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786T/c endothelial nitric oxide synthase gene polymorphism and coronary collateral circulation

Polimorfizm 786T/C genu endotelialnej syntazy tlenku azotu a oboczne krążenie wieńcowe

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction: In this study, we investigated the association between -786T/C polymorphism of the endothelial nitric oxide (*NOS3*) gene in which thymidine is replaced by a cytosine at nucleotide -786 (rs 2070744) and coronary collateral circulation (CCC) in patients with stable coronary artery disease.

Materials & Methods: 286 patients having a critical stenosis (> 95%) in at least one major epicardial coronary vessel were included in the study. CCC was defined according to the Rentrop classification (R). Patients with R0-1 CCC were included in the poor CCC group and subjects with R2-3 CCC were assigned to the good CCC group. The polymerase chain reaction method was used for genotyping. 152 patients with poor CCC and 134 patients with good CCC were examined.

Results: The frequency of cytosine-cytosine (CC) and thymidine-cytosine (TC) genotypes and allele C were higher in the poor CCC group, but the difference did not reach statistical significance. In the dominant model, the frequency of CC+TC vs. thymidine-thymidine (TT) genotypes was significantly higher in the poor CCC group (67.1% vs. 54.5%, respectively; χ^2=4.78; p=0.02). In multivariate regression analysis, the dominant model for -786T/C polymorphism of the *NOS3* gene remained as an independent correlate of poor CCC.

Discussion: -786T/C polymorphism of the *NOS3* gene (rs 2070744) may be associated with poor angiogenesis and the development of CCC in stable coronary artery disease.

Keywords: coronary collateral circulation • nitric oxide • eNOS gene polymorphism

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Introduction

Coronary collateral circulation (CCC) develops as an adaptive response to myocardial ischemia. It plays an important role in ameliorating the adverse outcomes of ischemic heart disease and reduces mortality [12,15]. The extent of collateral blood flow distal to the occluded vascular segment after myocardial infarction improves myocardial viability [28]. Endothelium-derived nitric oxide (NO) is synthesized from L-arginine by endothelial nitric oxide synthase (*NOS3*) enzyme in the vascular endothelium [18] and is suggested to stimulate collateral vessel growth and mediate vascular endothelial growth factor (VEGF) signaling [19,24]. It has been shown that *NOS3* inhibition accelerates atherosclerosis in animal models and that abnormalities in the NO pathway are present in humans with atherosclerosis [1,14]. Recently, several polymorphisms in the *NOS3* gene that result in abnormalities of NO metabolism have been defined. One of these polymorphisms is a result of thymidine (T) being replaced by a cytosine (C) at nucleotide -786 (rs 2070744). Relationships between the presence of -786T/C *NOS3* gene polymorphism and coronary artery disease (CAD) [4,26,39], coronary vasospasm [21], myocardial infarction [22] and hypertension [9] have been demonstrated. In this study, we hypothesized that -786T/C polymorphism of the *NOS3* gene could have an influence on the development of coronary collateral vessels and aimed to investigate the associations between -786T/C polymorphism of the *NOS3* gene and CCC growth in patients with critical coronary artery stenosis.

Materials and methods

Study population

This cross-sectional study was conducted in the Siyami Ersek Cardiovascular and Thoracic Surgery Center (Istanbul, Turkey). 286 Caucasian patients who had undergone diagnostic coronary angiography (CAG) between January 2011 and March 2012 with the diagnosis of stable angina pectoris were prospectively recruited. All patients had stenosis of > 95% in at least one major epicardial vessel with Thrombolysis in Myocardial Infarction (TIMI) flow grade < 1. Patients with unstable angina pectoris and a history of myocardial infarction within the last 3 months prior to recruitment were excluded from the study. Informed consent was obtained from each subject. Demographic and clinical properties and laboratory results of the participants were recorded and blood samples for genetic analysis were taken.

Definitions

Stable angina pectoris was defined as typical chest pain or angina equivalent symptoms triggered either by exercise or stressful conditions and relieved by rest. Hypertension was defined as a systolic pressure > 140 mmHg and/or a diastolic pressure >90 mmHg or the individuals already taking antihypertensive medications. Diabetes mellitus was defined as a fasting glucose level >126 mg/dL or the patients already taking anti-diabetic medications. Hyperlipidemia was defined by elevated total serum cholesterol levels > 200 mg/dl. Individuals who reported smoking of at least one cigarette per day during the year before examination were classified as smokers. Body mass index was calculated as weight divided by height (kg/m^2).

Angiographic study

Coronary angiography was performed by a percutaneous femoral approach and non-ionic, low-osmolarity contrast medium was used in all cases. Angiographic data of the patients were examined in a prospective manner, by two independent observers who were blinded to the clinical properties of the patients. To evaluate the level of coronary collateral circulation, we used the Rentrop (R) grading system from 0 to 3 based on angiographic opacification of the artery receiving collateral flow distal to the stenotic/occluded segment, and subdivided the patients according to the grade of collateral development. According to this grading system, CCC was graded as R 0 = no distal opacification; R 1= filling of side branches of the artery perfused by way of collateral vessels without visualization of the epicardial segment; R 2 = partial filling of the epicardial segment of the artery perfused by way of collateral vessels; R 3 = complete filling of the epicardial segment of the artery via collateral vessels [17]. Poor CCC was defined as the presence of R grade 0 to 1 CCC and patients with R grade 2 to 3 CCC were defined as having good CCC and served as the control group.

Genetic analysis

Genomic DNA was extracted from peripheral lymphocytes by using phenol chloroform methodology. Screening for the nitric oxide synthase 786 T to C substitution was performed by the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method. The PCR reaction was carried out in a total 50 µL volume including 0.1-1 µg template DNA, 10X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of each primer (ENOS 5' TGG AGA GTG CTG GTG TAC CCC A 3' (forward) and ENOS 5' GCC TCC ACC CCC ACC CTG TC 3' (reverse)) and 1 U of taq polymerase (MBI Fermentas, Lithuania). 35 cycles were performed as 2 minutes at 94°C for predenaturation, 1 min at 94°C for denaturation, 1 minute at 62°C for annealing, 1 minute at 72°C for extension and 7 minutes at 72°C for final extension. After PCR, the products obtained were digested with 10 U of Msp I restriction enzyme (MBI Fermentas, Li-

Table 1. Demographic, clinical, angiographic and laboratory findings of patients

Variables	Poor CCC Group (n=152)	Good CCC Group (n=134)	P
Age (years±SD)	57.7±10.9	57.9±11.7	0.41
Male (n %)	115 (75.7)	113 (84.3)	0.83
Hypertension (n %)	82 (53.9)	74 (55.2)	0.83
Diabetes mellitus (n %)	52 (34.2)	31 (23.1)	0.05
Hyperlipidemia (n %)	92 (60.5)	74 (55.2)	0.37
Smoking (n %)	72 (47.4)	65 (48.5)	0.84
LVEF (%±SD)	46.9±6.9	48.9±7.2	0.02
Angiographic findings			
LAD lesion (n %)	29 (19)	34 (25)	
Cx lesion (n %)	46 (30)	36 (26)	0.43
RCA lesion (n %)	77 (51)	64 (48)	
Diseased vessels (n±SD)	1.9±0.6	1.6±0.5	0.01
Laboratory findings			
Hemoglobin (g/dL±SD)	14.6±0.9	14.7±1.2	0.81
WBC (103/µL±SD)	7.68±2.09	7.81±2.42	0.63
Platelets (103/µL±SD)	244.4±62.1	231.4±58.2	0.18
MPV (fL±SD)	9.16±1.75	9.41±1.31	0.19
RDW (%±SD)	14.1±1.7	13.9± 1.3	0.28
Fasting glucose (mg/dL*)	119 [50]	124 [52]	0.41
Creatinine (mg/dL±SD)	0.94±0.34	0.89±0.31	0.06
Triglycerides (mg/dL*)	159 [87]	145 [82]	0.19
T. Cholesterol (mg/dL±SD)	204±48	198±51	0.46
LDL (mg/dL±SD)	123±44	117±38	0.24
HDL (mg/dL±SD)	42.4±10.8	44.1±12.1	0.61

*reported as median and [inter-quintile range] Cx: Circumflex Artery, HDL: High Density Lipoprotein, LAD: Left Anterior Descending Artery, LDL: Low Density Lipoprotein, MPV: Mean Platelet Volume, RCA: Right Coronary Artery, RDW: Red Cell Distribution Width, WBC: White Blood Cell, LVEF: Left ventricular ejection fraction, SD: Standard deviation, T. cholesterol: Total cholesterol

thuania) at 37°C overnight. After digestion, the fragments were run in 3% agarose gel electrophoresis at 90 V for 45 minutes. Subsequently the fragments of 40 bp (constant) and 140 (-786 T) or 90+50 bp (-786 C) were visualized with ethidium bromide under UV light.

Statistical analysis

All data are presented as a mean ± SD or a median for parametric variables and as percentages for categorical variables. Continuous variables were checked for the normal distribution assumption using Kolmogorov-Smirnov statistics. Categorical variables were tested by Pearson's χ^2 test or Fisher's exact test and parametric variables were tested using the Kolmogorov-Smirnov or Student t-test when appropriate. Correlations between two continuous variables were assessed with Pearson's test. Multivariate logistic regression analysis was used to assess the correlations between -786T/C polymorphism of the *NOS3* gene and the other study parameters together with CCC. The power analysis showed that at least 236 subjects should be recruited for the power to be equal to 0.80. The distribution of the -786T/C genotypes satisfied the Hardy-Weinberg equilibrium in the study groups. All statistical studies were carried out using Statistical Package for Social Sciences software (SPSS 16.0 for Windows, SPSS Inc., Chicago, Illinois) and significance was accepted at the $p< 0.05$ level.

Results

A total of 152 subjects with poor CCC and 134 subjects with good CCC were examined. The demographic and clinical characteristics and laboratory results of the study patients are presented in Table 1. In the poor CCC group, the frequency of diabetes mellitus was significantly higher. The other clinical properties including age, gender, smoking

Table 2. Genotype and allele distribution of T-786 C polymorphism

Genotypes (n %)	Poor CCC Group (n=152)	Good CCC Group (n=134)	P value
CC	31 (20.4 %)	19 (14.2 %)	0.07 (χ2=5.17)
TC	71 (46.7 %)	54 (40.3 %)	
TT	50 (32.9 %)	61 (45.5 %)	
Dominant model			
CC+TC	102 (67.1 %)	73 (54.5 %)	0.02 (χ2=4.78)
TT	50 (32.9 %)	61 (45.5 %)	
Recessive model			
TT+TC	121 (79.6 %)	115 (85.8 %)	0.17 (χ2=1.90)
CC	31 (20.4 %)	19 (14.2 %)	
Allele frequencies (%)			
C	44%	34%	0.14 (χ2=2.11)
T	56%	66%	

C: Cytosine, T: Thymidine

and the frequency of hyperlipidemia and hypertension, and also laboratory parameters including fasting glucose, creatinine, hemoglobin, red blood cell distribution width and mean platelet volume, were comparable between the two groups. However, the total number of diseased vessels was higher and left ventricular ejection fraction was lower in the poor CCC group (p=0.01 and p=0.02, respectively). The genotype distribution and allele frequencies of 786T/C polymorphism of the *NOS3* gene in the study population are represented in Table 2. The frequency of CC and TC genotypes was higher in the poor CCC group, but the difference did not reach statistical significance (χ^2=5.17; p=0.07). A significant difference was observed only in the dominant model, where the frequency of CC+TC was higher in the poor CCC group compared to the good CCC group (67.1 *vs.* 54.5%, respectively; χ^2=4.78; p=0.02). The frequency of allele C was not significantly higher in the poor CCC group compared to the good CCC group (χ^2=2.11; p=0.14). In univariate logistic regression analysis, none of the clinical properties was significantly correlated with presence of good CCC, except for 786T/C polymorphism of the *NOS3* gene in the dominant model (CC+TC *vs.* TT) (OR 1.71, 95% OR: 1.055-2.75; P=0.02). Using the multivariate model adjusted for age, gender, hypertension, diabetes mellitus and smoking status, only -786T/C polymorphism of the *NOS3* gene in the dominant model (CC+TC vs. TT) remained as an independent correlate of poor CCC (OR 1.75, 95% OR: 1.01-3.05; P=0.04). The other parameters did not differentiate between the groups in either the univariate or the multivariate model.

Discussion

The main finding of the study was that the presence of the C allele of the *NOS3* -786T/C gene polymorphism in the dominant model (CC+TC *vs.* TT) was associated with poor coronary collateral development in patients with stable angina pectoris. Development of collateral vessels is triggered by the pressure gradient between the coronary bed of arteries caused by a severe obstruction and myocardial ischemia [2,6]. Degree of coronary stenosis, proximal lesion location, longer duration of symptoms and occlusion are clinical factors that can influence the formation of coronary collateral vessel [16]. However, the absence of collateral vessels or poor collateral circulation development in some patients despite the presence of these factors and the evidence of myocardial ischemia suggest that additional factors such as genetic variations may contribute to the individual variability in the development of CCC in patients with coronary artery disease.

NO plays an important role in the coronary collateral growth via mediating the VEGF signaling in animal models [8,34]. VEGF also stimulates the release of NO from cultured human umbilical venous endothelial cells and upregulates the expression of *NOS3* [32]. In *NOS3* knockout mice, collateral growth is impaired in the ischemic hind limb due to limited angiogenesis [8]. Antagonists of *NOS3* inhibit the mitogenic effects of VEGF [24,34]. On the other hand, NO has several effects against atherosclerosis in the vascular endothelium such as relaxation of vascular smooth muscle, inhibition of adhesion of platelets and leucocytes to the endothelium, reduction of vascular smooth muscle cell migration and proliferation, and limitation of the oxidation of atherogenic low-density lipoproteins [29]. One of the polymorphism of the *NOS3* gene is a result of thymidine being replaced by a cytosine at nucleotide -786 (-786T/C), which was shown to be predictor of cardiovascular mortality in high-risk coronary artery disease [27]. Another study revealed that allelic polymorphism in the promoter region (-786T/C) of the *NOS3* gene is associated with acute coronary syndromes [5]. Coronary collateral vessel development was poorer in patients carrying the Asp 298 variant than in patients with *NOS3* Glu-Glu homozygosity, which results in the conversion of glutamate to aspartate for codon 298 (Glu-298-Asp) [7,11]. In vitro studies have suggested that the Asp 298 variant may result in decreased *NOS3* activity [31]. There are controversial results in the literature regarding the association of -786T/C polymorphism and NO concentrations [10,20]. Impaired collateral formation may be attributed the state of relative NO deficiency or functional changes secondary to polymorphism [7]. In this study, we established an independent correlation between the dominant model of -786T/C polymorphism of the *NOS3* gene and the degree of CCC in patients with stable CAD. Our results are consistent with current literature [13,33]. Even though our findings do not implicate a pathophysiological mechanism between this polymorphism and angiogenesis, it is plausible that eNOS gene polymorphisms may play a role in the development and

functioning of CCC, considering the wide spectrum of actions of NO on vascular cells. Other investigations into genetic links of collateral growth other than the *NOS3* gene have revealed associations with VEGF, hypoxia inducible factor, haptoglobin, angiotensin-converting enzyme and galectin [3].

Study limitations

We assessed the CCC by coronary angiography, but coronary angiography can define only vessels > 100 μm in size, while most collateral channels are much smaller. Myocardial contrast echocardiography or Doppler techniques would be more accurate in deciding the status of the CCC of the patients.

In conclusion, we have demonstrated that there is an independent correlation between *NOS3* 786T/C gene polymorphism in the dominant model (CC+TC vs. TT) and poor CCC in patients with stable CAD in this study. -786T/C polymorphism of the *NOS3* gene may be associated with poor CCC development.

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