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A polymorphism (G894T) in eNOS increases the risk of coronary atherosclerosis rather than intracranial atherosclerosis in Koreans

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Abstract

Objectives: The aim of this study was to investigate whether genetic variations in the eNOS gene were associated with intracranial and extracranial atherosclerosis in Koreans. Materials and methods: We analyzed data on 282 consecutive Korean patients with atherosclerosis in intracranial (ICAS) or extracranial vessels, and compared with 300 healthy individuals. The eNOS gene polymorphism was analyzed by PCR and direct sequencing.

Results: All genetic variants were single nucleotide polymorphism, two in the coding sequence (C774T and G894T) and one in intron 6. The frequency of the G894T polymorphism was significantly higher in the coronary atherosclerosis group than in the control group, but its presence did not correlate with other clinical risk factors. There was no significant difference in genotype and allele frequencies between coronary atherosclerosis and ICAS.

Conclusion: The G894T polymorphism in the eNOS gene might influence coronary atherosclerosis rather than ICAS in Koreans.

Keywords: Atherosclerosis, Intracranial, Extracranial, eNOS, Polymorphism, Korean.

Introduction

Atherogenesis is a systemic process, but atherosclerosis affects primarily large and medium sized elastic arteries, with critical changes usually occurring in single rather than multiple organs (1). In addition to contributing to myocardial and cerebral infarctions, atherosclerosis usually occurs in a single organ or relatively specific anatomic sites (2). In Asians, the incidence and mortality rate due to stroke is higher than that in whites, although the rate of coronary heart disease is lower in Asians (3). Angiographic and autopsy studies in stroke patients have shown that African American, Chinese, and Japanese individuals tend to have more intracranial vascular occlusions, whereas whites tend to have more extracranial lesions (3-6). The mechanisms that underlie this anatomic distribution of atherosclerosis remain unclear despite multiple studies on the topic (7-9).

Genetic polymorphisms contribute the extracranial atherosclerosis, particularly myocardial infarction, or intracranial atherosclerosis, such as ischemic stroke (10-15). One important product of endothelial cells is nitric oxide (NO), a principal factor involved in the anti-atherosclerotic properties of the endothelium (16-17). NO production is regulated by the Ca^{2+}-calmodulin-dependent endothelial isoform of the nitric oxide synthase enzyme (eNOS or NOS3) (18). The association between cardiovascular disease, including myocardial infarction or ischemic stroke, and eNOS polymorphisms has been tested (12-15), but only one report examined the effects of eNOS polymorphism between ischemic cerebrovascular disease and carotid atheroma in Caucasians (19).

The aims of this study were to investigate whether genetic variations of the eNOS gene are associated with extracranial or intracranial atherosclerosis in Koreans.

Materials and Methods

1. Study populations

We studied 282 consecutive Korean patients with coronary atherosclerosis or atherothrombotic stroke and compared them with 300 controls at the Chonnam National University Hospital. Because of the marked variation in common vascular candidate polymorphisms in different ethnic groups (20), only Koreans were studied.

1.1. *Coronary Atherosclerosis (CAS) patient group*

We recruited 168 patients with symptomatic coronary artery disease, including acute myocardial infarction, old myocardial infarction, and unstable angina pectoris. All patients were angiographically proven to have coronary artery disease, with more than 75% stenosis affecting at least one vessel.

1.2. *Atherothrombotic Ischemic Stroke (AIS) patient group*

We recruited 114 patients with neurological symptoms resulting from focal cerebral ischemia due to complete stroke and excluded patients with intracerebral or subarachnoid hemorrhage. All patients underwent carotid ultrasound sonosgraphy as well as brain-computed tomography or magnetic resonance imaging. On the basis of clinical symptoms and brain imaging findings, cases were classified into four subtypes according to the TOAST classification system: (1) large vessel disease; (2) small vessel disease; (3) cardiac embolism; and (4) stroke of undetermined etiology (21). The large vessel disease group was used for this study.

1.3. *Control Groups*

Three hundred control subjects without a history of coronary heart disease and stroke were recruited among hospitalized individuals for any reason other than heart or neurological diseases. Patients over 50 years of age were selected.

1.4. *Investigation of risk factors*

The included risk factors are age, gender, body mass index (BMI), hypertension, diabetes mellitus, hypercholesterolemia, and cigarette smoking. A patient was considered to have hypertension if elevated blood pressure (> 140/90 mmHg) had been documented on at least three separate occasions or antihypertensive medications had been prescribed. Diabetes mellitus (DM) was regarded as present if the subject was treated for diabetes at the time of inclusion, or if the fasting blood glucose level had been over 140 mg/dl on two or more separate occasions. All study patients were classified as smokers (including current and ex-smokers) or non-smokers. Total cholesterol was also determined at the time of entry.

2. Enos gene polymorphism identification

All molecular genetics studies were performed blinded to the case-control status. This study had used the eNOS genomic DNA sequence reported by Marsden *et al*. (22) (GenBank D26607) for nucleotide numbering in the sequence variations. To describe the variations we have adopted the nomenclature rules described by Dunnen (23).

The polymorphism identification was performed using the polymerase chain reaction (PCR) and direct sequencing using an ABI Prism 310 Genetic Analyzer. Genomic DNA was isolated from peripheral blood leukocytes and used as a template for PCR. Primers were derived from the eNOS genomic DNA sequence (22) and designed for a 497-base pair region, starting at base pair position 38 of exon 6 and ending at base pair 138 of exon 7. The sequences of the forward and reverse primers were CGAGGAGACTTCCGAATCCTG and AGGGGCACCTCAAGGACCAG, respectively.

PCR were performed in a final volume of 50 ml containing 100 mM KCl, 20 mM Tris-HCL (pH 7.9), 0.1 mM EDTA, 0.5 mM PMSF, 1 mM DTT, 50% glycerol, 10 pmol of each primer, 25 mM dNTP, and 250 ng (50 ng/ml) of genomic DNA in the presence of 2.5 U of Taq polymerase (Suwon, South Korea). After the initial denaturation step (94 °C for 5 min), each cycle (of an additional 35) consisted of 94 °C denaturation for 30 s, 62 °C annealing for 45 s, 72 °C extension for 60 s, and final extension lasting 10 min at 72 °C. The purified DNA was used as a sequencing template without further characterization.

Cycle sequencing was performed on the GeneAmp PCR system 2400 using the Applied Biosystems Prism™ BigDye® Terminator Sequencing Kit (Perkin-Elmer/Applied Biosystems Division, Foster City, CA, USA) according to the manufacturer's directions. DNA sequences were analyzed using sequencing analysis software Version 3.0 and Navigator 4.1 to compare with the reference sequence (GenBank accession numbers D26607).

3. Statistical analysis

The statistical analyses were made on a per-patient basis. Discrete variables are expressed as counts and compared with Chi-squared or Fisher's exact test as appropriate. Continuous variables are expressed as mean ± S.E. (standard error) and compared by means of the unpaired, two-sided *t*-test or analysis of variance for more than two groups. Odds ratios (approximating relative risk) were calculated as a measurement of the association of the eNOS genotype (wild homozygote type (*NN*), heterozygote (*Nn*), and mutant homozygote (*nn*)) with the effects of the mutant allele assumed to be additive (*NN, Nn*, and *nn*), dominant (*NN* vs *Nn* and *nn* combined), or recessive (*NN* and *Nn* combined vs. *nn*). In the additive effect, the values were predicted by the Hardy-Weinberg equilibrium. For each odds ratio, we

calculated two-tailed *P* values and 95% confidence intervals (CI). Statistical significance was assumed for *P*-values < 0.05. All statistical analyses were performed by SPSS Advanced Statistics 13.0 for Windows (SPSS Korea, Suwon, Korea).

Results

1. Comparison of control and atherosclerosis groups

We compared the control and atherosclerosis (AS) groups for atherosclerosis risk factors, including age, gender, body mass index, hypertension, cigarette smoking, diabetes mellitus, and serum cholesterol profile (Table 1).

2. Screening for the eNOS gene polymorphism

Among the 282 patients and 300 controls included in this study, DNA could be amplified and used in the analysis for 242 (85.8%) patients and 270 (90.0%) controls. Three polymorphisms were detected between exon 6 and exon 7 on the eNOS gene: two in exonic sequences (C774T in exon 6, G894T in exon 7), and one in intron [IVS(intervening sequence) 6+240A□G].

3. Comparison of eNOS gene polymorphism between controls and diseases

All three genetic variations were single nucleotide polymorphisms (SNPs), and allele frequencies in both control and patient populations were in the Hardy-Weinberg equilibrium. eNOS polymorphism frequencies in patients with atherosclerosis in coronary or intracerebral arteries are shown in Table 2. In patients, the frequencies of the eNOS C774T genotype were 72.0%, 0%, and 28.0% for CC, CT, and TT, respectively. The frequencies of the eNOS IVS6+240A□G genotype were 32.0%, 40.0%, and 28.0% for AA, AG, and GG, respectively. The frequencies of the eNOS G894T genotype were 83.5%, 15.0%, and 2.5% for GG, GT, and TT, respectively genotype and allele frequencies did not vary for the C774T and IVS6+240A□G polymorphism between controls and patients. The GT and TT combined genotype (dominant effect) and T allele frequency of the G894T polymorphism in exon 7 have a slightly higher incidence in all atherosclerosis patients than controls, but this did not reach statistical significance (Table 2).

There was no association between the control and coronary atherosclerosis groups (CAS) group in the genotype and allele frequency of C774T polymorphism. The IVS6+240A□G polymorphism showed slightly higher incidence of the A allele in the CAS group than in controls (57.1% vs 47.2%; p = 0.074). The GG, GT, TT genotype frequencies of the G894T

Table 1
Clinical Parameters of Control and Atherosclerosis (AS) Patients

Control Patients				
		Total	CAS	AIS
	(n = 300)	(n = 282)	(n = 168)	(n = 114)
Age, yr		62.7 ± 0.97	60.48 ± 1.22	66.00 ± 1.51
Men/women	172/128	180/102	102/66	78/36
BMI, kg/m^2	23.55 ± 0.89	23.78 ± 0.32	23.58 ± 0.39	24.30 ± 0.58
Hypertension	35	98	58*	40*
Smoking	135	176**	112*	64
Diabetes mellitus	35	60	32	28
T-cholesterol, mg/dl	170.83 ± 8.78	195.98 ± 3.22*	197.03 ± 4.19*	194.37 ± 5.06**
Triglyceride, mg/dl	122.00 ± 18.59	128.56 ± 8.09	127.71 ± 11.52	129.88 ± 10.48
HDL cholesterol, mg/dl	46.85 ± 3.18	46.27 ± 1.16	47.30 ± 1.60	45.18 ± 1.63
LDL cholesterol, mg/dl	99.57 ± 8.88	123.80 ± 3.26*	124.19 ± 4.29*	123.21 ± 5.05**
Lipoprotein B/A1	0.83 ± 0.14	0.94 ± 0.04	0.93 ± 0.04	1.06 ± 0.13
Lipoprotein (a), mg/dl	20.41 ± 3.38	29.78 ± 2.61	30.20 ± 2.72	20.83 ± 1.58

BMI, body mass index; T-cholesterol, serum total cholesterol; CAS, coronary atherosclerosis; AIS, atherothrombotic ischemic stroke. Data are mean ± SE, and *p < 0.01, **p < 0.05 being compared with controls.

Table 2
Genotype and allele frequency of eNOS gene Polymorphism in control and all atherosclerosis patients.

	Control Patients*		Odds Ratio	
	(n = 270)	(n = 242)	(95% CI)	p value
C774T				
Genotype				
CC	200 (74.1%)	174 (72.0%)		0.415
CT	0 (0.0%)	0 (0.0%)		
TT	70 (25.9%)	68 (28.0%)		
CC vs. CT and TT			1.16 (0.56 - 2.40)	0.415
CC and CT vs. TT			1.16 (0.56 - 2.40)	0.415
Allele			1.16 (0.70 - 1.94)	0.329
C	74.1	72.0%		
T	25.9%	28.0%		
IVS6 + 240A□G				
Genotype				
AA	80 (29.6%)	77 (32.0%)		0.362
AG	95 (35.2%)	97 (40.0%)		
GG	95 (35.2%)	68 (28.0%)		
AA vs. AG and GG			0.85 (0.43 - 1.71)	0.395
AA and AG vs. GG			1.34 (0.69 - 2.61)	0.242
Allele			0.75 (0.48 - 1.20)	0.140
A	47.2%	54.1%		
G	52.8%	48.9%		
G894T				
Genotype				
GG	250 (92.6%)	202 (83.5%)		0.233
GT	20 (7.4%)	36 (15.0%)		
TT	0 (0.0%)	4 (2.5%)		
GG vs. GT and TT			2.48 (0.80 - 7.64)	0.076
GG and GT vs. TT			2.18 (0.70 - 6.80	0.128
Allele			2.60 (0.87 - 7.74)	0.055
G	96.3%	90.9%		
T	3.7%	9.1%		

Coronary atherosclerosis and atherothrombotic stroke patients

polymorphism were 92.6%, 7.4%, and 0.0% in the control and 80.8%, 16.6%, and 2.6% in CAS group, which was significant for GG versus GT and TT genotype (odds ratio, 2.09; 95% CI, 0.93 to 9.53; p = 0.026 for dominant effect). The mutant T allele frequency was higher in the CAS group than in the control group (odds ratio, 3.18; 95% CI, 1.04 - 9.73; p = 0.017). Clinical and laboratory values were similar regardless of G894T polymorphism genotype, as was severity of coronary atherosclerosis as determined by the number of involved vessels (p = 0.307). No genotype or allele frequency differences were noted between controls and the atherothrombotic ischemic stroke (AIS) group.

Finally, we compared genotype and allele frequency of eNOS gene polymorphism between CAS and intracranial atherosclerosis. However, there was no statistically difference between two groups in all polymorphisms (Table 3).

Table 3
Comparing of genotype and allele frequency of eNOS gene polymorphism between CAS and AIS groups

	CAS (n = 168)	AIS (n = 114)	Odds Ratio (95% CI)	p value
C774T				
Genotype				
CC	116 (69.2%)	85 (74.4%)		0.350
CT	0 (0.0%)	0 (0.0%)		
TT	52 (30.8%)	29 (25.6%)		
CC vs. CT and TT			0.77 (0.34 - 1.79)	0.350
CC and CT vs. TT			0.77 (0.34 - 1.79)	0.350
Allele			0.77 (0.43 - 1.40)	0.242
C	69.2%	74.4%		
T	30.8%	25.6%		
IVS6 + 240A□G				
Genotype				
AA	65 (38.5%)	27 (23.3%)		0.198
AG	62 (37.2%)	58 (51.2%)		
GG	41 (24.4%)	29 (25.6%)		
AA vs. AG and GG			2.06 (0.89 - 4.79)	0.065
AA and AG vs. GG			1.77 (0.83 - 3.76)	0.097
Allele			1.39 (0.82 - 2.36)	0.137
A	57.1%	48.8%		
G	42.9%	51.2%		
G894T				
Genotype				
GG	136 (80.7%)	101 (88.4%)		0.413
GT	28 (16.7%)	13 (11.6%)		
TT	4 (2.6%)	0 (0.0%)		
GG vs. GT and TT			0.55 (0.19 - 1.64)	0.207
GG and GT vs. TT			0.66 (0.22 - 1.99)	0.322
Allele			0.51 (0.18 - 1.42)	0.139
G	89.1%	94.2%		
T	10.9%	5.8%		

Discussion

The G894T polymorphism in exon 7 of the eNOS gene, which changes glutamic acid to an aspartic acid at amino acid 298, was associated with coronary atherosclerosis but not with atherothrombotic ischemic stroke. This Glu298Asp change shows an increased risk for myocardial infarction (13, 19, 24-25) and stroke (7, 14-15) in some, but not all, studies (26-29). The GT, TT genotype was 16.7%, 2.6%, respectively, in CAS, and 7.4%, 0% in controls, with mutant T allele rates of 10.9% in CAS and 3.7% in controls. In Japanese populations, Shimasaki *et al*. (30) and Hibi *et al*. (31) discovered that the frequencies of the G894T polymorphism (TT genotype) had a prevalence of 0.4%, 2.2% in patients and 0.2%, 0.0% in controls, similar to rates in Koreans (32). Caucasians show a higher frequency of the mutant T allele in angiographically proven coronary artery disease (33-35). This difference in genotype and allele frequency indicates that this marker is ethnic difference. And the Glu298Asp polymorphism is a risk factor for coronary atherosclerosis, but its low

prevalence (2.6% for TT genotype) only explains part of the genetic susceptibility to coronary atherosclerosis in Koreans.

We did not observe a relationship between the G894T polymorphism and general risk factors for atherosclerosis or disease severity, as estimated by coronary angiography. In the multiple logistic regression analysis, eNOS gene polymorphisms were not independent risk factors ($p = 0.74$) for coronary atherosclerosis, although others showed that this polymorphism and LDL cholesterol may interact to determine the risk of brain infarction[7]. Quyyumi *et al*. (36) reported that no correlation existed between the angiographic severity of coronary atherosclerosis and the magnitude of depression in basal NO activity. Guzik *et al*. (37) tested Glu298Asp polymorphisms and vascular NO production in human blood vessels from patients with atherosclerosis, and found that the presence of the Asp298 variant in eNOS did not affect vascular NO bioactivity. Our findings and previous reports (36-37) suggest that the effect of the Asp298 variant on eNOS activity is small compared with other factors that affect vascular NO bioactivity.

Atherogenesis is a systemic process, but the clinical outcomes of atherosclerosis have distinct features; some cause myocardial infarctions and others cause strokes. Shochina *et al*. (38) reported that the NO system plays different roles in the coronary and cerebral circulations during renal hypertension, and Lembo *et al*. (39) suggested that the Asp298 variant of the eNOS contributes mainly to focal vascular phenomena such as the atherosclerotic plaques, rather than to the generalized architecture of the blood vessels. We did not find any differences between coronary and intracerebral atherosclerosis using the clinical profile including eNOS gene polymorphisms, except age distribution, suggesting that other factors can determine the clinical distinct of intracranial and extracranial atherosclerosis.

This study was a case-control study to define the effect of genomic DNA variations on atherogenesis. To better categorize patients for this study, we enrolled patients with angiographically confirmed atherosclerotic lesions in the coronary artery and definitive atherosclerotic lesions in the intracerebral artery by MR angiography or Color Doppler ultrasonography. We could not completely exclude the presence of diseased vessels among the controls, but only included control patients over 50 with no phenotypes of atherosclerosis. We only recruited Korean patients, which could limit the generalizability of the study. Finally, we could not compare control and concurrent atherosclerosis patients in coronary and intracerebral regions.

In conclusion, the G894T polymorphism in exon 7 of the eNOS gene is associated with coronary atherosclerosis, but only explains a small part of genetic susceptibility to atherosclerosis in Koreans. This eNOS polymorphism may not be critical in distinguishing between coronary (extracranial) and intracranial atherosclerosis. Because atherosclerosis is a multifactorial disease, several genes with weak or moderate effects are likely to be involved, and other candidate genes should also be investigated. Linkage analyses are also needed to evaluate the interaction between these polymorphisms and other functional variants.

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