VOLUME 7 • NUMBER 3 • MARCH 2016

JOURNAL OF CLINICAL AND EXPERIMENTAL INVESTIGATIONS

RESEARCH ARTICLE

The association between MGP gene polymorphisms and coronary artery disease

Mehmet Zihni Bilik[1], Ali Fuat Kara[2], Bülent Göğebakan[3], Mehmet Ata Akıl[1], Ferhat Özyurtlu[4], Halit Acet[1], Sait Alan[1]

[1]Dicle University , Faculty of Medicine , Department of Cardiyology, Diyarbakır, Turkey
[2]Genesis Hospital, Department of Cardiyology, Diyarbakır, Turkey
[3]Mustafa Kemal University, Faculty of Medicine, Department of Medical Biology, Hatay, Turkey
[4]Grandmedical Hospital, Department of Cardiyology, Manisa, Turkey

ABSTRACT

Objective: In this respect, we aimed to establish the relationship between the distributions of nucleotide alterations found in promoter and coding regions of the Matrix Gla protein (MGP) gene in patients with coronary artery disease (CAD) and patients with normal coronary.
Methods: DNA samples (n = 115) were obtained from 58 patients with CAD and 57 healthy controls. The DNA samples obtained were analyzed by a Polymerase Chain Reaction (PCR) method using 3 sets of primer pairs, which cover the coding (Thr83Ala in exon 4) and promoter regions (T-138C and G-7A) of the MGP gene. Amplified regions were analyzed by a Restriction Fragment Length Polymorphism (RFLP) method for possible polymorphisms.
Results: The chi-square analysis of the results revealed that there is no relationship between the observed polymorphisms and CAD.
Conclusion: In this study, we investigated the relationship between MGP gene polymorphism and CAD. However, according to our findings, there was no statistically significant difference between the CAD and the control group.
Key Words: Coronary artery disease, vascular calcification, matrix Gla Protein, genetic polymorphism

Correspondence:
Dr. Mehmet Zihni Bilik,
Dicle Üniversitesi Tıp Fakültesi,
Kardiyoloji Anabilim Dalı, Kalp Hastanesi, Diyarbakır, Türkiye

E-mail: mzbilik@mynet.com
Received: 03 June 2016,
Accepted: 13 July 2016
DOI: 10.5799/jcei.328614

INTRODUCTION

Coronary artery disease (CAD) is one of the most common causes of mortality and morbidity in the world. The main cause of CAD is atherosclerosis [1].

Atherosclerosis affects most of the arterial system especially aorta, carotid, and coronary and cerebral arteries. Inflammation, endothelial injury, oxidative stress, and calcification are important in the pathogenesis of atherosclerosis [2]. Calcification is caused by calcium deposition in tissues [3]. In the coronary arterial plaques, calcium deposition makes an important contribution to the plaque volume [4]. Coronary artery calcification (CAC) has an important role in the formation of atherosclerosis and there is a strong relationship between CAC and plaque burden [5]. CAC is an active process. It has been revealed that CAC is an independent factor for cardiovascular mortality and morbidity [6]. Clinically, CAC leads to stiffening of the arterial vessel wall and this leads to a reduction in arterial compliance. Thus, the perfusion in the coronary artery decreases as a result of fatal complications that arise [7, 8]. CAC is commonly seen with aging, in patients with chronic kidney disease, diabetes mellitus, and atherosclerosis [9, 10].

Matrix Gla protein (MGP) is one of the protective proteins, which inhibit arterial calcification [11]. MGP contains 84 amino acids and molecular weight of mature MGP is 10-kDa [12]. It has been shown that MGP deficient rat models have an excessive mineralization in the cartilage and severe calcification in the main arteries. As a result, vessel occlusion occured and the rats died within 8 weeks after they were born due to damage of the thoracic and abdominal

aorta [11]. Normally, the vascular smooth muscle cells and chondrocytes have non-calcified extracellular matrix. However, it has been shown that both of them have severe calcification when MGP was deficient. According to the data, MGP was suggested to be a potent calcification inhibitor macromolecule [13].

In this study, we aimed to investigate the relationship between three single nucleotide polymorphisms of MPG (T-138C, G-7A and Thr83Ala) and CAD.

METHODS

Patients

The study included a total of 115 patients who were admitted to the cardiology department and underwent a coronary angiography between January 2009 and January 2010. Fifty eight patients with CAD (66% male) were registered as the patient group, and 57 cases with normal coronary artery without CAD (53% male) proven angiographically, were registered as control group. The study was approved by the Local Ethics Committee and informed consent was obtained from each patient.

All patients received a complete physical examination. Hypertension was defined as systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, or active use of antihypertensive medication. Diabetes mellitus (DM) was considered when fasting plasma glucose levels were above 126 mg/dL in at least two different measurements or active use of anti-diabetic drugs. Smoking was defined as currently smoking or ex-smokers who forwent smoking in the past 6 months. Patients with severe liver and renal diseases, heart failure, cancer, severe valvular diseases were excluded from the study. Blood was collected under sterile conditions into 5 mL tubes containing EDTA potassium salt as an anticoagulant (samples were frozen and stored at -20°C). Total cholesterol, HDL-cholesterol, and LDL-cholesterol were measured by an Abbott Architect C16000 autoanalyzer (Abbott Laboratory, Abbott Park, IL, USA) with original kits. Fasting lipid panels were obtained after an overnight fast.

Coronary angiography was routinely performed using the Allura Xper FD10 (Philips, Amsterdam, The Netherlands) through femoral artery by Judkins technique. The contrast agent was Iopamiro 370 (Bracco, Milan, Italy) that used in all patients. Coronary artery disease was defined as stenosis greater than 50.0% at least in one of the major coronary arteries. Transthoracic echocardiography was performed to determine left ventricular ejection fraction (LVEF) (Vivid S6, GE Medical Systems, USA).

Statistical Analysis

Statistical analysis was performed using statistical software package (SPSS 18.0, Chicago, USA). The quantitative parameters were reported as mean and standard deviation. The differences between groups were evaluated by student t-, chi-square, and Fisher's Exact tests. A p-value of less than 0.05 was considered to be significant. The genotype frequencies for the polymorphisms were compared with under conditions of Hardy-Weinberg equilibrium using the chi-square test.

DNA extraction and Genotyping

Five milliliters of blood was collected into EDTA containing tubes to use for DNA isolation and blood was stored at -20°C. The genomic DNA was extracted from WBC using salting out method [14].

DNA Analysis

Polymerase Chain Reaction (PCR) technique was used for DNA analysis. Reproduction process of the relevant regions for MGP gene was performed by in-house type MJ Research PTC-200 PCR machine.

PCR Process

Reference sequences were taken from NCBI database. For MGP structural gene region, gi:37543832 numbered sequence which is in the NT_009714 numbered contigs region, for promoter region, gi:3172535 numbered sequence, which is in the AF067176 accession numbered region were used to design the primers.

The base pairs which are used to analyze the MGP gene polymorphism were designed by using the online primer design program (http://workbench.sdsc.edu/).

Firstly, a gradient PCR was performed for 1 DNA sample and 3 base pairs to check the annealing temperature for the base pairs used in PCR. Optimized annealing temperature was obtained for T-138C and Thr83Ala as 59°C for G-7A as 65°C.

PCR was performed in a total volume of 20 µL of a buffer solution containing the following: distilled water (13.9 µL), 10X Taq buffer (2.4 µL), 25mM $MgCl_2$ (1.2 µL), 2mM dNTP mix (1.6 µL), 100mM F-Primer (0.16 µL), 100mM R-Primer (0.16 µL), Taq polimerase-5u/µL (0.08 µL), and genomic DNA (0.5 µL).

For G-7A F-primer (5'-CTAGTTCAGTGCCAACCCTTCCCCACC-3') and the R-primer (5'-TAGCAGCAGTAGGGAGAGAGGCTCCCA-3').

For T-138C F-primer (5'-AAGCATACGATGGCCAAAACTTCTGCA -3') and R-primer

(5'- GAACTAGCATTGGAACTTTTCCCAACC–3').

For Thr83Ala F-prime (5'-CACGAGCTCAATAGGGAAGC–3') and R-primer

(5'- GCTGCTACAGGGGGATACAA- 3').

PCR for three polymorphisms was run as following: Denaturation at 94°C for 5-min followed by 35 cycles of 94°C for 30 s, 65°C (G-7A), and 59°C (Thr83Ala and T-138C) for 60 s, and 72°C for 60 s and final extension at 7 min.

Variables of the reaction except the primers' annealing temperature were same in all reactions and obtained products

were analyzed by Restriction Fragment Length Polymorphism (RFLP).

Agarose Gel Elektrophoresis

Agarose gel electrophoresis was used to check the PCR reaction. Samples were run on agarose gel (1.0%) for 40 minutes at 90 volts (range 37-501 bp) in the presence of pUC19 DNA/Mspl marker. Then it was visualized by UV transillumination after ethidium bromide staining.

Restriction Process

Ten µl PCR product was placed into microcentrifuge tube (0.2 ml) along with distilled water (dH2O) (3.4 µl) and 10X RE buffer (1.5 µl) and RE [BseNI, HaeIII, NcoI (10 u/µl)] to obtain a 0.1 µl mixture. Five µl of a prepared mixture was added to each tube containing the PCR products. These were then subjected to restriction digestion. After incubation, samples were run on agarose gel (3.0%) for 40 minutes at 90 volts (range 37-501 bp) in the presence of pUC19 DNA/Mspl marker for analyzing the restricted products. Subsequently, the digested DNA was visualized by UV transillumination by staining with ethidium bromide. The genotype of each sample was determined by analyzing the obtained bands.

RFLP Process

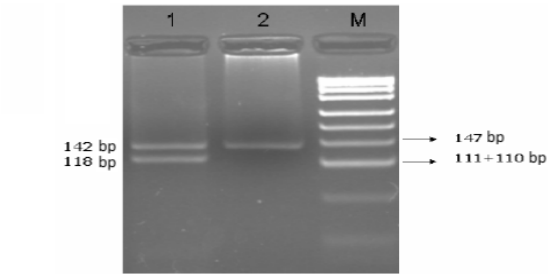

Figure 1. The band images of nucleotide exchange for T-138C polymorphism obtained by the RFLP method

By PCR, 142 bp sized product was obtained for T-138C. Then, it was restriction digested with BseNI and ran on an agarose gel using electrophoresis (3.0%). The nucleotide was found in the -138. If the position was C, then BseNI cannot digest the 142bp fragment. If the nucleotide was T, the enzyme performed the restriction digest. By the restriction process, 2 DNA fragments (118 and 24 bp in length) were obtained. According to this, sample 1 was TC and 2 was CC. M was the pUC19 DNA/Mspl marker.

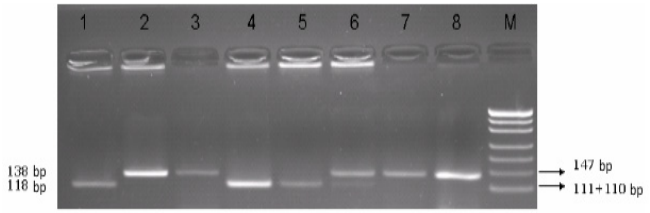

Figure 2. The band images of nucleotide exchange for G-7A polymorphism obtained by the RFLP method

By PCR, the 138 bp sized product was obtained for G-7A and subjected to NcoI restriction digestion. Samples were genotyped, and restriction digested products were run using agarose gel electrophoresis (3.0%) in the presence of pUC19 DNA/Mspl marker. The nucleotide in the -7 position was G, if the NcoI enzyme did not digest the sample. If the nucleotide was A, the enzyme digested the DNA. By restriction process, 2 DNA fragments were obtained (118 and 20 bp in length). According to this, samples 2, 3 and 7 are GG; samples 1, 4 and 5 are AA; sample 6 was GA; and sample 8 is used as negative control.

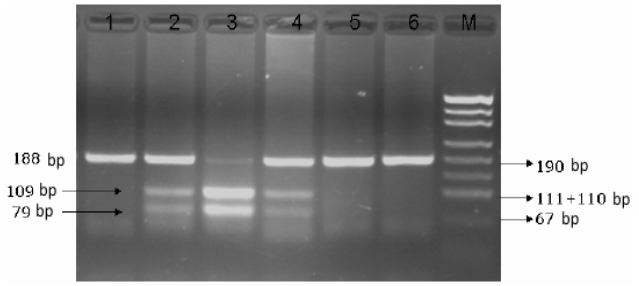

Figure 3. The band images of nucleotide exchange for Thr83Ala polymorphism (A→G) obtained by the RFLP method

By PCR, a 188 bp sized product was obtained for Thr83Ala. Then, it was restricted with HaeIII enzyme and was run on an agarose gel electrophoresis (3.0%). If A, HaeIII did not digest the DNA. If the nucleotide was G, the enzyme performed the restriction digestion. By the restriction process, 2 DNA fragments were obtained (109 and 79 bp in length). According to this, samples 1 and 5 are AA; samples 2, 3 and 4 are GA; and sample 6 was a negative control. M was DNA marker (pUC19 Mspl).

RESULTS

The clinical characteristics of 58 patients with CAD and 57 controls were summarized in Table 1. In the CAD group 66% of patients and in control group 53% were male. The mean age was significantly ($p = 0.048$) higher in CAD group (63.6 ± 12.3 years) than in the control (47.7 ± 11.7 years). There was no significant differences between the two groups with respect to LDL-C, HDL-C, total cholesterol, cigarette smoking, and LVEF. Diabetes mellitus ($p = 0.012$) and hypertension ($p = 0.032$) were significantly more prevalent in the patient group.

Results of patient genotyping of the MGP genes were summarized in Table 2. As shown, TT, TC, and CC genotype distributions for T-138C polymorphism in CAD group were 39.7%, 37.9% and 22.4%, respectively. In the control group, they were 42.1%, 45.6%, and 12.3%, respectively. There was no statistically significant difference between groups ($p=0.762$ for TC, $p=0.289$ for CC). T and C allele frequencies were not significant between controls and patients ($p= 0.326$)

GG, GA and AA genotype distributions for G-7A polymorphism were as following. In CAD group were 39.7%, 39.7% and 20.6%

respectively. In the control group, they were 38.6%, 49.1% and 12.3%, respectively. The difference between groups was not significant (p=0.556 for GA and p=0.422 for AA). The G and A allele frequencies were not significant (p=0.567).

AA, AG, and GG genotype distributions for Thr83Ala polymorphism also were not significant (p=0.120 for AG and p=0.777 for GG). Distributions in CAD group were 36.2%, 46.6% and 17.2%, respectively. In the control group, they were 24.6%, 61.4% and 14.0%, respectively. The A and G allele frequencies were not significant (p=0.518)

Table 1. Baseline characteristics of the study population

	CAD group (n=58	Control (n=57)	p value
Male, n(%)	38 (66%)	30 (53%)	0.185
Age, year	63.6 ± 12.3	47.7 ± 11.7	0.048
Diabetes Mellitus,n(%)	22 (38%)	3 (5.3%)	0.012
Hypertension, n(%)	37 (64%)	20 (35%)	0.032
Smoking, n(%)	14 (24%)	19 (33%)	0.112
LDL-C, mg/dl	132.7 ± 37.3	124.2 ± 35.3	0.298
HDL-C, mg/dl	40.1 ± 8.3	42 ± 9.2	0.381
Total Cholesterol, mg/dl	161.7 ± 58.3	153.5 ± 57.9	0.212
LVEF, %	57.3 ± 9.5	58.6 ± 7.1	0.874

Data were presented as mean ± SD or %. CAD: coronary artery disease, HDL-C: high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol, LVEF: left ventricular ejection fraction.

Table 2. The distribution of genotypes and alleles in CAD and control groups for T-138C, G-7A and Thr83Ala (4A-G) polymorphisms

Genotypes MGP 138 (T-C)	CAD (*n* = 58), *n* (%)	Control (*n* = 57), *n* (%)	P	OR (95% CI)
T/T	23 (39.7)	24 (42.1)		
T/C	22 (37.9)	26 (45.6)	0.762⁺	0.883 (0.394-1.977)
C/C	13 (22.4)	7 (12.3)	0.289*	1.938 (0.656-5.721)
Alleles				
T	68 (58.6)	74 (64.9)		
C	48 (41.4)	40 (35.1)	0.326+	1.306 (0.766-2.226)
Genotypes MGP 7 (G-A)				
G/G	23 (39.7)	22 (38.6)		
G/A	23 (39.7)	28 (49.1)	0.556+	0.786 (0.352-1.755)
A/A	12 (20.6)	7 (12.3)	0.422*	1.640 (0.546-4.929)
Alleles				
G	69 (59.5)	72 (63.2)		
A	47 (40.5)	42 (36.8)	0.567+	1.168 (0.686-1.987)
Genotypes MGP 4 (A-G)				
A/A	21 (36.2)	14 (24.6)		
A/G	27 (46.6)	35 (61.4)	0.120+	0.514 (0.222-1.194)
G/G	10 (17.2)	8 (14.0)	0.777*	0.833 (0.264-2.632)
Alleles				
A	69 (59.5)	63 (55.3)		
G	47 (40.5)	51 (44.7)	0.518+	0.841 (0.499-1.420)

OR: Odds ratio, CI: Confidence interval, ⁺Chi-square test, *Fisher's Exact Test

DISCUSSION

Atherosclerosis is the major cause of CAD, and is characterized by endotelial dysfunction, vascular inflammation, calcification, and accumulation of lipid and inflammatory cells in the intima [1]. In the development and progression of atherosclerosis, genetic risk factors can play an important role especially in young patients adding to classic risk factors such as hypertension, hyperlipidemia, smoking, and diabetes mellitus [15]. Some of the patients with CAD are come up with acute myocardial infarction or sudden cardiac death without any pre-specified symptoms. At this point, determination of the risk profile for cardiovascular disease and the initiation of appropriate prevention programs for patients before symptoms are highly important.

At present, frequently used protection algorithms including classic cardiovascular risk factors may be inadequate to determine the person's risk profile. Recently some methods such as coronary artery calcium scoring are thought to be helpful in this regard. Coronary artery calcification by computed tomography scan is a noninvasive measure of the extent of atherosclerosis. CAC progression is strongly associated with most CAD risk factors [16]. There is a relationship between coronary arterial calcification and CAD [17]. CAC burden is a major determinant of subclinical atherosclerosis and gives prognostic information, which adds to the conventional risk factors about a person's cardiovascular risk. After it has been demonstrated that CAC is a reversible process [18], attention to arterial calcification inhibitors was increased.

One of the major calcification inhibitors is MGP. Although the molecular mechanism of MGP function is not fully understood, the firstly collected information has revealed that its major role was the inhibition of soft tissue calcification. The first data about this effect of MGP has come from a study which rats were treated with warfarin an antagonist of the K-vitamin [19]. Massive cartilage calcification was developed especially in epiphysis and facial bones in rats [20]. After MGP has been identified in cartilage, the cartilage calcification was thought to be associated with the loss of MGP function [21]. The primary function of MGP was found to inhibit arterial media calcification. MGP-knockout rats died within 6-8 weeks due to main artery rupture and resulted in elastic lamellae calcification in the tunica media [22].

In MGP knockout rats, calcium-phosphate deposition ratios were similar to hydroxyapatite in arterial calcification-like bone mineralization. By histochemical studies, it has been shown that arterial calcification was together with change of vascular smooth muscle cells to chondrocyte-like cells [19]. MGP inhibits the conversion of vascular muscle cells to chondrocyte and osteoblast-like cells. This finding was also observed by Shanahan et al. They reported that MGP expression in normal vessels was less than in diabetic patient's vessel media with presenting Mönckeberg sclerosis [18].

In normal vessels, MGP synthesis is low because the inhibition of calcification is less needed. In calcified vessels, there was increased MGP values by MGP synthesis. But, it has been shown that increased MGP containing mostly an inactive form of MGP (uncarboxylated MGP-ucMGP) [18]. ucMGP accumulate in atherosclerotic and calcified arteries. Carboxylated MGP (cMGP) is the active form of MGP and it is usually not presenting in calcified arteries. Sweat et al. reported that calcified arterial lesions in rats were included high MGP values and this MGP form was ucMGP [23]. Another study showed massive ucMGP around calcified lesions in rat's arteries which were treated with warfarin [18].

CAC is an important predictor of cardiovascular mortality and recent data present that MGP is a major calcification inhibitor. Although it is known CAC's this affect, Huang et al. showed that massive coronary arteries calcification was not associated with plaque stress [24].

In vitro and clinical studies had been performed about MGP gene polymorphism have different or opposite results. Ferzaneh-Far et al. reported that -138C molecular variety was more effective than -138T up to 4 times in an expression study by using vascular smooth muscle cells in rats. In this study -138CC homozygotes had 30% higher serum MGP values. Therefore, they considered that the -138C allele could provide protection against tissue calcification. And indicated that *in vitro* G-7A allele did not lead to significant differences in the expression of gene [25]. On the other hand, Hermann et al. reported that the minor -138C allele was decreased and the promoter regio activity was 20% in rats' vascular smooth muscle cells and 50% in human fibroblast cell line when compared with the -138T allele. The G-7A allele were more common in patients with previous myocardial infarction, and the –7A allele was more frequent in patients with femoral atherosclerotic calcification. They concluded that the –7A or Ala 83 alleles of the MGP gene may confer an increased risk of plaque calcification and myocardial infarction [26]. Brancaccio et al. found that the –138 TT genotype was more frequent in hemodialysis patients and the –7 AA genotype was more frequent in both chronic kidney disease and hemodialysis patients when compared with control group. They suggested that patients with –7A allele had an increased risk of calcification and cardiovascular events [27].

Cassidy-Bushrow reported that the MGP Thr83Ala polymorphism was associated with CAC progression [28]. Garbuzova et al. compared 115 acute coronary syndrome (ACS) patients with 115 healthy subjects in their study and they revealed that a polymorphism of MGP G–7A was significantly associated with ACS. However, they did not find any significant relation between ACS and T–138C and Thr83Ala [29]. Najafi et al. showed in their study that the MGP promoter polymorphic variants (MGP promoter rs 1800801 G-7A, rs1800802 T-138C and rs1800799 polymorphisms) and its serum levels were not associated with the stenosis of coronary arteries [30].

In this study, we aimed to establish the relationship between the distributions of nucleotide alterations found in promoter and coding regions of the MGP gene in patients with CAD. According to the literature CAC is more prevalent in patients

with older age, DM, and hypertension. In this study, the patient group was older than the control group; and DM and hypertension were more prevalent in patient group. However, there was no significant difference between the patient and control group. In these patients, the vessel calcification may be related to other molecular activated pathways including passive calcium deposition.

Study Limitations

The population of the study was relatively small. This was a limitation of the study. Furthermore, larger studies are needed to investigate the relationship between MGP gene polymorphism and CAD.

In conclusion, in this study, we investigated the relationship between MGP gene polymorphism and CAD. However, according to our findings, there was no statistically significant difference between the CAD and the control group.

Conflict of Interest: The authors declare no conflict of interest.

Financial Disclosure: No financial support was received.

Acknowledgement: We are grateful to Dicle University DUBAP for their sponsorship about this study and English editing of this manuscript.

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