ORIGINAL ARTICLE

Eastern Journal of Medicine
East J Med 24(4): 472-477, 2019
DOI: 10.5505/ejm.2019.36036

Association Between Endothelial Nitric Oxide Synthase Polymorphisms T786C and G894T and Ischaemic Stroke

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Abstract

Endothelial nitric oxide synthase (*eNOS*) gene polymorphisms are suspected to increase the risk of ischaemic stroke (IS). *eNOS*-synthesized NO regulates vascular tone and inhibits the progression of atherosclerosis. The present study aimed to determine the association between *eNOS* polymorphisms G894T and T786C and IS.

Sixty acute IS patients (32 male, 28 female) were included and classified in accordance with the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification. Genotypes of patients with *eNOS* G894T and *eNOS* T786C polymorphisms were determined through polymerase chain reaction.

Significant differences were observed in the distribution of *eNOS* T786C polymorphism among IS subgroups. The *eNOS* T786C polymorphism heterozygote (TC) and homozygote (CC) genotypes more frequent in patients in the large artery atherosclerosis (LAA) subgroup. Considering a dominant model of inheritance for *eNOS* T786C polymorphism, the risk of IS was higher for the LAA subgroup than for other IS subgroups. Among potential haplotypes, the *eNOS* 786C+ *eNOS* 894G haplotype was associated with an increased risk of LAA; however, this finding was not statistically significant.

eNOS gene polymorphisms are suspected to increase the risk of ischaemic stroke. Our results suggest that the *eNOS* T786C gene polymorphism is associated with LAA in IS patients.

Key Words: Endothelial nitric oxide synthase; polymorphisms; genetics; ischemic stroke; TOAST classification; large artery atherosclerosis

Introduction

Ischaemic stroke (IS), a multifactorial disorder, usually resulting from atherosclerosis, hypertension, valvular heart disease, diabetes, smoking, etc. Other than conventional risk factors, genetic factors may also contribute to the risk of stroke development (1). Atherosclerosis involves multiple pathogenic processes initiated by endothelial dysfunction, with consequent formation of atheromatous plaques and its clinical manifestations. Nitric oxide (NO) synthesized from L-arginine by endothelial nitric oxide synthase (*eNOS*), is an important mediator of endothelial function and regulates cerebral blood flow and thrombogenesis (2). NO also protects against atherosclerosis by relaxing smooth muscle cells, inhibiting endothelial adhesion of platelets, and inhibiting vascular smooth muscle cell growth and proliferation (3). *eNOS* is produced in platelets, endothelial cells, its gene, comprising 26 exons, is located on choromosome 7q35 to 7q36 (GenBank D26607). Several *eNOS* gene polymorphisms have been reported, which affect its expression levels or activity; these include T786C (also referred to as rs2070744), localized in the promoter region and G894T (also referred to as rs1799983), located in exon 7.

T786C may cause significant reductions in eNOS gene promoter activity, thereby altering its expression (4) and is associated with ischaemic stroke in black women (5) and myocardial infarction in Koreans (6), but not to myocardial infarction and coronary artery disease in Caucasians (7).

G894T located in exon 7 results in Glu298Asp substitution. Its functional relevance has been suggested to be attributable to the observed genotype-dependent alterations in basal and shear-induced activation of the enzyme (8). The mutant T is associated with coronary artery disease and stroke (9,10).

Classically, acute thrombosis at the site of a ruptured lipid-rich atherosclerotic plaque results from the transition from stable or subclinical atherosclerotic disease to acute myocardial infarction or IS (11).

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Received: 25.04.2019, **Accepted:** 04.09.2019

Increasing evidence suggests atherosclerosis is a complex multifactorial disorder involving multiple gene-gene and gene-environment interactions. Considering the function of endothelial NO, its primary antithrombotic role in the vasculature is easily discernable. Hence, the present study aimed to investigate the possible association between eNOS polymorphisms and IS in IS subgroups classified in accordance with the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification.

Material and Methods

Ethics Statement: The study was approved by the Yuzuncu Yil University Faculty of Medicine Ethical Committee and written informed consent was obtained from participant.

Subjects: This prospective study involved 60 unrelated consecutive hospitalized patients with first IS in neurology clinic of Yuzuncu Yil University Faculty of Medicine Hospital. Patients were those with new onset IS registered in stroke centre with clinical evidence lasting more than 24 h. Only patients with first-ever IS were included in this analysis. Patients with recurrent stroke, transient ischemic attack, cancer, hematological diseases, autoimmune disease including antiphospholipid antibody syndrome, renal or liver failure were excluded.

All cases were grouped using the Trial of Org 10172 in Acute Stroke Treatment criteria (TOAST) classification. According to TOAST classification cases were divided into five categories: large-artery atherosclerosis (LAA), small vessel occlusion, cardioembolism, other determined etiology, and stroke of undetermined etiology. Stroke of undetermined etiology group consists of patients who no etiologic factor is found despite sufficient investigation and those with incomplete assessment (12,13). Stroke severity was assessed on admission using the validated Canadian Neurological Scale (CNS); higher scores in this scale indicate lower severity (14). The CNS evaluates level of consciousness; aphasia; orientation; facial paresis; and power in arm, hand, and leg.

Data Collection, Defining Risk Factors: A question survey was completed by the participants involving the demographic characteristics and history of hypertension, diabetes, myocardial infarction, smoking, family history and other risk factors. The examination findings and results of investigation including electrocardiography, echocardiography, blood tests, brain imaging and cranial arterial imaging were recorded. Diabetes was assessed according to fasting blood glucose, and /or use of glucose-lowering drugs. Hypertension was defined as high blood pressure on two separate occasions or a history of treated hypertension. Presence of myocardial infarction, angioplasty, or lower limb arterial disease history was defined as positive cardiovascular history.

eNOS G894T, T-786C Genotyping: Polymorphisms are invastigated with PCR technic by using reverse hybridisation based stripassay kits (ViennaLab Diagnostika GmbH, Austria). Stripassays are based on reverse-hybridization of biotinylated PCR products working with immobilized oligos on teststrip generate results by enzymatic color reaction visible to the naked eye. By Cardio Vascular Disease Ateroscleroz StripAssay tests eNOS G894T, T-786C polymorphisms are searched on genetic material related genetic region. This procedure involves three grades: (1) genomic DNA isolation from peripheral lymphocites, (2) PCR amplification using biotinylated primers, (3) hybridization of amplification products to a test strip containing allele-specific oligonucleotide probes. Bound biotinylated sequences are detected using color substrates.

Statistical Analysis: Descriptive statistics for the continuous variables were presented as mean, standard deviation, minimum and maximum values, with count and percentages for categorical variables. The chi-square test was used to determine whether the allelic frequencies were in accordance with Hardy– Weinberg equilibrium (HWE) or not. One-way analysis of variance (ANOVA) was used to compare group means. The frequencies of the alleles and genotypes were compared between patient groups by the chi-square test. The conditional logistic regression analysis was used to estimate Odds Ratio (OR) and 95% confidence intervals (CIs) for the strength of association between *eNOS* gene polymorphism and the risk of IS subgroups. The statistical significance level was measured as 5%; the Statistical Package for the Social Sciences (SPSS) was used for all statistical computations (version 22, IBM SPSS, Armonk, NY, USA).

Results

The demographic characteristics of 60 IS patients (53.3% male; mean age, 63.6 years) are shown in table 1, along with the prevalence of conventional risk factors for vascular disease such as history of hypertension, diabetes, smoking, hyperlipidaemia, etc. IS patients were classified into the following subgroups (Table 2) in accordance with the TOAST classification: small vessel occlusion (n=12), large artery atherosclerosis (LAA; n=22), cardioembolic infarct (n=8), unknown etiology (n=7), and other etiologies (n=1). Disease severity was rated in accordance with CNS guidelines. The distribution of

Table 1: Demographic and clinical characteristics of ischemic stroke subjects

Characteristics	n	%
Females /Males	28/32	46.7/53.3
Hypertensives	41	68.3
Diabetics	17	28.3
Heart disease	25	41.7
*TIA	5	8.3
Atrial Fibrillation	9	15
LDL- cholesterol >130	17	28.3
Carotid Artery Atherosclerosis	33	54.1
Smokers	25	41.7

*:Transient ischemic attack

Table 2: Distubution of the patients according to TOAST classification

	n (60)	%
small vessel occlusion	12	20,0
large-artery atherosclerosis	22	36,7
cardioembolism	18	30,0
stroke of undetermined etiology	7	11,7
other determined etiology	1	1,7

CNS points of IS patients in accordance with TOAST classification is shown in table 3. Ten IS patients died during hospitalisation.

Genotype Distributions: All genotypes were found to be in Hardy-Weinberg equilibrium in study group. A significant difference was observed in the distribution of T786C (Table 4). *eNOS* -786TC (heterozygote) and *eNOS* -786CC (homozygote) genotypes were more frequent in the LAA subgroup (LAAS) than in other IS subgroups (p=0.03). In other words, the frequency of the C allele was increased in the LAAS (Table 4). There was no significant difference in the distribution of G894T among IS subgroups.

As shown in Table 5, T894 and C786 were considered rare alleles and their association with IS subgroups was also analysed using dominant and recessive models. Interestingly, logistic regression analyses revealed that the dominant model phenotype for the polymorphism in the promoter region (TC+CC) was a risk factor for LAAS, although this finding was not statistically significant (OR:2.68; p:=0.07; Table 5).

Haplotype Analysis: All potential haplotypes are designated by the allele at positions -786 and 894 table 6. Logistic regression analysis revealed that compared with other haplotypes, the 786C 894G haplotype was associated with an increased risk of LAA; however, this finding was not statistically significant (p=0.06; OR:2.77; Table 6).

Discussion

Atherosclerosis and thromboembolisms are major causes of morbidity and mortality worldwide. Increasing evidence indicates that several genetic polymorphisms are risk factors for the progression and for complications of these diseases. Polymorphisms in *eNOS*, responsible for the production of endothelial NO an important arterial vasodilator, can determine pathogenesis, and predisposition to atherosclerosis.

Endothelium-derived NO plays several predominant roles in arteries, including inhibition of endothelial adhesion of platelets and leukocytes, endothelial vasodilation, and inhibition of the formation of oxidized low-density lipoprotein to prevent atherogenesis (15,16)

In the present study, we investigated the association between two *eNOS* gene polymorphisms and IS in IS patients classified into subgroups in accordance with TOAST classification. Previous studies have reported that individuals with *eNOS* polymorphisms are susceptible to cardiovascular diseases; however, the correlation between *eNOS* polymorphisms and susceptibility to stroke is unclear. The present results report an association between LAAS in IS and *eNOS* T786C. The frequency of C allele of *eNOS* T-786C polymorphism was significantly higher in the LAAS subgroup than in other IS subgroups. Furthermore,

Table 3: Canadian Neurologic Scala values to TOAST classification

	n	Mean	SD (±)
SVO	12	8,87	1,860
LAA	22	6,68	3,084
Cardioembolism	8	6,88	3,393
SUE	7	3,00	3,640
ODE	1	9,00	.
Total	60	6,79	3,372

SVO: small vessel occlusion LAA: large-artery atherosclerosis SUE: stroke of undetermined etiology ODE: other determined etiology

Table 4: eNOS T786C genotype distribution in ischemic stroke subgroups

	LAA	SVO	Cardio embolism	ODE	SUE	TOTAL
	n (%)	n (%)	n (%)	N (%)	N (%)	n (%)
Wild type TT	8 (36,4)	7 (58,3)	14 (77,8)	0	2 (28,6)	31 (51,7)
Heterozygote TC	9 (40,9)	4 (33,3)	4 (22,2)	0	4 (57,1)	21 (35,0)
Homozygote CC	5 (27,2)	1 (8,3)	0	1 (100)	1 (14,3)	8 (13,3)
TOTAL	22 (100)	12 (100)	18 (100)	1 (100)	7 (100)	60 (100)

LAAS: Large-artery atherosclerosis SVO: small vessel occlusion ODE: other determined etiology SUE: stroke of undetermined etiology

logistic regression analysis revealed that T786C increases the risk of LAAS; however, this finding is not statistically significant.

Larger epidemiological studies have attempted to determine the association between T786C and susceptibility to IS; however, the results are inconsistent (17). A meta-analysis by Niu et al. reported that T786C was significantly associated with IS in Asian populations, rather than in Caucasians and blacks (18), while that by Liu et al. suggested that T786C may alter the risk of IS in all analysed ethnic groups (17); however, that by Guo et al. suggested that *eNOS* T786C is not a risk factor for IS for both Asians and Caucasians (19).

Despite conflicting data in the literature regarding the association of *eNOS* polymorphisms and IS, our results are consistent with the role of endothelium-derived NO in atherogenesis. eNOS-synthesised NO exerts an atheroprotective effect (20). T786C was previously reported to be associated with lower eNOS mRNA and serum nitrite/nitrate levels and with an increased risk of stroke (21,6). It is well-established that endothelial dysfunction potentiates atherogenesis, concurrent with our findings, wherein the -786C allele appears more frequently in the LAAS subgroup than in other subgroups. Findings of Salimi et al are concurrent with the present findings, wherein the frequency of the C allele in individuals with T786C polymorphism is higher in coronary artery disease patients than in controls (22). On the contrary, Jaramillo et al. suggested that eNOS T786C was not associated with coronary artery disease in Chilean individuals (23). Reduced NO production is associated with vascular dysfunction and smooth muscle cell proliferation in the arterial endothelium, thereby resulting in the pathogenesis of atherosclerosis (24). Further analysis of eNOS polymorphisms in IS subgroups is required, which would yield more consistent results.

Numerous studies have supported the possible association between eNOS G894T and IS (25,26,19). eNOS G894T in exon 7 renders eNOS more susceptibility to degradation and cleavage (27,28) and is associated with reduced NO synthesis and endothelial dysfunction (29).

Another important finding of the present study is that G894T distribution was not significantly different among all IS subgroups. Kumar et al. suggest that eNOS G894T is an important risk factor for IS, specially for LAAS in IS in the North Indian population (24). Furthermore, Campedelli reported that the GG genotype is associated with greater susceptibility to atherosclerosis (30). Moreover, Abdel-Aziz et al. recently reported that the TT genotype of *eNOS* Glu298Asp is an independent risk factor for premature coronary artery disease in Egyptians (31). The present results do not support these findings, since there are several potential explanations for the differences in these associations among different populations. For instance, Zhang et al. reported that people from different ethnicities, with the same genotype, might have different, even

Table 5: Association of eNOS polymorphisms recessive and dominant models with LAAS

Recessive and dominant models	P	OR (95% CI)
eNOS 786C recessive		
CC vs TT+TC	0,103	3,43 (0,73-16,07)
eNOS 786Cdominant		
TT vs TC+CC	0,071	2,68 (0,90-7,94)
eNOS 894T recessive		
TT vs GG+GT	0,616	0,556 (0,54-5,69)
eNOS 894Tdominant		
GG vs GT+TT	0,282	1,80 (0,62-5,33)

Table 6: Association of eNOS haplotypes with LAAS

Haplotype	P	OR (95% CI)
786C/894T	0,100	2,59 (0,81-8,22)
786C/894G	0,061	2,77 (0,94-8,19)
786T/894T	0,815	1,14 (0,36-3,57)
786T/894G	0,599	0,68 (0,16-2,85)

inverse, susceptibility to the same diseases, e.g., the ACE DD genotype, as they reported, was associated with a 2.20-fold risk of IS when compared with two other genotypes in Asians; however, the ACE DD genotype was not associated with the increased risk of IS in Caucasians (32).

After identification of the susceptibility haplotypes 786C+894T, 786C+894G, 786T+894T, and 786T+894G, regression analysis comparing the LAAS subgroup and the other IS subgroups revealed no significant difference; however, the 786C+894G haplotype tended towards statistical significance.

The pathogenesis of arterial thrombotic disease is complex and involves multiple genetic and environmental factors related to atherosclerosis and thrombosis, as well as their interaction. Consistent with the important role of NO in endothelial regulation, further analysis of *eNOS* polymorphisms in IS subgroups is required. This study was limited by its small sample size, which deters the deduction of any significant conclusions to be extrapolated to the population. However, our results have important implications, suggesting that *eNOS* polymorphisms may increase the risk of vascular disease among different IS subgroups.

This study has a number of limitations worthy of discussion. It was underpowered to generalize the current study findings to all population because of the limited number of patients. Moreover this study was restricted to a single geographical region within a single country therefore caution must be exerted in evaluating the study findings particularly in countries other than Turkey.

In conclusion, the present results indicate that eNOS T786C is a risk factor for LAAS in IS. Further studies with larger sample sizes and heterogeneous populations are required for independent validation. Our findings may facilitate further, more detailed studies on polymorphisms in etiologic subgroups of IS rather than the disease in its entirety.

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

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