Lack of association between the Glu298Asp polymorphism of endothelial nitric oxide synthase and slow coronary flow in the Turkish population

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BACKGROUND: Coronary endothelial dysfunction plays an important pathogenetic role in patients with slow coronary flow (SCF). No data exist regarding the possible contribution of the Glu298Asp polymorphism genotype of the endothelial nitric oxide synthase (*eNOS*) gene to human SCF in the literature.
OBJECTIVE: To investigate the association between SCF and the Glu298Asp polymorphism of the *eNOS* gene.
METHODS: The study population consisted of 85 consecutive patients. The patient group included 66 patients with angiographically proven normal coronary arteries with SCF, and 19 subjects with normal coronary arteries with no SCF. The thrombolysis in myocardial infarction frame count was used for the diagnosis of SCF. The Glu298Asp polymorphism was determined by polymerase chain reaction and restriction fragment length polymorphism.
RESULTS: The baseline characteristics were similar between the two groups, except for high-density lipoprotein cholesterol, which was higher in the SCF group than in the controls. The genotype distribution of Glu298Asp was as follows: GG 26%, GT 56% and TT 12%, where G is guanine and T is thymine. There was no difference in the frequency of the various genotypes or the alleles in patients with SCF versus normal controls.
CONCLUSIONS: The Glu298Asp polymorphism genotype of the *eNOS* gene is not a risk factor for SCF in the present study population.

Key Words: *Genetics; Receptor; Renin-angiotensin system; Slow coronary flow*

L'absence de lien entre le polymorphisme Glu298Asp de la synthase endothéliale du monoxyde d'azote et le débit coronaire lent au sein de la population turque

HISTORIQUE : La dysfonction endothéliale coronaire joue un rôle pathogène important chez les patients ayant un débit coronaire lent (DCL). Les publications scientifiques ne contiennent aucunes données sur l'apport possible du génotype du polymorphisme Glu298Asp du gène de la synthase endothéliale du monoxyde d'azote (*eNOS*) au DCL chez les humains.
OBJECTIF : Explorer l'association entre le DCL et le polymorphisme Glu298Asp du gène *eNOS*.
MÉTHODOLOGIE : La population à l'étude se composait de 85 patients consécutifs. Ce groupe de patients était formé de 66 patients ayant des artères coronaires normales démontrées par angiographie et un DCL et de 19 patients ayant des artères coronaires normales démontrées par angiographie, sans DCL. Pour diagnostiquer le DCL, on a utilisé le compte du nombre d'images de thrombolyse dans l'infarctus du myocarde. Le polymorphisme Glu298Asp était déterminé par réaction en chaîne de la polymérase et polymorphisme de restriction.
RÉSULTATS : Les caractéristiques de départ étaient similaires entres les deux groupes, sauf pour ce qui est du cholestérol à lipoprotéines de haute densité, plus élevé dans le groupe ayant un DCL que dans le groupe témoin. La répartition génotypique du Glu298Asp s'établissait comme suit : GG 26 %, GT 56 % et TT 12 %, où G désigne la guanine et T, la thymine. Il n'y avait pas de différence dans la fréquence des divers génotypes ou des allèles chez les patients ayant un DCL par rapport aux sujets témoins.
CONCLUSION : Le génotype du polymorphisme Glu298Asp du gène *eNOS* n'est pas un facteur de risque de DCL au sein de la population à l'étude.

Nitric oxide (NO), an important endothelium-derived relaxing factor, is synthesized from L-arginine by at least three isoforms of NO synthase (NOS) (inducible NOS, constitutive neuronal NOS and constitutive endothelial NOS [eNOS]) (1). Evidence that NO plays a protective role in various important activities during atherogenesis has been presented in several studies. It has been demonstrated that deficiency in NO activity is involved in the pathogenesis of coronary spasms (2).

NO production can be influenced by polymorphisms of the *eNOS* gene. The gene is located on chromosome 7q35-36 and consists of 26 exons with a total size of 21 kb (3). The *eNOS* gene is expressionally and functionally regulated through multiple regulatory steps (4,5), and entails several polymorphisms (6), some of which bear functional consequences. A point mutation of guanine (G) to thymine (T) at nucleotide 1917 in exon 7 of the *eNOS* gene has been described (7). This variant results in the replacement of glutamic acid by aspartic acid at codon 298 (Glu298Asp). Various studies have stated that this gene polymorphism is associated with coronary spasm (8), essential hypertension (9) and the risk of acute myocardial infarction (6,10,11). Controversial results were obtained with respect to coronary artery disease (CAD). Whereas previous studies failed to detect a link between the gene variation and CAD (11-13), another study reported the Glu298Asp gene polymorphism to be a major risk factor for CAD in individuals in the United Kingdom (6).

Slow coronary flow (SCF) is an angiographic observation characterized by normal coronary arteries with delayed opacification of the distal vasculature (14,15). It has been reported that coronary microvascular endothelial dysfunction plays an important pathogenetic role in SCF (16,17). However, to date, only a limited number of studies have focused on the etiology of SCF.

We hypothesized that SCF may be associated with the *eNOS* gene polymorphism because the major pathophysiological mechanism in

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TABLE 1
Baseline characteristics of patients

Characteristic	SCF (n=66)	Controls (n=19)	P
Male:female, n	58:8	14:5	NS
Age, years	52±13	49±12	NS
Smoking,%	54	57	NS
Hemoglobin, mmol/L	9.1±1.3	8.8±0.9	NS
WBC,×10^9/L	8.4±2.3	8.6±3.3	NS
Thrombocyte, ×10^9/L	255±78	253±73	NS
Total cholesterol, mmol/L	5.3±0.9	5.2±1.2	NS
Triglyceride, mmol/L	2.2±1	2.31±0.6	NS
HDL cholesterol, mmol/L	1.21±0.3	0.98±0.2	<0.05

Data presented as mean ± SD unless specified otherwise. HDL High-density lipoprotein; NS Nonsignificant; SCF Slow coronary flow; WBC White blood cell count

SCF is endothelial dysfunction, and the *eNOS* gene polymorphism is associated with endothelial dysfunction.

METHODS

Study population

A total of 85 patients in whom CAD was suspected were prospectively selected for the study group. Elective coronary angiography was performed between April and September 2005 at the Cardiology Department of Erciyes University in Kayseri, Turkey. The patient group included 66 patients with angiographically proven normal coronary arteries and SCF in a coronary vessel and 19 subjects with normal coronary arteries and no SCF. Normal coronary arteries were defined as completely normal coronary arteries with no obstructive or nonobstructive lesions. Coronary angiograms were analyzed by two blinded observers. The present study was approved by the Erciyes University Medical Faculty Ethics Committee. All patients gave informed consent. Blood samples obtained were used to investigate genetic polymorphisms in the present study only.

Exclusion criteria

Patients with valvular heart disease, a prosthetic heart valve, diabetes mellitus, hypertension, CAD history, coronary ectasia, atrial fibrillation, complete bundle branch block and serious conduction defect, mitral valve prolapse, hypertrophic, restrictive and dilated cardiomyopathies, left ventricular hypertrophy, ejection fraction of less than 50%, connective tissue diseases, and pulmonary, renal, hepatic and hematological disorders were excluded from the study.

Blood analyses

Serum fasting blood glucose, total cholesterol, triglyceride, high-density lipoprotein cholesterol, and serum and urine creatinine levels were determined by enzymatic methods after 12 h of fasting. Lipid parameters were measured according to Konelab 60i (Thermo Clinical Labsystems, Finland) with original Thermo kits. Low-density lipoprotein cholesterol levels were calculated using Friedewald's formula. Blood glucose was analyzed using the glucose oxidase method.

Coronary angiography

Selective coronary arteriography was performed with a 7 Fr Judkins catheter. Iopromide (Ultravist-370; Schering AG, Germany) was used as the contrast agent during coronary angiography in all patients and control subjects. Approximately 6 mL to 8 mL of contrast medium was administered in each dose by manual injection. All images were obtained on a Philips Integris H5000 (Philips Medical Systems, USA).

Thrombolysis in myocardial infarction frame count

Thrombolysis in myocardial infarction frame count (TFC) was used for the diagnosis of SCF. TFC was determined for each major coronary artery in each patient and control subject as previously described (18). The first frame used for TFC is the first frame in which dye fully enters the proximal coronary artery lumen. The last frame is counted, or included, and is defined as the frame when dye first enters the distal landmark branch. The measurement of frame count for each artery was performed by subtracting the first frame from the last frame. To obtain a corrected TFC for the left anterior descending (LAD) coronary artery, TFC values were divided by 1.7. The mean TFC for each patient and control subject was calculated by adding the TFC for the LAD, the left circumflex artery and the right coronary artery and then dividing the value obtained by 3.

Diagnostic criteria for SCF

Patients with a corrected TFC greater than 2 SDs from the normal published range for the particular vessel were considered to have SCF. Therefore, the mean (± SD) normal values as previously defined were accepted (36.2±2.6 for the LAD, 22.2±4.1 for the left circumflex artery and 20.4±3.0 for the right coronary artery). An artery that had TFC values above the predicted normal values was considered to have SCF (18).

Glu298Asp polymorphism on exon 7 of the *eNOS* gene

Venous blood was collected in 6 mL EDTA tubes, and genomic DNA was isolated using the NucleoSpin Blood Kit (Macherey-Nagel Inc, USA) and stored at 4°C. The Glu298Asp mutation in the *eNOS* gene was analyzed with polymerase chain reaction, followed by restriction fragment length polymorphism.

Statistical analysis

Data are expressed as mean ± SD or as proportions. Differences between groups (continuous variables) were compared using Student's *t* test. The relationship between NO genotypes, and clinical and biochemical findings in the patient group were evaluated by one-way ANOVA. Pearson's χ^2 test was used to analyze the correlation between variables. The differences in genotype or allele distributions of the *eNOS* gene Glu298Asp polymorphisms were examined by χ^2 analysis. $P<0.05$ was considered to be statistically significant. Data were analyzed using SPSS 12.0 (SPSS Inc, USA).

Risk estimations for the association of SCF with the G/T polymorphisms were calculated using ORs and 95% CIs by comparing the genotypic combinations. Variables are presented as mean ± SD. To test independent relationships between variables, the χ^2 test and Fisher's exact test were performed. The genotypic OR was calculated for SCF and the 95% CI with two-tailed P values, using adjusted multiple logistic regression analysis.

RESULTS

The mean age was 52±13 years in the patient group and 49±12 years in the control group. Baseline characteristics are shown in Table 1. HDL cholesterol was higher in the SCF group than in the controls. The TFC count was 42±9 in the SCF group.

The genotype distributions and allele frequencies of the Glu298Asp polymorphism in SCF patients and control subjects in the Turkish population are shown in Table 2. The distribution of the three genotypes in the present study was as follows: GG 26%, GT 56% and TT 18%. The frequency of the variant allele (aspartic acid) was 0.41 in the control group and 0.38 in the patient group. There was no statistical difference in genotype distribution between the SCF group and the controls.

DISCUSSION

SCF syndrome is an angiographically and clinically unique phenomenon with no evidence of disease. The precise mechanism for this phenomenon is not clear. SCF may be observed in the absence of any obstructive lesion on coronary angiography. Furthermore, coronary flow rate may be normal in patents with severe stenotic coronary vessels. Therefore, in addition to known risk factors, other possible pathopsychological mechanisms that may cause SCF should be considered.

Endothelial dysfunction may play an active role in the pathophysiology of SCF (16), and the Glu298Asp gene polymorphism is

considered to be a major risk factor for CAD (19). However, to date no studies have examined the association between the Glu298Asp polymorphism and/or the T allele frequency of the *eNOS* gene and SCF. The aim of the present study was to investigate a possible association between the Glu298Asp polymorphism and SCF. The major finding of the present study was the lack of association of the Glu298Asp polymorphism and T allele frequency of the *eNOS* gene, with the presence of SCF in the Turkish population.

Because the mutation is not localized within the functional domains postulated within the *eNOS* gene sequence, the *eNOS* Glu298Asp polymorphism has been speculated to represent a marker mutation (20,21). However, it also has been assumed (22) that the gene variation could be involved in a conformational change in the NOS protein that consequently modifies NOS activity. Philip et al (23) demonstrated that the T allele was associated with an enhanced vascular responsiveness to the alpha-1 adrenergic vasoconstrictor phenylephrine. Because it was shown that NO reduced the maximal response to phenylephrine (24), Philip et al (23) suggested a lesser production of NO in *eNOS* T allele carriers. Tesauro et al (25) demonstrated that *eNOS* isoforms were processed differently depending on the presence of aspartate or glutamate at position 298. Therefore, it can be speculated that the Glu298Asp gene polymorphism may be perceived by an endogenous protease as a target for cleavage.

In previous studies, conflicting results were obtained concerning CAD and the Glu298Asp gene polymorphism. Several studies have shown a possible association between CAD and the Glu298Asp gene polymorphism (6,26,27). Contrary to these studies, Karvonen et al (28) stated that the Glu298Asp variant of the *eNOS* gene was not a major risk factor for cardiovascular alterations. In addition, it has been demonstrated that there is no evidence concerning the association between the G894T polymorphism and premature CAD in various populations (29).

Camsari et al (16) showed that endothelial function (assessed by endothelin-1 and NO concentrations) and its response to exercise were abnormal in SCF patients compared with healthy subjects. Also, it was reported that vascular endothelial function is impaired in patients with SCF using the brachial artery flow-mediated dilation technique (17). No relationship between the G894T polymorphism and SCF was observed in our study. Consistent with the results of the present study, Aras et al (30) showed that the *eNOS* gene polymorphism is not associated with CAD in the Turkish population. The frequency of the G894T polymorphism shows ethnic variety. In addition, the interaction between gene polymorphisms is an important factor in the evaluation of the effects of any gene polymorphism. It has been demonstrated that the G894T gene polymorphism is associated with different gene polymorphisms and other factors (31,32). Therefore, a possible cause of the lack of association between SCF and the G894T polymorphism in the present study may be the ethnic variety of the population and other interactions of the G894T polymorphism.

TABLE 2
Genotype distribution and allele frequencies (n=85)

	Genotype, n (%)			Allele frequency		Total alleles on chromosomes	
	GG	GT	TT	G	T	G	T
SCF (n=66)	17 (26)	37 (56)	12 (18)	0.54	0.46	71	61
Control (n=19)	8 (42)	8 (42)	3 (16)	0.59	0.41	24	17
χ^2	1.9			1.9		0.2	
P	0.38			0.38		0.65	
OR	1.1			1.1		–	
95% CI	0.5–2.3			0.5–2.3		–	

G Guanine; T Thymine; SCF Slow coronary flow

The present study has several limitations. First, the coronary arteries were only angiographically evaluated. Coronary angiography does not reveal the atherosclerotic plaque burden and endothelial functions. Second, the study lacked functional investigation. It should be determined whether the GluAsp298 polymorphism functionally underlies mechanisms leading to SCF. Third, the study group sample was not large. Thus, a larger sample population should be examined to confirm the relationship between gene polymorphisms and SCF.

In future studies, it will be necessary to identify a larger number of polymorphisms throughout the *eNOS* gene in the Turkish and other populations, and to perform association studies between these polymorphisms and SCF. In these investigations, gene interaction with different factors should be considered.

CONCLUSION

In the present study, the Glu298Asp polymorphism genotype of the *eNOS* gene was not found to be a risk factor for SCF in the Turkish population.

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