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Are P-glycoprotein (ABCB1/MDR1) and endothelial nitric oxide synthase (eNOS) polymorphisms related to severity of the coronary artery disease?

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Ethics Committee Approval
The approval of the Çanakkale Onsekiz Mart University, Faculty of Medicine Ethics Committee was obtained for this study (09.04.2014/07-04).
All procedures in this study involving human participants were performed in accordance with the 1964 Helsinki Declaration and its later amendments.

Conflict of Interest
No conflict of interest was declared by the authors.

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Abstract

Background/Aim: Atherosclerotic cardiovascular disease is one of the most common causes of morbidity and mortality in developed countries. Genetic and environmental factors are associated with atherosclerosis development. Some single nucleotide polymorphisms have also been directly related to atherosclerosis, for example, polymorphisms that reduce nitric oxide (NO) levels and/or activity have been linked to atherosclerotic diseases. However, the multidrug resistance gene 1 (MDR-1) polymorphism is related to repeated cardiovascular events. This study aimed to investigate the relationship between MDR-1 and endothelial NO synthase (e-NOS) polymorphism and severe coronary artery disease (CAD).
Methods: In total, 90 patients presenting with acute coronary syndrome were included in this cross-sectional study. Patients with at least > 70% stenosis in ≥ 2 coronary vessels were defined as severe CAD (Group 1), while those with this same level of involvement in < 2 vessels were diagnosed with single-vessel disease (Group 2). MDR 3435C-T and eNOS T-786C were determined by polymerase chain reaction (PCR) amplification. Comparison of parametric and nonparametric values between the two groups was performed using the Student's t- or Mann–Whitney U test. Categorical variables were analyzed using the χ^2 test. Risk estimations for the association of severe CAD with the polymorphisms were calculated using odds ratio (OR) and 95% confidence intervals by comparing the genotypic combinations.
Results: Baseline demographic parameters were similar in both groups, except for the presence of diabetes mellitus and glucose level. T allele of MDR was 42% and 40% in groups 1 and 2, respectively (OR = 1.12). The C allele of eNOS was 34% and 30% in groups 1 and 2, respectively (OR = 1.16). Fourteen and 15 patients (40% and 27%, respectively) had both T and C alleles in patients in groups 1 and 2, respectively (OR = 1.77). All *P*-values were > 0.05.
Conclusion: This study is the first one that shows that MDR1 and e-NOS polymorphisms are frequent in patients with ≥ 2 vessel disease and may be associated with severe CAD.

Keywords: MDR-1, eNOS, Coronary artery disease, Polymorphism, P-glycoprotein

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Introduction

Atherosclerotic cardiovascular disease is one of the most common causes of morbidity and mortality in people in developed countries. Atherosclerosis is a chronic, multifocal, immuno-inflammatory, and fibro-proliferative disorder that results from lipid accumulation in medium and large vessels. Endothelial dysfunction is an important change that is often seen in the pathogenesis of atherosclerosis [1, 2]. Many molecules and cytokines play important roles in endothelial functions. Genetic and environmental factors can affect endothelial functions through different mechanisms. Nitric oxide (NO) is a vasoactive substance synthesized from L-arginine via NO synthase. NO affects vascular small vessel tonus and also plays a protective role in atherogenesis because it causes a decrease in low-density lipoprotein (LDL) oxidation, promotes adhesion of thrombocytes and leukocytes to the endothelial surface, and leads to an increase in migration and proliferation of small vessel cells [3, 4]. Genetic polymorphisms on the endothelial NO synthase (eNOS) gene can cause a decrease in the release of NO and/or a deficiency in NO activity [5, 6]. A thymidine mutation in which replacement by cytosine at the nucleotide 786 (T-786C) gene can lead to a significant reduction in eNOS gene promoter activity and is associated with hypertension (HT), acute coronary syndrome (ACS), and coronary vasospasm [7, 8].

P-glycoprotein (Pgp) functions as an adenosine triphosphatase (ATP)-dependent pump of metabolic residual products, xenobiotics, many drugs, and some inflammatory mediators out of the cell [9]. The Pgp-encoded multidrug resistance gene 1 (MDR-1) 3435C-T polymorphism was shown to be related to repeated cardiovascular events in patients who are being treated with clopidogrel [10]. However, the relationship between MDR-1 polymorphism and atherosclerosis remains unclear.

In this study, the association between MDR-1, e-NOS polymorphisms, and severe coronary artery disease (CAD) was investigated.

Materials and methods

Patient population

In total, 90 patients with ACS who were admitted to the hospital and who underwent coronary angiography between November 2014 and April 2015 (mean age: 62 (9) years) were consecutively enrolled in this cross-sectional study. Diagnosis of ACS was defined as unstable angina pectoris (UA), non-ST-segment elevated myocardial infarction (NSTEMI), and/or ST-segment elevated myocardial infarction (STEMI) according to the European Society of Cardiology guidelines [11,12]. Possible biases were avoided by including all consecutive patients who fulfilled the specified diagnostic criteria within the specified time interval.

Definitions

A complete history, physical examination findings, risk factors for atherosclerotic heart disease, and current medications were recorded. Patients who were treated with antihypertensive drugs or those whose baseline blood pressure exceeded 140/90 mmHg were diagnosed with HT. Diabetes mellitus (DM) was defined as a fasting blood glucose level > 126 mg/dl or the use of anti-diabetic medication. Hyperlipidemia was defined as a total cholesterol level > 200 mg/dl and/or an LDL level > 160 mg/dl. Patients with known atherosclerotic disease, visible coronary artery plaque(s) on coronary angiography, peripheral artery disease, malignancy, renal or hepatic insufficiency, and/or chronic inflammatory disease, and those who were younger than 18 or older than 90 years of age were excluded from the study. All participants agreed to take part in the research, and the approval of the Çanakkale Onsekiz Mart University, Faculty of Medicine Ethics Committee was obtained for this study (09.04.2014/07-04).

Coronary angiography

Coronary angiography was performed with a femoral approach using Judkins catheters and the contrast agent, Iopramide (Ultravist-370, Bayer Schering Pharma, Germany) along with angiographic equipment (GE Medical Systems, Innova 2100, USA). Two independent cardiologists quantitatively evaluated the severity of coronary atherosclerotic lesions based on at least three projections in all patients. Cohen's kappa was calculated for intra-observer agreement test ($K = 0.94$; $P = 0.001$). Any patients with > 70% lumen stenosis in at least two projections in ≥ 2 vessels were described as having severe CAD (Group 1), while those with this same level of involvement in < 2 vessels were diagnosed with the single-vessel disease (Group 2).

Laboratory analysis

All routine biochemical tests were carried out with the Cobas 6000 Integra (Roche Diagnostics, IN, USA) auto-analyzer device using the chemiluminescence method. Venous blood was collected in 6-ml ethylenediaminetetraacetic acid (EDTA) tubes for isolation of the genomic DNA and stored at –20 °C. A total of 90 DNA samples were genotyped by real-time polymerase chain reaction (PCR) analysis.

The total genomic DNA was extracted using MagnaPure Compact (Roche) and Invitek kit extraction techniques (Invitek®; Invisorb spin blood, Berlin, Germany). Target genes were amplified by real-time PCR (qPCR), LightCycler 2.0 methods (Roche). LightCycler FastStart DNA Master HybProbes, master mix (water, PCR-grade, $MgCl_2$, stock solution, primer mix, HtbProbe mix), and template DNA from patients were used for real-time amplification for each target gene. MDR 3435C-T and eNOS T-786C were determined by PCR amplification.

The amplification protocol for eNOS T-786C consisted of a denaturation step of 10 min at 95 °C. The amplification conditions for 40 cycles consisted of several steps: (1) denaturation at 95°C for 5 s, (2) annealing at 62 °C for 10 s, (3) extension at 72 °C for 6 s, (4) melting curve step with denaturation at 72 °C for 30 s, (5) annealing at 95 °C for 20 s, (6) melting at 40 °C for 1 s, and (7) cooling step at 40 °C for 30 s.

The amplification protocol for MDR 3435C-T consisted of a denaturation step of 30 minutes at 95°C. The amplification conditions for 45 cycles consisted of several steps: (1) denaturation at 95 °C for 5 s, (2) annealing at 55 °C for 5 seconds, (3) extension at 72 °C for 8 s, (4) melting curve step with denaturation at 95 °C for 30 s, (5) melting at 40 °C for 2 s, (6) 80 °C 0.1 s, and (7) cooling step at 40 °C for 30 s. A software program (LightCycler 2.0, Roche) was used for the

detection of the mutated profiles for target single nucleotide polymorphism (SNP) analysis.

Statistical analysis

All statistical studies were carried out with the SPPS program (version 19.0, SPSS, Chicago, Ill., USA). Quantitative variables were expressed as median (minimum–maximum), and qualitative variables were expressed as percentages. All measurements were evaluated with the Kolmogorov–Smirnov test and the Shapiro–Wilk test, and a comparison of parametric and nonparametric values between the two groups was performed with the Student's t- or Mann–Whitney U test. Categorical variables (risk factors and polymorphisms) were analyzed using the χ^2 test. Risk estimations for the association of severe CAD with the polymorphisms were calculated using odds ratio and 95% confidence intervals (CIs) by comparing the genotypic combinations.

Results

Clinical and laboratory findings for the subjects are given in Table 1. Baseline demographic parameters were similar in both groups, except for the presence of DM and glucose level were higher in group 1. When coronary artery involvement was examined, stenosis rates of > 70% for each vessel, and the number of patients with the three-vessel disease was higher in group 1. Genotype properties and allele frequencies are given in Table 2. The T alleles of MDR were 42% and 40% in groups 1 and 2, respectively (OR = 1.12; *P* = 0.70). The C alleles of eNOS were 34% and 30% in groups 1 and 2, respectively (OR = 1.16; *P* = 0.63). Fourteen and 15 patients (40% and 27% in groups 1 and 2, respectively) had both T and C alleles (OR = 1.77; *P* = 0.21).

Table 1: Baseline demographic parameters of patients

Clinical characteristics	≥ 2 vessel disease (n = 35)	< 2 vessel disease (n = 55)	*P*-value
Male/Female	21/14	41/14	0.25
Diabetes mellitus, n (%)	12 (48)	18 (27.7)	0.06
Hypertension, n (%)	18 (72)	36(55.4)	0.15
Hyperlipidemia, n (%)	11 (44)	18(27.7)	0.138
Smoking, n (%)	13 (52)	38(58.5)	0.58
Family history, n (%)	2 (8)	6 (9.2)	0.85
Age, mean (SD)	63 (9)	61 (8)	0.17
Glucose, (mg/dl)	132 (84–280)	110 (72–263)	0.012
LDL, (mg/dl)	109 (53–200)	110 (45–194)	0.551
Troponin I, (ng/ml)	1.0 (0.11–25)	1.43 (0.1–25)	0.402
Hemoglobin, (g/dl)	12.75 (9.3–17.1)	13.7 (8.3–18)	0.17
Creatinine, (mg/dl)	0.9 (0.5–2)	0.8 (0.5–1.7)	0.38
eGFR, mean (SD)	80.8 (25.6)	89 (26.4)	0.151
Vessel stenosis ≥70%			
LAD, n (%)	10 (28.6)	15 (25.5)	0.02
Cx, n (%)	6 (17.1)	5 (9.1)	<0.001
RCA, n (%)	5 (14.3)	5 (9.1)	<0.001
Gensini score	94 (30–174)	52.7 (10–132)	0.001

SD: standard deviation, LDL: Low density lipoprotein, eGFR: Estimated glomerular filtration rate, LAD: Left anterior descending artery, Cx: Circumflex artery, RCA: Right coronary artery.

Table 2: The polymorphic SNPs, genotypic, and allele frequencies of the target MDR-1 and eNOS genes in both groups

Gene/Genotype	Patients		*P*-value	OR	CI (95%)
	≥ 2 vessel disease (n:35) n/%	< 2 vessel disease (n:55) n/%			
MDR-1 3435C<T					
C/C	9/25.7	19/34.5			
C/T	22/62.9	28/51.0			
T/T	4/11.4	8/14.5			
Alleles					
C	40/0.571	66/0.600			
T	30/0.429*	44/0.400	0.70	1.12	(0.61–2.06)
eNOS T786C					
T/T	16/45.7	28/51.0			
T/C	14/40.0	20/36.3			
C/C	5/14.3	7/12.7			
Alleles					
T	46/0.657	76/0.691	0.63	1.16	(0.61–2.02)
C	24/0.343**	34/0.309			
MDR-1 T + eNOS C alleles, patient (%)	14 (40)***	15 (27)	0.21	1.77	(0.72–4.37)

OR: odds ratio, CI: confidence interval, *: The T allele for MDR-1 3435C<T; OR = 1.12 (0.61–2.06), *P*=0.70, **: The C allele for eNOS T786C; OR = 1.16(0.61–2.02); *P* = 0.63, ***: The T allele for MDR-1 + C allele for eNOS T786C were significant; OR = 1.77 (0.72–4.37); *P*=0.21

Discussion

This study showed that coexistence of the eNOS T-786C and MDR-1 3435C-T polymorphisms may be related to the severity of CAD in ACS patients.

Endothelial dysfunction is an important mechanism in atherosclerosis pathogenesis. NO is one of many molecules that regulate endothelial functions. NO has a regulatory effect on vascular tonus; also, reduced activity of NO results in an increase of leukocyte adhesion, vascular small muscle cell migration and proliferation, and platelet aggregation [5–7, 13]. The T786C variation of the eNOS gene is associated with a reduction in gene promoter activity and therefore a decrease in NO levels. Fatini et al. [14] reported that the eNOS T-786 C/C genotype is related to a 9.1-fold higher risk of CAD in patients with homocysteinemia. Tangurek et al. [15] found that the incidence of eNOS T-786C polymorphism was 2.1-fold higher in patients with CAD than it was in controls; this trait was also related to CAD severity. Other studies have suggested that the T-786C CC genotype is an independent risk factor for both the presence and severity of atherosclerosis [13], and this risk is currently increasing in younger patients [16]. In our study, the C allele frequency for eNOS was higher in patients with severe CAD, but the difference was not statistically significant. This non-significant result may have been caused by our study's small sample size.

The MDR-1 (also called ABCB1) gene encodes Pgp, which is responsible for cellular efflux of many medications, inflammatory mediators, and metabolic remnants. Pgp also affects the absorption of drugs along the gastrointestinal tract [10]. Platelet-activating factor (PAF) is a pro-inflammatory molecule that stimulates the release of inflammatory mediators from endothelial and inflammatory cells [17]. A high PAF level or inappropriate PAF transport can cause an increase in inflammatory mediators and endothelial dysfunction [18]. Endothelial dysfunction is one component in the process of atherosclerosis onset [19]. PAF levels were found to be higher in patients with atherosclerosis and acute myocardial infarction than in controls [20–22]. The body's PAF level is regulated by metabolization or transmembrane transport mechanisms that include the Pgp transporter system. Because Pgp is responsible for the efflux of some phospholipid molecules, such as PAF,

PAF levels are related to Pgp expression and activity [23]. It is known that the SNP of MDR-1 can cause an increase in Pgp expression and activity. A few studies evaluating the relationship between CAD and MDR-1 polymorphisms can be found in the literature. Ayaz et al. [24] reported that the PAF levels are not related to MDR-1 polymorphism in both the CAD and control groups. Most MDR-1 studies have investigated antiplatelet resistance in atherosclerotic CAD and acute ischemic events. Spiewak et al. [25] demonstrated that C3435T polymorphism of the MDR-1 gene may influence platelet function as assessed using CADP-CT with PFA-100 in patients with ACS who were treated with percutaneous coronary intervention with stenting. A subgroup analysis of the TRITON-TIMI 38 study reported repeated coronary events in patients who required antiaggregant treatment [10]. To our knowledge, no study evaluating the presence of both eNOS and MDR-1 polymorphisms in ACS patients has been published.

We evaluated two polymorphisms of eNOS and MDR-1 that are associated with endothelial functions. The presence of the C allele in both eNOS and MDR-1 appears to be related to the severity of CAD in patients with ACS.

Limitations

Some limitations to this study should be discussed. First, our study population consisted of a small sample size because our funding was limited. The results will most likely be statistically significant if the number of patients is high enough. Second, whether inflammatory and coagulation markers were affected by polymorphisms was not examined. Last, our study population includes ACS patients, but a healthy control group was not included. More studies are needed to evaluate different genetic mechanisms and environmental factors in atherosclerosis pathogenesis.

Conclusion

This study showed that MDR1 and e-NOS polymorphisms frequently occur in patients with ≥ 2 vessel diseases and may be associated with severe CAD.

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