ± 32.96	0.188*
Triglyceride, mg/dL	176.28 ±128.11	177.65 ± 113.96	0.736*
CRP, mg/L	22.88 ± 4.51	7.17 ± 3.17	<0.001****
Smoking (+/-), (%)	(24) / (76)	(10.2) / (89.8)	0.200[a]
Alcohol (+/-), (%)	(14.7) / (85.3)	(7.4) / (92.6)	0.384[b]
DM (+/-), (%)	(26.5) / (73.4)	(7.1) / (92.9)	0.010[a]
MI (+/-), (%)	(62.38) / (37.62)	(0.0) / (100)	<0.001[a]
Stroke (+/-), (%)	(16.5) / (83.5)	(0.0) / (100)	0.429[b]

CAD: Coronary artery disease, BMI body mass index, BP blood pressure, HDL- plasma high-density lipoprotein, LDL plasma low density lipoprotein - CRP plasma level of C-reactive protein, DM- diabetes mellitus MI-myocardial infarction

*: Mann-Whitney U Test, **: Student's t Test, ***: Pearson Chi-Square Test ****: Student T Test

[a] Yates' Chi-Square Test, [b] Fisher's Exact Test.

The genotype and allele frequencies of the MTHFR 677 C/T polymorphism in CAD patients and controls are also summarized in Table 2. We found genotype frequencies of MTHFR 677 C/T polymorphism as 57.8% CC, 36.7% CT and 5.5% TT for controls and 64.2% CC, 35.8% CT and 0% TT for patients. T allele frequencies were found as 23.9% for controls and 17.9% for patients, while C allele frequencies as 76.1% for controls and 82.1% for the patients. There were no statistically significant differences between control and patient groups in terms of numbers and percentages of genotypes and alleles of MTHFR 677 C/T polymorphism (p=0.398, p=0.581 respectively). Genotype distributions were in Hardy Weinberg equilibrium for both MMP-9 -1562 C/T and MTHFR 677 C/T polymorphisms.

Table 2. Analysis of MMP9 -1562 C/T and MTHFR 677 C/T polymorphisms genotype and allele frequencies in healthy controls and coronary artery disease patients

Genotype	CAD patients (n=109) n (%)	Healthy controls (n=108) n (%)	p[†]
MMP9 - 1562 C/T			
CC	83 (76.1)	83 (76.8)	0.222
CT	26 (23.9)	25 (23.2)	
TT	0 (0)	0 (0)	
C	192 (88.1)	191 (88.4)	0.581
T	26 (11.9)	25 (11.6)	
MTHFR 677 C/T			
CC	70 (64.2)	63 (57.8)	
CT	39 (35.8)	40 (36.7)	0.398[†]
TT	0 (0)	6 (5.5)	
C	179 (82.1)	166 (76.1)	0.581[†]
T	39 (17.9)	52 (23.9)	

CAD: Coronary artery disease, [†]Pearson Chi Square test

No statistically significant differences were found in combination of MMP-9 -1562 C/T (rs3918242) and MTHFR gene 677 C/T (rs1801133) polymorphisms in terms of haplotype (MMP9/MTHFR; C/C, C/T, T/C, T/T) analysis between controls and CAD (p> 0.05) (Table 3). We found haplotype frequencies of MMP9-1562 C/T /MTHFR 677 C/T polymorphisms as 70.4% C/C, 4.9% C/T, 18.9% T/C and 5.8% T/T for healthy controls and 69.7% C/C, 6.9% C/T, 20.2% T/C and 3.2% T/T for patients.

Table 3. Haplotype analysis of MMP9-1562 C/T and MTHFR 677 C/T gene polymorphisms in healthy controls and the patients

Haplotype	CAD patients (109) n (%)	Healthy controls (108) n (%)	p	OR (%95 CI)
MMP9 /MTHFR				
C/C	131 (69.7)	145 (70.4)		1.396 (0.590-3.300)
C/T	13 (6.9)	10 (4.9)	0.513	1.135 (0.672-1.917)
T/C	38 (20.2)	39 (18.9)		0.537 (0.195-1.475)
T/T	6 (3.2)	12 (5.8)		

CAD: Coronary artery disease, OR: Odds ratio

DISCUSSION

Coronary artery disease is a multifactorial disease that influenced by both genetic and environmental factors. SNPs in the candidate genes produce susceptibility to such multifactorial diseases. Therefore, investigations of SNPs in the genes that may play role in etiopathogenesis of CAD become crucial. In the present study we investigated the both independent and synergistically effects of MMP9 -1562 C/T and MTHFR 677 C/T polymorphisms that are known to have important role in the etiopathogenesis of CAD in consequence of atherosclerosis, on the CAD occurrence in Turkish population from Southeast Anatolia.

Among risk factors for cardiovascular disease; Age, DM, MI, total cholesterol, HDL and CRP were found to be different between patient and control groups. However, there were no differences in terms of gender, stroke, BMI, and other cardiovascular risk factors as LDL, triglyceride, systolic and diastolic blood pressure between groups (Table 1).

CAD increases mortality and still remains as the most frequent cause of death in developed countries, therefore knowing the risk factors, taking preventive measures and effective treatment of this disease is mandatory. Hence, understanding of the contribution of SNPs in candidate genes and their effects on predisposition to atherosclerosis becomes important in development of new diagnostic and effective therapeutic approaches.

In this study we found no significant association between MMP-9 -1562 C/T genotype distributions and allele frequencies and CAD in comparison to patients and controls (Table 2). There are numerous studies that support our findings [9,11,22-24]. Alberia et al. have indicated in their published review, being a meta-analysis as well, although some studies of they have used in meta-analysis have shown associations between MMP gene polymorphisms including MMP9 -1562 C/T and coronary atherosclerosis, in their meta-analysis they could not find any significant relation [11]. The polymorphism -1562 C/T in the promoter region of MMP9 gene has been firstly defined by Zhang et al.[10]. They found association between this polymorphism and severity of coronary atherosclerosis [10]. Morgan et al. and Li et al. indicated that the CC genotype poses a low risk while the TT genotype constitutes a high risk for CAD [1,25]. Zhi et al. have suggested that MMP9 -1562 C/T polymorphism T allele is more likely to play role in CAD development in patients with DM and MI [4].

In the present study we found predominantly CC genotype of MMP9 -1562 C/T polymorphism whereas TT genotype was not identified in total 201 individuals. In some studies with similar findings; CC genotype was found predominantly while TT genotype had rarely occurrence [9,22,26,27]. Cho et al. concluded that homozygote CC genotype is more likely to be as a protective genetic effect against CAD [22]. Because we found in this study

high incidence of -1562 CC genotype even in patients group and no significant difference in terms of its distribution between CAD patients and controls, we assume that this point of view gives clues about this genotype that may not be having protective effects against CAD occurrence.

In this study, we found also no statistically significant association between MTHFR 677 C/T genotype distributions and allele frequencies and CAD (Table 2). Whereas some studies are consistent with our study [16,21,28-31], some other studies reported significant associations between MTHFR 677 T allele and CAD occurrence [15,19,20,32,33].

We are of the opinion, that our these results with no relation between both polymorphisms and CAD may be related to geographic and ethnic differences rather than sample size, because the results of different wide-scale studies carried out as meta analysis are: The study by Wu et al. on 1373 CAD patients and 695 healthy controls, in a total 2068 individual revealed CC, CT, and TT genotypes for MMP9 -1562 C/T polymorphism 80% / %19 / %1 in patients and 79% / 21% / 0.00% in controls respectively and produced no significant association between the polymorphism and CAD [34]. The study by Clarke et al. on 48,175 CAD cases and 67,961 controls revealed no evidence regarding to increased risk of CAD in TT versus CC homozygotes for the MTHFR 677C/T polymorphism [21].

We have found no statistically significant differences in combination of MMP-9 -1562 C/T (rs3918242) and MTHFR gene 677 C/T (rs1801133) polymorphisms in terms of haplotype (MMP9/MTHFR; C/C, C/T, T/C, T/T) analysis between controls and CAD (Table 3). Although both polymorphisms are known to be common in their own genes (MMP9 and MTHFR genes) we found them rarely in our study. Therefore they may not have influence on predisposition of CAD independently or synergistically in Turkish population from Southeast Anatolia region.

In conclusion, we found that there is no association between MMP9 -1562 C/T and MTHFR 677 C/T polymorphisms and coronary artery disease both independently and synergistically and the C allele and CC genotype are predominant for both polymorphisms in Turkish population from Southeast Anatolia region in Turkey, whereas the TT genotype is very rare.

Declaration of Conflicting Interests: The authors declare that they have no conflict of interest.

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