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THE RELATIONSHIP BETWEEN GLU298ASP AND T786-C GENE POLYMORPHISMS OF ENDOTHELIAL NITRIC OXIDE SYNTHASE AND CORONARY SLOW-FLOW PHENOMENON

KORONER YAVAŞ AKIM FENOMENİ İLE ENDOTEL NİTRİK OKSİT SENTAZ'IN GLU298ASP VE T786-C GEN POLİMORFİZMLERI ARASINDAKİ İLİŞKİ

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ÖZET

Giriş: Çalışmada Endotelyal Nitrik Oksit Sentaz Glu 298-Asp Ve T786-C gen polimorfizmleri ile koroner yavaş akım arasındaki ilişkinin belirlenmesi amaçlanmıştır.

Yöntemler: Çalışmaya anginal yakınmalar, efor testi veya miyokard perfüzyon sintigrafisi sonucuna göre koroner anjiyografi yapılan 148 birey alındı. Koroner akım TIMI çerçeve sayısına göre hesaplandı. Hastalardan, gen analizi ve diğer biyokimyasal parametreler için venöz kan örneği alındı.

Bulgular: Yapılan koroner anjiyografi işlemi sonucunda TIMI çerçeve sayısına göre koroner yavaş akım saptanan 74 birey hasta olarak ve normal koroner saptanan 74 birey kontrol grubu olarak alındı. Hasta ve kontrol grubunun genotipik polimorfizm incelemesinde T786-C polimorfizminde CC, CT ve TT genotipine sahip bireyler arasında (p=0.941) ve Glu298-Asp polimorfizminde GG, GT ve TT genotipine sahip bireyler arasında (p=0.070) anlamlı fark saptanmadı. Ayrıca koroner yavaş akımın tek damar tutulumu (LAD, CX veya RCA'dan yalnızca birinde) veya çok damar tutulumuna (LAD, CX veya RCA'dan en az iki veya daha fazlasında) göre dağılımı incelendiğinde T-786 C (p=0.220) ve Glu298-asp (p=0.378) polimorfizminin genotiplerinin dağılımı açısından istatiksel anlam bulunmadı.

Sonuç: Çalışmamız, Türk toplumunda eNOS geninin T786-c ve Glu298-asp polimorfizmleri ile koroner yavaş akım fenomeni arasında ilişki olmadığını göstermiştir.

Anahtar Kelimeler: Koroner yavaş akım, endoteliyal nitrik oksit sentaz, genetik polimorfizm

ABSTRACT

Introduction: This study aims to determine the association between Glu 298-Asp and T786-C gene polymorphisms of endothelial nitric oxide synthase (eNOS) and coronary slow-flow (CSF) phenomenon.

Methods: 148 individuals who underwent coronary angiogram (CAG) based on anginal symptoms, exercise testing, or myocardial perfusion scintigraphy were included in the study. Coronary flow was calculated based on TIMI frame counts (TFCs) on CAG. Venous blood samples were drawn from the patients for gene analysis and other biochemical parameters.

Results: 74 patients with a CSF pattern based on TFCs were included as patients and 74 individuals with normal coronary arteries were included as the control group. In the genotypic polymorphism analysis of the patient and control groups, no significant difference was found among individuals with CC, CT, and TT genotypes in terms of T786-C polymorphism (p=0.941) and among individuals with GG, GT, and TT genotypes (p=0.070) in terms of Glu298Asp polymorphism. Also, there was no statistically significant difference with regard to the distribution of the T786-C (p=0.220) and Glu298Asp (p=0.378) polymorphisms between those with single (only one of LAD, CX, or RCA) and those with multi-vessel (at least two or more of LAD, CX, or RCA) CSF patterns.

Conclusion: Our study demonstrated a null relationship between T786-C and Glu298Asp polymorphisms of the eNOS gene and CSF phenomenon in the Turkish population.

Keywords: Coronary slow-flow, endothelial nitric oxide synthase, genetic polymorphisms

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INTRODUCTION

CSF phenomenon is defined as the slow progression of contrast material to distal coronary structures during conventional CAG usually in the absence of any critical stenosis in epicardial coronary arteries (1). It has been reported that CSF phenomenon is encountered in 1-7% of patients who undergo CAG (2, 3). Patients with CSF usually present with exercise angina, acute coronary syndromes (ACSs) without ST-segment elevation (NSTE-ACS), or ST-elevation myocardial infarction(STEMI) (4). Initial studies on this phenomenon highlighted the particular implications of increased blood viscosity and disturbed thrombocyte functions (5-7). With this, a potential imbalance between vasoconstrictor and vasodilator factors was also suggested as a possible causative factor in the setting of CSF phenomenon (1, 8).

Nitric oxide (NO) is a potent endothelium-derived vasodilator molecule. NO is synthesized from L-arginine via nitric oxide synthase (NOS) (9). NOS is a family of isoenzymes consisting of three components. These are called endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), and neuronal nitric oxide synthase (nNOS). NO is the common product of all NOS isoenzymes (9). NO affects both blood pressure and blood flow rate through induction of smooth muscle relaxation (2, 3). Previous studies have demonstrated associations of certain eNOS gene polymorphisms with coronary artery disease and coronary artery spasm (10, 11). Moreover, it was also previously reported that one of the alleles of the Glu298Asp (894G/T) polymorphism of the eNOS gene decreases the activity of eNOS potentially leading to a reduction in NO production (12). Studies in Turkey reported significantly lower NO levels in patients with a CSF pattern in comparison to those with normal coronary flow (on CAG) (13, 14). A previous study by Gupta et al. (conducted in Northern India) revealed a particular association of Glu298Asp polymorphism of the eNOS gene with the CSF phenomenon (15). Conversely, another study in Turkey failed to exhibit any relationship between CSF phenomenon and Glu298Asp polymorphism of the eNOS gene (16). Notwithstanding the plenitude of studies on this issue, results have been mostly inconsistent, and have significantly varied from population to population. Accordingly, we aimed to investigate the potential relationship between CSF phenomenon and Glu298Asp-T786-C polymorphisms of the eNOS gene.

METHODS

Patients with typical angina and ischemia detected on exercise testing or myocardial perfusion scintigraphy between 01/01/2015 and 31/08/2016 in Trakya University Faculty of Medicine Cardiology Clinic were included in the study. Patients with heart failure (EF<50%), acute myocardial infarction, thyroid dysfunction, coronary ectasia, autoimmune disease, coronary artery stenosis of > 50%, and chronic kidney or liver failure were excluded from the study. The study was conducted with 74 patients with CSF who met the inclusion criteria among the patients who underwent CAG within the specified dates, and 74 patients with similar demographic characteristics having normal coronary artery. CAG through femoral artery cannulation was performed using the Judkins technique in all subjects. CSF was determined based on the evaluation of TFCs as defined by Gibson et al. (17). Blood samples drawn from patients with CSF and those with normal coronary flow (control group) were studied in two stages. In the first stage, DNA isolation was performed from blood samples of the patient, and control groups. In the second stage, Glu298Asp or T786-C polymorphisms of the eNOS gene were determined with fluorescent-labeled (with Taqman Props) Real-Time-Polymerase Chain Reaction (QT-PCR). Blood tests and analyses of the patients were performed by the clinicians blind to the CAG data. The study protocol was in accordance with the Declaration of Helsinki and was approved by the Trakya University Scientific Research Ethics Committee with the decision of TUTF-BAEK 2016/199.

Extraction of samples:

200 µl of the whole blood sample was placed in 2 ml Eppendorf and 20 µl proteinase K solution and 400 µl lysis solution were added. After the solutions were added, they were vortexed briefly to obtain a homogeneous mixture. The mixtures were incubated at 56°C for 10 minutes until the cell membranes were completely disrupted. 200 µl of ethanol (96-100%) was added and vortexed briefly. After the prepared mixture was added to the column, it was centrifuged at 6000xg for 1 min. The collection tube containing the waste was discarded and a column was placed in the new collection tube. 500 µl Wash Buffer I (ethanol added) was added and centrifuged at 8000xg for 1 min. The waste in the collection tube was separated and placed back into the column. 500 µl Wash Buffer II (with ethanol added) was added and after centrifugation at 12000xg for 3 min, the column was placed in 2 ml Eppendorf. 100 µl of Elution Buffer was added and after waiting 2 minutes at room temperature, it was centrifuged at 8000xg for 1 minute. Finally, the column was discarded, and pure DNA was stored at -20°C.

Analysis of the Glu298Asp Polymorphism

Primary sense 5′-AAG GCA GGA GAC AGT GGA TGGA-3', antisense 5′- CCC AGT CAA TCC CTT TGG TGC TCA-3' (Product length 248 base pairs).

Initially, a total of 40 cycles were repeated for 5 minutes at 94oC, 20 seconds at 94oC, 20 seconds at 57oC, 22 minutes at 72oC. In the end, 10 minutes of the final extension was performed at 72oC.

Analysis of the T786-C Polymorphism:

Primer sense 5′- CACCTGCATTCTGGGAACTGTA -3′, anti sense 5′- GGCAGAGGCGGTAGACCC -3′ (Product length 250 base pairs).
Initially, a total of 40 cycles were repeated for 5 minutes at 94oC, 20 seconds at 94oC, 20 seconds at 62oC, 22 seconds at 72oC. In the end, 5 minutes of scanning was performed at 72oC.

Statistical Analysis:

Statistical analysis was performed using SPSS version 21.0 software. Kolmogorov-Smirnov and Shapiro-Wilk tests, which are called normality tests, were used in paired comparisons of continuous variables. Student's t-test, which is suitable for normal distribution and called the parametric method; The Mann-Whitney U test, which is called the non-parametric method, were performed depending on the result of the applied test. The Chi-square ($\chi 2$) - 2x2 table test, suitable for categorical variable comparison, was applied to the genotypes that were determined to be qualitative according to the data characteristics. According to the p-values obtained from the test results of the Chi-Square test and applied methods, $p<0.05$ was accepted as the significance limit.

RESULTS

The demographic characteristics of both groups are shown in Table 1. The difference in mean age between the two groups was not significant (p=0.156). There was no significant difference between the two groups with regard to the incidences of hypertension (p=0.511), diabetes (p=0.408), smoking (p=0.868), and family history (p=0.096). There was no significant difference with regard to values of fasting blood glucose and lipid profiles between the two groups (Table 2).

Table 1. Demographic data of coronary slow flow and control group

		Patient Group (n=74)	Control Group (n=74)	P*
Age, years, mean ± SD		53.6± 10.7	51.1± 10.2	0.156
Gender (n)	Male	54 (%73.0)	52 (%70.3)	0.715
	Female	20 (%27.0)	22 (%29.7)	
Diabetes mellitus (n)		17 (%23.0)	12 (%16.2)	0.408
Hypertension (n)		38 (%51.4)	33 (%44.6)	0.511
Smoking (n)		31 (%41.9)	32 (%43.2)	0.868
Family History (n)		19 (%25.7)	10 (%13.5)	0.096

SD: standart deviation, n: number of patients, p*: Student's t-test and The Mann-Whitney U test

Regarding the evaluation of genotypic polymorphisms, no significant difference was found among individuals with CC, CT, and TT genotypes in terms of T786-C polymorphism (p=0.941). Regarding the Glu298Asp polymorphism, no significant difference was found among individuals with GG, GT, and TT genotypes (p=0.070) (Table 3)

Table 2. Biochemical results of coronary slow flow phenomenon and control group

	Patient Group (n=74)	Control Group (n=74)	P*
Fasting blood sugar (mg/dl)	110.5± 47.0	105.4± 28.1	0.420
Total cholesterol (mg/dl)	184.1± 40.1	193.6± 46.4	0.189
HDL (mg/dl)	39.9± 9.1	42.2± 11.3	0.168
LDL (mg/dl)	106.4± 30.1	116.1± 35.8	0.079
Triglyceride (mg/dl)	185 (55-858)	175 (65-753)	0.554

HDL: High density lipoprotein, LDL: Low density lipoprotein, mg/dl: milligram/deciliter, n: number of patients, p*: Student's t-test and The Mann-Whitney U test

The distribution of genotypic polymorphisms according to the coronary artery involvement was particularly examined: the distributions of LAD, CX, and RCA TFCs according to T786-C and Glu298Asp polymorphisms are presented in Figure 1a, b, c, d, e and f (with no significant differences, p=0.708 and p=0.377, respectively).

Table 3. Relationship between coronary slow flow phenomenon and the control group's genotype polymorphism

	Patient Group (n=74)	**Control Group (n=74)**	**P***
T786-c			
CC	15 (%20.3)	16 (%21.6)	0.941
CT	31 (%41.9)	32 (%43.2)	
TT	28 (%37.8)	26 (%35.2)	
GLU 298-ASP			
GG	33 (%44.6)	46 (%62.2)	0.070
GT	33 (%44.6)	20 (%27.0)	
TT	8 (%10.8)	8 (%10.8)	

CC: Cytosine-Cytosine, CT: Cytosine-Thymine, TT: Thymine-Thymine, GG: Guanine-Guanine, GT: Guanine-Thymine, n: number of patients, p*: Chi-Square test

Table 4. Distribution of genotypes of T-786C and Glu298-asp polymorphisms by slow flow vascular disease

T786-C GENOTYPE	**Single vessel (n=30)**	**Multi-vessel (n=44)**	**P***
CC	7 (23,3%)	8 (18,2%)	0,220
TC	9 (30,0%)	22 (50,0%)	
TT	14 (46,7%)	14 (31,8%)	
GLU298-ASP GENOTYPE	Single vessel(n=30)	Multi-vessel (n=44)	P*
GG	16 (53,3%)	17 (38,6%)	0,378
GT	12 (40,0%)	21 (47,7%)	
TT	2 (6,7%)	6 (13,6%)	

CC: Cytosine-Cytosine, CT: Cytosine-Thymine, TT: Thymine-Thymine, GG: Guanine-Guanine, GT: Guanine-Thymine, n: number of patients, p*: Chi-Square test

There was no statistical significance in terms of distribution of genotypes of the T786-C (p=0.220) and Glu298Asp (p=0.378) polymorphisms according to the extent of coronary artery involvement (single (CSF involving one of the major

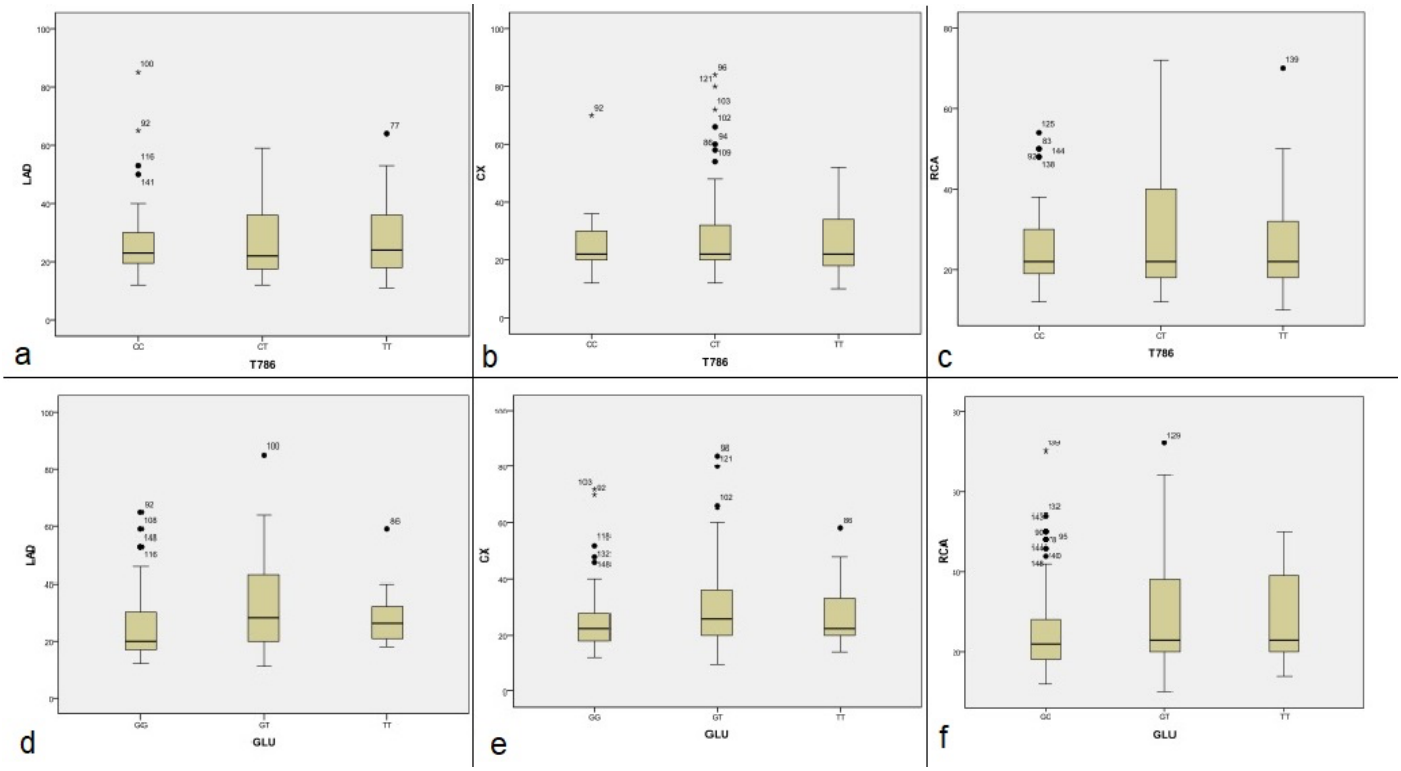

Figure 1a. Change of LAD TIMI frame count according to T786-c genotype polymorphism group. b. Change of CX TIMI frame count according to T786-c genotype polymorphism group. c. Change of RCA TIMI frame count according to T786-c genotype polymorphism group. d. Change of LAD TIMI frame count according to Glu298-asp genotype polymorphism group. e. Change of CX TIMI frame count according to Glu298-asp genotype polymorphism group. f. Change of RCA TIMI frame count according to Glu298-asp genotype polymorphism group.

coronary arteries (LAD, CX, or RCA)) or multi-vessel (CSFs in at least two of these coronary arteries) involvement) (Table 4).

DISCUSSION

Initial studies on CSF phenomenon particularly suggested inflammation as a potential causative factor. However, subsequent studies focused on the disruption of the balance between vasoconstrictor and vasodilator factors in the pathogenesis of this phenomenon (13, 14, 18). NO is a powerful vasodilator substance, and has a pivotal role in the orchestration of endothelial functions and blood pressure along with maintenance of vascular integrity (18). A causal relationship between NO levels and CSF evolution was first shown in the study by Chauhan A et al. (19). Subsequent studies have shown that patients with CSF phenomenon might not only have decreased NO levels but might also suffer an impaired NO response to exercise (13, 14, 20, 21). Since NO is a mediator synthesized by the NOS, recent studies have focused on the potential relation between CSF evolution and this enzyme. However, NOS is not a single enzyme, but a complex of 3 isoenzymes where the end-product is NO (2, 3).

Interestingly, it was previously reported that T786-C base polymorphism in the promoter domain of the eNOS gene might cause a 50% reduction in eNOS transcription with consequent decreases in NO levels (12). However, the results of studies investigating the relationship between T786-C polymorphism of the eNOS gene and CSF evolution seem to be contradictory. In one of these studies conducted in the Turkish population, it was shown that there was a significant role of this polymorphism in CSF evolution (22). However, this finding was not confirmed in another study (23). Similarly, Glu298Asp polymorphism of the eNOS gene was previously demonstrated to be associated with reduced NO levels potentially serving as a risk factor for the evolution of diffuse coronary spasm (24). The association of Glu298Asp polymorphism with CSF evolution was not previously shown in the Turkish population, while another study conducted in Northern India reported a causal relationship (15, 16). The only study investigating the effects of T786-C and Glu298Asp polymorphisms on vascular blood flow velocity was reported by Rossi et al. This study demonstrated that forearm blood flow velocity was significantly reduced in patients with essential hypertension having both polymorphisms compared with normotensive individuals (25). In addition, the relationship between these genes and coronary artery disease was investigated in the Turkish population, but no relationship was found (26).

On the other hand, the present study indicates a null relationship between T786-C and Glu298Asp polymorphisms and CSF evolution. The results of our study appear to be consistent with those of the study by Caglayan et al. (16) and Gazi et al. (22). The study by Nurkalem et al. was also conducted in the Turkish population (23), but had a smaller

sample size. Similarly, the study by Gupta et al. (15) also had a smaller sample size, and was conducted in patients with different ethnic origins. Contradictory results in the studies might be attributable to the diversities in geographical and ethnic characteristics as well as sample sizes.

Our study has also a variety of limitations: first results from single-center data in the Thrace region might not possibly reflect the profile of the whole country. Second, even though the present study has a larger sample size as compared with most of previous studies, it might still be regarded as a small-medium scale study, and hence cannot definitely suggest absolute implications in this setting.

CONCLUSION

In conclusion; we have demonstrated that there was no association between T786-C and Glu298Asp polymorphisms of the eNOS gene and CSF phenomenon. However, further large-scale studies are still warranted to illuminate absolute implications of eNOS gene polymorphisms in the setting of CSF phenomenon.

Ethics Committee Approval: Ethical approval was received from Trakya University Faculty of Medicine Ethics Committee (TÜTF-BAEK 2016/199).

Informed Consent: Informed consent was obtained from all individual participants included in the study.

Authorship Contributions: CÖ, MB, GT, AKY were involved in the collection of the data and the clinical follow-up of the patients, design and conception of the study, writing the manuscript. All authors read and approved the final manuscript.

Conflict Of Interest: The authors declare that they have no conflict of interest.

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