Endothelial nitric oxide synthase gene polymorphisms and the risk of silent brain infarction

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Received December 22, 2009; Accepted February 25, 2010

DOI: 10.3892/ijmm_00000410

Abstract. Silent brain infarction (SBI), a unique cerebrovascular disorder, is frequently detected on magnetic resonance imaging (MRI) of apparently healthy elderly persons, and it is known to increase the risk of stroke and other related diseases. Although detailed mechanisms of SBI pathogenesis have yet to be determined, recent studies suggest that SBI is significantly influenced by genetic factors. In this study, we investigated polymorphisms in the endothelial nitric oxide synthase (*eNOS*) gene (i.e., -786T>C, 4a4b and 894G>T) as possible risk factors for SBI. We enrolled 269 patients with SBI and 234 control subjects and examined their fasting plasma homocysteine and folate levels, and analyzed for *eNOS* polymorphisms and haplotypes. The prevalence of SBI was shown to be significantly higher in patients with the *eNOS* 894GT genotype (OR, 2.00; 95% CI, 1.30-3.08) and 894GT+TT genotype (OR, 2.05; 95% CI, 1.34-3.16), compared with the 894GG genotype. However, in the case of -786T>C and 4a4b polymorphisms, no significant difference was observed between SBI patients and normal subjects. Interestingly, we found that the prevalence of SBI can increase twice as high when either -786T>C or 4a4b polymorphism was combined with 894G>T polymorphism, -786TC+CC/ 894GT+TT (OR, 3.83; 95% CI, 1.24-11.80) and 4a4b+4a4a/894GT+TT (OR, 4.08; 95% CI, 1.34-12.40), respectively. According to haplotype analysis, we found that three haplotypes (-786T-4b-894G, -786T-4b-894T and -786C-4a-894T) were shown to be significantly different between SBI patients and control subjects. These results indicate that *eNOS* polymorphisms and haplotypes serve as risk factors for SBI, and three different polymorphic loci in the *eNOS* gene play interactively, thereby leading to synergistic effects for the generation of SBI.

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Key words: endothelial nitric oxide synthase, silent brain infarction, polymorphism, haplotype, risk factors

Introduction

Silent brain infarction (SBI) is defined as a unique form of cerebral infarction, which can be evident by brain imaging but does not exhibit overt clinical manifestations, such as rapidly developing clinical symptoms and signs of focal and at times global loss of brain function. It is relatively common in the geriatric population with a prevalence of 10-40%, which gradually increases with age (1-6). Although the clinical significance of SBI has received much attention in recent years, detailed mechanisms of its pathogenesis have yet to be determined. Recent studies strongly suggest that SBI can be significantly influenced by many risk factors, such as hypertension (5), homocysteine (7,8), cigarette smoking (9), and metabolic syndrome (10). However, relatively little is known about the genes responsible for causing SBI. Among various candidate genes that might be involved in SBI pathogenesis, we have first focused on the *eNOS* gene, which is known to be responsible for causing hyperhomocysteinemia that was found to be a risk factor for SBI in our previous study (8).

eNOS, an essential enzyme catalyzing the formation of soluble NO from L-arginine, possesses various physiological roles, including the maintenance of basal cerebral blood flow, cerebral vasodilation, vascular integrity, as well as the inhibition of smooth muscle proliferation (11-13). Therefore, the dysregulation of eNOS can lead to atherosclerosis, ischemic stroke, and various other vessel diseases (14-18). Related to these pathogenic conditions, three representative polymorphisms of the *eNOS* gene have been identified: i) -786T>C within the 5'-flanking region (14); ii) 27-bp deletion(a)/insertion(b) within the intron 4 (collectively designated as '4a4b') (19); iii) 894G>T within the exon 7 (20-22).

Considering the significance of SBI, which is known to increase the risk of developing into serious vascular diseases, such as stroke and dementia, there has been no epidemiological study on the *eNOS* polymorphisms in the SBI patients to our knowledge, although very few polymorphic studies of other genes have been reported (8,23-25). Therefore, in the present study, we investigated the role of polymorphisms in the

eNOS gene (-786T>C, 4a4b and 894G>T) as a risk factor for SBI. Interestingly, we found combinatorial effects caused by interactions among three loci, which lead to a significant increase in the risk of SBI. We have also carried out a haplotype analysis on three loci in SBI patients.

Materials and methods

Subjects. The study population was composed of 269 patients with SBI (124 men, 145 women) and 234 control subjects (116 men, 118 women). The patients were enrolled from July 1, 2000 to February 28, 2004 in the Neurology Department at Bundang CHA General Hospital by consecutive referral. A diagnosis of SBI was made by MRI examination and by agreement between two independent experienced neurologists. SBI was excluded when an agreement was not reached and patients with cerebral hemorrhage were excluded in advance. All patients underwent a brain MRI scan and electrocardiography, and the criteria for selecting SBI patients were used as we have previously reported (8).

For control subjects, we selected healthy individuals matched for sex and age within 5 years, from patients undergoing health examinations at our hospitals, which included biochemistry, electrocardiogram, and brain MRI, during the same period, and have no history of cerebrovascular disease or myocardial infarction. Exclusion criteria were the same as those used in the patient group, as mentioned above. Among the study population, some patients were diagnosed as having hypertension, diabetes mellitus or hyperlipidemia after the diagnostic criteria were fulfilled at the time of enrollment. Hypertension was defined as systolic pressure >140 mmHg and/or diastolic pressure >90 mmHg on more than one occasion, according to the Joint National Committee (JNC 7) report guidelines and currently taking hypertensive medication. Diabetes was defined as fasting plasma glucose >126 mg/dl (7.0 mmol/l) and included patients with diabetic medication (26,27).

Informed consent was obtained from all study participants after being given a full explanation of the study. In this study, all study populations were in Hardy-Weinberg equilibrium. The institutional review board of Bundang CHA Hospital approved this genetic study in June 2000.

Laboratory analysis. Venous blood samples were collected in tubes containing genetic analysis on *eNOS* polymorphisms and was carried out using polymerase chain reaction (PCR)-restriction fragment length polymorphism method, according to the method of Wilcox *et al* (28). Plasma homocysteine levels were determined as total homocysteine by fluorescence polarization immunoassay (FPIA), according to the manufacturer's instructions (IMx, Abbott Laboratories, Abbott Park, IL).

Statistical analysis. To estimate the magnitude of the association between *eNOS* polymorphisms and SBI, we used odds ratio (OR) and 95% confidence interval (CI). For crude analysis of baseline characteristics, we used the Chi test for categorical data (gender, hypertension, diabetes mellitus, and hyperlipidemia), and the two-sample t-test for continuous data (age and homocysteine). For multivariate analysis, we used logistic regression to adjust for possible confounders, i.e., age, gender, hypertension, diabetes mellitus and hyperlipidemia. Analysis was performed using SPSS for Windows, version 11.0 (SPSS Inc., Chicago, IL, USA). Haplotype frequencies for multiple loci were estimated using the Expectation-Maximization (EM) algorithm with the SNPAlyze program (Dynacom, Yokohama, Japan, http://www.dynacom. co.jp/).

Table I. Demographic and clinical characteristics of SBI patients and controls.

Characteristic	Patients (n=269)	Controls (n=234)	P-value
Male (%)	124 (46.1)	116 (49.6)	0.474
Age (years, mean ±SD)	62.22±11.90	59.55±11.75	0.012
tHcy (μmol/l, mean ±SD)	11.30±6.35	9.68±3.96	0.001
Hypertension (%)	142 (52.8)	118 (50.4)	0.655
Diabetes mellitus (%)	38 (14.1)	42 (17.9)	0.272
Hyperlipidemia (%)	88 (32.7)	43 (18.4)	0.000

tHcy, total plasma homocysteine; SD, standard deviation.

Results

The demographic characteristics of 269 SBI patients and 234 control patients are shown in Table I. Each population consists of 46.1 and 49.6% males, and the mean age of each population was 62.22±11.90 and 59.55±11.75 years, respectively. Between the two groups, there was no significant difference in gender composition but significant difference was detected in age composition, for which adjustment was made in the subsequent analyses (Table II). Nevertheless, there were no overall changes in values even after adjustments were made. Interestingly, as shown in Table I, we observed significant differences according to the levels of total plasma homocysteine and in hyperlipidemia patients, but no significant difference was observed in hypertension or diabetes mellitus (DM) patients.

We first investigated the *eNOS* gene -786T>C, 4a4b and 894G>T polymorphisms, the genotype distribution and allele frequency in SBI patients and controls are shown in Table II. The distribution of the *eNOS* -786T>C, 4a4b, and 894G>T genotypes and allele frequencies in patients and controls were compatible with Hardy-Weinberg's equilibrium. As for the *eNOS* 894G>T polymorphism, OR at 95% CI of 894GT and 894GT+TT were 2.00 (1.30-3.08) and 2.05 (1.34-3.16), respectively, showing significant differences, compared to 894GG. However, there were no significant differences in either -786T>C or 4a4ab polymorphisms between the SBI patient and control groups. We also examined the values of adjusted OR (AOR), according to age, gender, HTN, DM and hyperlipidemia. However, no significant differences were detected between AOR and crude OR (COR).

We next carried out combinatorial analysis on the effects of different genotypes in *eNOS* polymorphisms, i.e., *eNOS*

Table II. *eNOS* genotype distributions in SBI patients and controls.

Genotype	Patients (n=265)	Controls (n=232)	COR	(95% CI)	AOR	(95% CI)
-786TT (%)	218 (82.3)	193 (83.2)	1.00	-	-	-
-786TC (%)	43 (16.2)	39 (16.8)	0.98	(0.61-1.57)	0.78	(0.43-1.41)
-786CC (%)	4 (1.5)	0 (0.0)	-	-	-	-
-786TC+CC (%)	47 (17.7)	39 (16.8)	1.07	(0.67-1.70)	0.84	(0.47-1.51)
C allele	0.096	0.084				
4b4b (%)	214 (80.8)	181 (78.0)	1.00	-	-	-
4a4b (%)	43 (16.2)	41 (17.7)	0.89	(0.55-1.42)	0.60	(0.32-1.09)
4a4a (%)	8 (3.0)	0 (0.0)	-	-	-	-
4a4b+4a4a (%)	51 (19.2)	41 (17.7)	1.05	(0.67-1.66)	0.73	(0.41-1.30)
A allele	0.111	0.089				
894GG (%)	185 (69.8)	190 (81.9)	1.00	-	-	-
894GT (%)	78 (29.4)	40 (17.2)	2.00	(1.30-3.08)	2.27	(1.34-3.83)
894TT (%)	2 (0.8)	0 (0.0)	-	-	-	-
894GT+TT (%)	80 (30.2)	40 (17.2)	2.05	(1.34-3.16)	2.35	(1.39-3.96)
T allele	0.165	0.086				

Numbers of corresponding controls for -786T>C, 4a4b, 894G>T polymorphisms were 232, 222, and 230, respectively. Adjusted odds ratio (AOR) was made according to the combinations of age, gender, hypertension, DM and hyperlipidemia. COR, crude odds ratio; CI, confidence interval; DM, diabetes mellitus.

Table III. Combinatorial effects observed in SBI patients and controls, according to the genotypes in *eNOS* polymorphisms (*eNOS* -786T>C, 4a4b and 894G>T).

Genotype	Patients (%)	Controls (%)	COR	(95% CI)	AOR	(95% CI)
-786/4a4b	(n=263)	(n=220)				
TT/4b4b	211 (80.2)	178 (80.9)	1.00	-	-	-
TT/4a4b+4a4a	6 (2.3)	4 (1.8)	1.26	(0.35-4.56)	0.65	(0.15-2.90)
TC+CC/4b4b	1 (0.4)	1 (0.5)	0.84	(0.05-13.60)	0.81	(0.05-13.49)
TC+CC/4a4b+4a4a	45 (17.1)	37 (16.8)	1.03	(0.64-1.66)	0.72	(0.39-1.33)
-786/894	(n=263)	(n=230)				
TT/GG	152 (57.8)	155 (67.4)	1.00	-	1.00	-
TT/GT+TT	65 (24.7)	36 (15.7)	1.84	(1.16-2.93)	2.11	(1.21-3.70)
TC+CC/GG	31 (11.8)	35 (15.2)	0.90	(0.53-1.54)	0.68	(0.35-1.32)
TC+CC/GT+TT	15 (5.7)	4 (1.7)	3.82	(1.24-11.80)	3.59	(0.91-14.08)
4a4b/894	(n=263)	(n=220)				
4b4b/GG	149 (56.7)	143 (65.0)	1.00	-	1.00	-
4b4b/GT+TT	63 (24.0)	36 (16.4)	1.68	(1.05-2.69)	1.96	(1.11-3.47)
4a4b+4a4a/GG	34 (12.9)	37 (16.8)	0.88	(0.52-1.48)	0.59	(0.30-1.16)
4a4b+4a4a/GT+TT	17 (6.4)	4 (1.8)	4.08	(1.34-12.40)	3.80	(0.95-15.28)

Adjusted OR (AOR) was made according to the combinations of age, gender, hypertension, DM and hyperlipidemia. OR, odds ratio; CI, confidence interval; DM, diabetes mellitus; COR, crude OR.

-786T>C, 4a4b and 894G>T. When the effects of -786T>C and 4a4b polymorphisms were combined, significant differences were observed in the genotypes -786TT/894GT+TT (COR, 1.84; 95% CI, 1.16-2.93) and -786TC+CC/894GT+TT (COR, 3.82; 95% CI, 1.24-11.8), compared to those examined individually (Table III). A similar phenomenon was detected in the genotypes 4b4b/894GT+TT (COR, 1.68; 95% CI, 1.05-2.69) and 4a4b+4a4a/894GT+TT (COR, 4.08; 95% CI, 1.34-12.4) when the effects of 4a4b and 894G>T polymorphisms were combined (Table III). Interestingly, in the case of AOR, corresponding combined genotypes also exhibited increased values in both -786/894 (2.11 and 3.59) and 4a4b/894 (1.96 and 3.80) polymorphisms. However, with respect to the values for 95% CI in AOR, significance was observed in TT/GT+TT and 4a4b/GT+TT genotypes, but no significance was detected in TC+CC/GT+TT and 4a4b+4a4a/ GT+TT

Table IV. Frequencies of eNOS -786T>C, 4a4b and 894G>T haplotypes in SBI patients and controls.

eNOS -786/4a4b/894	Overall	Patients	Controls	P-value
T-4b-G	0.778	0.748	0.814	0.014
T-4b-T	0.116	0.138	0.091	0.024
C-4a-G	0.080	0.077	0.084	0.703
T-4a-G	0.013	0.016	0.009	0.327
C-4a-T	0.009	0.016	7.42E-7	0.008
C-4b-G	0.002	0.002	0.002	0.905
T-4a-T	0.002	0.003	0.0	0.263

genotypes. However, in the latter case, it was found that the values can be misleading, since AORs for individual variables were shown to be significant in most cases, with a few exceptions (data not shown). Notably, it was shown that the 894GT+TT genotype may confer synergistic effects in OR values when combined with -786TC+CC (COR, 1.07→3.82; AOR, 0.84→3.59) or 4a4b+4a4a (COR, 1.05→4.08; AOR, 0.73→3.80) genotypes, respectively (Tables II and III).

Since multiple loci may affect the functions of the *eNOS* gene, it will be important to perform a haplotype analysis, which would allow us to understand the effects of allelic changes on the *eNOS* gene simultaneously. There are six alleles in the *eNOS* gene, i.e., -786(T or C), Intron 4(4a or 4b), 894(G or T), and the combination of each allele can constitute eight haplotypes in theory. However, we detected only seven haplotypes in this study (Table IV). According to the hyplotype analysis, there were significant differences between SBI and control patients in three haplotypes, as follows: T-4b-G (p=0.014), T-4b-T (p=0.024) and C-4a-T (p=0.008). Among them, it is not surprising, the normal genotype, T-4b-G, displays a higher frequency in the control group.

Discussion

In recent years, clinical significance of SBI has had much attention, but the details of its pathogenesis and epidemiology are relatively unknown. At the genetic level, only a few genes have been reported as risk factors for SBI to date, which include apolipoprotein (a), methylenetetrahydrofolate reductase (MTHFR) angiotensinogen (AGT) and angiotensin II type 1 receptor (AT1R) (23,24,29). We have recently studied the role of angiotensin-converting enzyme (ACE) polymorphism in the development of stroke and SBI, and found that it is associated with stroke but not with SBI (25).

In the present study, we demonstrate that the polymorphisms and haplotypes of the endothelial nitric oxide synthase (*eNOS*) gene are involved in the development of SBI at a statistically significant level. It is well known that eNOS has various vascular functions, including maintenance of basal cerebral blood flow, cerebral vasodilation, vascular integrity and the inhibition of smooth muscle proliferation (11-13). Therefore, it is not surprising that the aberration of eNOS function can lead to critical vascular diseases, such as atherosclerosis, myocardial and acute cerebral infarction (14-18). As for the cerebrovascular diseases, it has been previously reported that *eNOS* polymorphism is associated with cerebral infarction and small vessel diseases (14-16). Scarce previous knowledge exists whether it is related to SBI, another important type of cerebrovascular disease, but hard to diagnosed. In the present study, we carefully selected the SBI patients, according to the results of brain MRI and neurological examination.

According to our analysis, among three polymorphisms in the *eNOS* gene (-786, 4a4b and 894), 894G>T polymorphism was shown to give a rise to the highest level of SBI risk (Table II). To coincide with our findings, it has been previously reported that 894G>T polymorphism alone acts as a risk factor for brain infarction, especially in the lacunar type (16). Therefore, it is conceivable to propose that 894G>T polymorphism in *eNOS* gene may be involved in various types of cerebrovascular diseases. Interestingly, Hassan *et al* (14) previously reported a significant association between the *eNOS* 4a4b polymorphism and small vessel disease, but in the current study we showed that 894G>T polymorphism is associated with SBI. To address the relationship between small vessel disease and SBI with respect to *eNOS* polymorphism, further studies using larger sample populations will be needed.

In addition, we found that the prevalence of SBI increased significantly when either -786T>C or 4a4b polymorphism was combined with 894G>T polymorphism (Table III), indicating that three different polymorphic loci in the *eNOS* gene (-786T>C, 4a4b and 894G>T) play interactively, thereby leading to synergistic effects for the generation of SBI. Lastly, we carried out haplotype analysis, in which significant differences were detected between SBI patients and control in three haplotypes (Table IV).

Taken together, the present study provides the first evidence that *eNOS* polymorphisms and haplotypes are closely associated with the development of SBI. However, further studies using larger and heterogeneous populations would be of a great value to firmly validate their association with SBI, which will, in turn, provide a basis for developing a useful tool for diagnosing patients who have risk of developing SBI.

Acknowledgements

This work was partly supported by the Korea Research Foundation grant from the Korean Government (MOEHRD) (KRF-2008-521-E00121) and partly supported by Priority Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0093821). The authors report no conflict of interest.

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