The relationships between endothelial nitric oxide synthase polymorphisms and the formation of intracranial aneurysms in the Korean population

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Object. Some genetic factors are known to be associated with the formation of cerebral aneurysms in the Caucasian population. One of these factors is endothelial nitric oxide synthase (eNOS) gene polymorphisms. Endothelial nitric oxide synthase genes encode eNOS, which synthesizes NO from L-arginine. There continues to be controversy about the relationships between eNOS gene polymorphisms and the formation of intracranial aneurysms. In this study, the authors evaluated these relationships in the Korean population.

Methods. Three eNOS polymorphisms (eNOS 27VNTR, T786C, and G894T) were genotyped in 96 patients with ruptured aneurysms, 53 patients with unruptured aneurysms, and in 121 volunteers via polymerase chain reaction-restriction fragment length polymorphism analysis.

Results. The mean ages of the patients and healthy volunteers were 52.9 ± 12.3 years and 55.2 ± 9.1 years, respectively. The patient group was composed of 56 men and 93 women, and the healthy volunteer group was composed of 46 men and 75 women. Only the incidence of smoking history was significantly higher in the patient group than in the control group (p = 0.001). The genotypic frequencies for the 3 eNOS gene polymorphisms were in agreement with those predicted by Hardy-Weinberg equilibrium. There were no significant associations between the eNOS recessive models and the formation of an aneurysm. The authors found no genotypic differences between similar races among patients with aneurysms.

Conclusions. The present study shows that eNOS 27VNTR, T786C, and G894T polymorphisms cannot be used as indicators of the formation of intracranial aneurysms in Korean patients. To confirm these findings an additional analyses might need to be performed using a larger sample size. There were no differences in the genotypic distributions and allelic frequencies between similar races among patients with aneurysms, which were the same in previously reported normal populations. (DOI: 10.3171/2011.2.FOCUS10227)

Key Words • cerebral aneurysm • 27VNTR polymorphism • T786C polymorphism • G894T polymorphism • endothelial nitric oxide synthase

The incidence of subarachnoid hemorrhages due to ruptured cerebral aneurysms is 10 to 11 per 100,000 people per year. The prevalence of unruptured cerebral aneurysms has increased due to the development of diagnostic imaging techniques such as MR angiography, and it has been reported to be more than 2% of the population.32 The hemodynamic mechanisms of aneurysmal formation are well known, and aneurysms located in the posterior circulation as well as larger aneurysms are known to be more prone to rupture than others.32 In addition, there are some reports about environmental and genetic factors associated with the formation, growth, and rupture of cerebral aneurysms. Endothelial nitric oxide synthase gene polymorphisms are among the genetic factors known to be associated with aneurysms.

Nitric oxide is synthesized by the catalyzing action of the NOS family enzymes via the conversion of L-arginine, nicotinamide adenine dinucleotide phosphate, and O2 to NO and citrulline.29 Nitric oxide was originally known as “endothelial-derived relaxing factor,” which is responsible for the potent vasodilating properties of stimulated endothelia.29 Since then, NO has been widely implicated as a biological mediator of activities ranging from neuronal function to immune system regulation, including relaxation of the vascular smooth muscle, vasodilation, maintenance of the vessel wall geometry,24 inhibition of vascular smooth muscle cell proliferation and platelet and monocyte adhesion,41 and protection from vascular dis-
ease.9 Therefore, altered NO levels have been reported to be related to many conditions and activities such as sepsis,26 reproduction,39 infection,4 hypertension,22 exercise,22 diabetes,44 hypoxia,23 and cancer.57 In particular, reduced NO levels can result in cardiovascular disease, carotid atherosclerosis, hypertension, and aortic aneurysm.27

There are 3 types of NOS: neuronal (nNOS/NOS1), endothelial (eNOS/NOS3), and inducible (iNOS/NOS2).2 Neuronal NOS is mainly expressed in neurons of the central31 and peripheral nervous systems, but can also be found in other cells such as skeletal muscle myocytes,30 lung epithelial cells,1 and skin mast cells.36 Endothelial NOS is mainly expressed by endothelial cells and can be found in other cells such as skeletal muscle myocytes,30 dermal fibroblasts,40 epidermal keratinocytes,36 thyroid follicular cells,5 hepatocytes, and smooth muscle cells.4 Inducible NOS is expressed in many cell types, including chondrocytes,22 epithelial cells,2 hepatocytes,11 glial cells, and some types of immune cells.4 Endothelial NOS and nNOS are normally expressed and regulated by Ca2+/calmodulin, whereas iNOS is relatively insensitive to Ca2+ and is usually expressed by endotoxin and inflammatory cytokines.2

Endothelial NOS is encoded by eNOS genes on chromosome 7q35–36. Polymorphisms of the eNOS genes may alter the structure and stability of the eNOS enzyme42 or modify the interactions of eNOS genes with other components.32 These alterations can result in decreased expression or activities of the eNOS enzyme and eventually decrease NO production.39 Polymorphisms of eNOS genes might be related to the formation or rupture of intracranial aneurysms because decreased NO production can result in cardiovascular disease and carotid atherosclerosis. However, there is still some controversy about the relationships between eNOS gene polymorphisms and plasma NO levels. Veldman et al.39 reported that the G894T eNOS gene polymorphism, a transversion of guanine (G) to thymine (T) at a locus 894 base pairs in exon 7 of the eNOS gene, is associated with reduced basal NO production and might have functional implications in the development of atherosclerosis or hypertension. Tsukada et al.38 reported that the 27VNTR eNOS gene polymorphism, a 27-base pair variable-number-tandem-repeat polymorphism in intron-4 of the eNOS gene, had a strong association with plasma NO metabolites. On the other hand, Moon et al.42 indicated that there was no substantial effect of the G894T eNOS gene polymorphism on the variance of plasma NO metabolites in a healthy Korean population. Schneider et al.45 reported no biological effect of the G894T eNOS gene polymorphism on the cardiovascular system through its influence on endothelium-dependent vasodilation.

Three types of eNOS genes were recently studied: 1) eNOS 27VNTR,41 2) eNOS T786C, a substitution of thymine (T) to cytosine (C) at a locus 786 base pairs upstream of the eNOS gene;23 and 3) eNOS G894T.43 The relationship between the formation of an intracranial aneurysm and eNOS gene polymorphisms is controversial. This study examined 3 eNOS gene polymorphisms in patients with cerebral aneurysms (including both RAs and URAs), and volunteers. The aim of this study was to evaluate the relationships between the formation of an aneurysm and eNOS gene polymorphisms in the Korean population.

Methods

Study Participants

The study population consisted of 96 patients with ruptured subarachnoid hemorrhages, 53 patients with URAs, and 121 healthy volunteers. The patients were enrolled in the neurosurgery department at Bundang CHA Hospital between May 2008 and April 2010. The diagnosis of an RA was based on each patient’s history and radiological findings, such as brain CT and conventional angiography. A diagnosis of a URA was based on the radiological findings, such as MR angiography or conventional angiography. The 121 volunteers were healthy individuals who visited the Bundang CHA General Hospital for a health examination and had no history of cerebrovascular disease. The volunteers all underwent MR angiography, which revealed no cerebrovascular disease in any of the volunteers. The institutional review board of Bundang CHA General Hospital approved this study. All study participants were Korean, and each participant provided informed consent before being enrolled (Table 1).

Genetic Analysis

A single blood sample was collected from patients with RAs, patients with URAs, and healthy controls. Genomic DNA was extracted from peripheral blood leukocytes using the G-DEX blood extraction kit (Intron). The nucleotide changes were determined by PCR-restriction fragment length polymorphism analysis using the isolated genomic DNA as a template.

Endothelial NOS 27VNTR Polymorphism. The primer sequences used to detect the eNOS intron 4 polymorphism were 5'-AGGCCCTATGTAGTGCTTTT-3' (sense) and 5'-TCTCTTTAGTGCTGTGGTCAC-3' (antisense). The PCR reaction was performed in a 20-μl reaction volume including 100 ng of genomic DNA, 10 pmol of each primer, and 10 μl of HotStarTaq master mix (Qiagen). The PCR reaction began with an initial denaturation step for 15 minutes at 95°C. This step was followed by 35 cycles at 94°C for 1 minute, 49°C for 40 seconds, and 72°C for 40 seconds, with a final extension at 72°C for 7 minutes. The PCR products were separated by electrophoresis (Mupid-2Plus) on a 3% agarose gel stained with ethidium bromide. Fragments of 393 bp and 420 bp corresponded to the eNOS alleles 4a and 4b, respectively.

Endothelial NOS T786C Polymorphism. The PCR was performed using primers 5'-ATGCTCCCACCGG GCATCA-3' (sense) and 5'-TCTCTTTAGTGCTGTGTCAC-3' (antisense). A 236-bp fragment was amplified by PCR in a final volume of 20 μl containing 100 ng of genomic DNA, 10 pmol of each primer, and AccuPower Hot-Start PCR Premix (Bioneer). Amplification was performed in 35 cycles consisting of denaturation (94°C, 30 seconds), annealing (51°C, 40 seconds), and extension (72°C, 40 seconds). The 236-bp PCR products were digested with 5 units of the restriction enzyme NgoMIV (New England Biolabs Inc.) at 37°C for 16 hours, producing fragments of...
Table 1: Demographic and clinical characteristics of the patients with aneurysms and healthy controls

<table>
<thead>
<tr>
<th>Variable</th>
<th>RA &amp; URA</th>
<th>RA</th>
<th>URA</th>
<th>Controls</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>no. of patients</td>
<td>149</td>
<td>96</td>
<td>53</td>
<td>121</td>
<td></td>
</tr>
<tr>
<td>mean age ± SD</td>
<td>52.9 ± 12.3</td>
<td>51.2 ± 12.7</td>
<td>55.8 ± 10.9</td>
<td>55.2 ± 9.1</td>
<td>0.07</td>
</tr>
<tr>
<td>male (%)</td>
<td>56 (37.6)</td>
<td>39 (40.6)</td>
<td>17 (32.1)</td>
<td>46 (38.0)</td>
<td></td>
</tr>
<tr>
<td>female (%)</td>
<td>93 (62.4)</td>
<td>57 (59.4)</td>
<td>36 (67.9)</td>
<td>75 (62.0)</td>
<td>0.942</td>
</tr>
<tr>
<td>smoking (%)</td>
<td>40 (26.8)</td>
<td>30 (31.2)</td>
<td>10 (18.9)</td>
<td>13 (10.7)</td>
<td>0.001*</td>
</tr>
<tr>
<td>hypertension (%)</td>
<td>76 (51.0)</td>
<td>44 (45.8)</td>
<td>32 (60.4)</td>
<td>60 (49.6)</td>
<td>0.816</td>
</tr>
<tr>
<td>diabetes (%)</td>
<td>11 (7.4)</td>
<td>7 (7.3)</td>
<td>4 (7.5)</td>
<td>20 (16.5)</td>
<td>0.019*</td>
</tr>
</tbody>
</table>

* Statistically significant (patients vs controls).

Statistical Analysis

The demographics are reported as the mean ± SD for continuous variables and as percentages of the totals for the categorical variables. The univariate relationships between the demographic variables and conditions were assessed using an independent samples t-test for the continuous variables and Pearson chi-square test for the categorical variables. The genotypic distribution and allelic frequencies were assessed using the Pearson chi-square test. The allelic frequency distribution at each polymorphism locus was tested against Hardy-Weinberg equilibrium under the Mendelian biallelic expectation by performing the Pearson chi-square test. Multivariate associations were assessed using logistic regression analysis for the categorical variables. All data were analyzed using SPSS for Windows, version 11.0 (SPSS). A probability value < 0.05 was considered significant.

Results

Table 1 describes the demographic and clinical characteristics of the 149 patients with aneurysms (96 with RAs, 53 with URAs) and 121 healthy controls. The mean ages of the patients and controls were 52.9 ± 12.3 years and 55.2 ± 9.1 years, respectively. The patient group was composed of 56 men and 93 women, and the control group was composed of 46 men and 75 women. Comparisons of age, sex, and hypertension revealed no significant differences between the patient and control groups. The incidence of smoking history was significantly higher in the patient group than the control group (p = 0.001), and the incidence of diabetes was significantly higher in the control group than in the patient group (p = 0.019).
There were no significant associations between the eNOS recessive models and the formation of an aneurysm. This was the same for all 3 genes (eNOS T786C, p = 0.99; eNOS 27VNTR, p = 0.643; eNOS G894T, p = 0.532) and for all 3 alleles (T allele, p = 0.996; b allele, p = 1.000; G allele, p = 0.996).

The adjusted ORs for vascular risk factors (age, sex, smoking, hypertension, diabetes) and eNOS gene polymorphisms with regard to aneurysm formation were obtained (Table 3). Smoking history (OR 3.464, p = 0.001) and no history of diabetes (OR 0.393, p = 0.025) were significantly associated with the formation of an aneurysm. The other factors, including eNOS gene polymorphisms, were not associated with the formation of an aneurysm (Table 3).

In normal populations, genotypic and allelic differences between races have been examined.1,16 This study compared the genotypic and allelic differences between Asian and Caucasian patients with aneurysms (Table 4). The American (a) group was composed of patients from the Mayo Stroke Center, Mayo Clinic, in Rochester, Minnesota, and the American (b) group was composed of patients from the University of California, San Francisco. There were no significant differences in the distribution of the eNOS T786C, 27VNTR, and G894T genes between Korean and Japanese patients (p = 0.882, 0.895, and 0.18, respectively), but there were significant differences between these 3 distributions in Korean and American (a) patients (p = 0, 0.002, and 0, respectively), Koreans and American (b) patients (p = 0, 0.027, respectively), and Koreans and Germans (p = 0, 0.032, and 0, respectively). There were even some differences between American (a) and German patients (p = 0.065, 0.439, and 0.017, respectively) and American (b) and German patients (p = 0.001, 0.001, and 0, respectively).

### Discussion

Despite recent medical developments, approximately 50% of patients with a ruptured cerebral aneurysm die or become severely disabled, and the cost of treatment is very high. If a URA can be detected before rupture, the patient can be treated more safely with a better prognosis and at a lower cost. Recently, diagnostic imaging techniques, such as CT angiography or MR angiography, have been developed, and a URA can be detected more easily. However, these imaging studies are relatively difficult and expensive.

There is some controversy about the relationships between the formation of an intracranial aneurysm and

### TABLE 3: Adjusted ORs for vascular risk factors and eNOS gene polymorphisms in relation to aneurysm formation

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR (95% CI)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>age</td>
<td>0.659 (0.388–1.121)</td>
<td>0.124</td>
</tr>
<tr>
<td>sex</td>
<td>1.409 (0.788–2.519)</td>
<td>0.248</td>
</tr>
<tr>
<td>smoking</td>
<td>3.464 (1.622–7.396)</td>
<td>0.001*</td>
</tr>
<tr>
<td>hypertension</td>
<td>1.41 (0.829–2.398)</td>
<td>0.205</td>
</tr>
<tr>
<td>diabetes</td>
<td>0.393 (0.173–0.892)</td>
<td>0.025*</td>
</tr>
<tr>
<td>eNOS 27VNTR recessive model (4b4b vs 4a4b+4a4a)</td>
<td>no frequency difference</td>
<td>0.999</td>
</tr>
<tr>
<td>eNOS T786C recessive model (TT vs TC+CC)</td>
<td>no frequency difference</td>
<td>0.999</td>
</tr>
<tr>
<td>eNOS G894T recessive model (GG vs GT+TT)</td>
<td>0.832 (0.429–1.611)</td>
<td>0.585</td>
</tr>
</tbody>
</table>

* Statistically significant.

### TABLE 4: Genotypic and allelic differences of eNOS gene polymorphisms in patients with aneurysms according to race

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Korean (149 patients)</th>
<th>Japanese* (405 patients)</th>
<th>American (a)† (107 patients)</th>
<th>American (b)‡ (319 patients)</th>
<th>German§ (142 patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td>786TT (%)</td>
<td>122 (81.9)</td>
<td>326 (80.5)</td>
<td>50 (46.7)</td>
<td>167 (52.4)</td>
<td>48/135 (35.6)</td>
</tr>
<tr>
<td>786TC (%)</td>
<td>24 (16.1)</td>
<td>72 (17.8)</td>
<td>46 (43.0)</td>
<td>119 (37.3)</td>
<td>60/135 (44.4)</td>
</tr>
<tr>
<td>786CC (%)</td>
<td>3 (2.0)</td>
<td>7 (1.7)</td>
<td>11 (10.3)</td>
<td>33 (10.3)</td>
<td>27/135 (20)</td>
</tr>
<tr>
<td>T allele frequency</td>
<td>0.9</td>
<td>0.89</td>
<td>0.68</td>
<td>0.71</td>
<td>0.58</td>
</tr>
<tr>
<td>4b4b (%)</td>
<td>122 (81.9)</td>
<td>325 (80.2)</td>
<td>68 (63.6)</td>
<td>141/236 (59.7)</td>
<td>98 (69.0)</td>
</tr>
<tr>
<td>4a4b (%)</td>
<td>24 (16.1)</td>
<td>70 (17.3)</td>
<td>38 (35.5)</td>
<td>63/236 (26.7)</td>
<td>41 (28.9)</td>
</tr>
<tr>
<td>4a4a (%)</td>
<td>3 (2.0)</td>
<td>10 (2.5)</td>
<td>1 (0.9)</td>
<td>32/236 (13.6)</td>
<td>3 (2.1)</td>
</tr>
<tr>
<td>b allele frequency</td>
<td>0.9</td>
<td>0.89</td>
<td>0.81</td>
<td>0.73</td>
<td>0.84</td>
</tr>
<tr>
<td>894GG (%)</td>
<td>125 (83.9)</td>
<td>349 (86.2)</td>
<td>67 (62.6)</td>
<td>184/245 (75.1)</td>
<td>64 (45.1)</td>
</tr>
<tr>
<td>894GT (%)</td>
<td>24 (16.1)</td>
<td>50 (12.3)</td>
<td>32 (29.9)</td>
<td>53/245 (21.6)</td>
<td>67 (47.2)</td>
</tr>
<tr>
<td>894TT (%)</td>
<td>0</td>
<td>6 (1.5)</td>
<td>8 (7.5)</td>
<td>8/245 (3.3)</td>
<td>11 (7.7)</td>
</tr>
<tr>
<td>G allele frequency</td>
<td>0.92</td>
<td>0.92</td>
<td>0.78</td>
<td>0.86</td>
<td>0.69</td>
</tr>
</tbody>
</table>

* From data by Krischek et al., 2006.
† From data by Khurana et al., 2005. American (a) group composed of patients from the Mayo Stroke Center, Mayo Clinic, Rochester, Minnesota.
‡ From data by Ko et al., 2008. American (b) group composed of patients from the University of San Francisco, California.
§ From data by Krex et al., 2006.
eNOS gene polymorphisms. In a recent study, Khurana et al. found the following positive correlations: between the eNOS 27VNTR polymorphism and the formation of a cerebral aneurysm, between the eNOS T786C polymorphism and cerebral vasospasm after an aneurysmal subarachnoid hemorrhage in Caucasian patients, and between eNOS polymorphisms and a tendency for aneurysmal rupture. Ozum et al. suggested that the G894T polymorphism of the eNOS gene has a positive relationship with the formation of an intracranial aneurysm. On the other hand, Krischek et al. reported no relationship between eNOS polymorphisms and rupture of a cerebral aneurysm in Japanese patients. Moreover, Akagawa et al. reported the same findings of negative association between the T786C polymorphism of the eNOS gene and the formation of an intracranial aneurysm.

In this study, we could not find any evidence of a positive relationship between 3 eNOS gene polymorphisms and the formation of an intracranial aneurysm. The same results were obtained in the relationships between the allelic frequencies and the formation of an intracranial aneurysm. Our study group was relatively small (149 patients with aneurysms and 121 healthy controls). Therefore, additional studies with a larger study group will be needed to confirm the relationship between the genotypic distribution of eNOS gene polymorphisms and sex in the patient group. Univariate and multivariate logistic regression analyses revealed a relationship between smoking history and the formation of an intracranial aneurysm. This is consistent with previous reports showing that smoking is related to the formation of an aneurysm. In our study, diabetes history had a negative relationship with the formation of an intracranial aneurysm, which is not consistent with previous reports showing that diabetes is not related to the formation of an aneurysm. We cannot explain the reason of a negative relationship between diabetes history and the formation of an aneurysm. One possible cause is the small number of patients and controls with diabetes history. In a larger study, a positive relationship might be revealed.

This study compared the genotypic and allelic differences in aneurysmal patients of different races. The outcome was similar to the previously reported findings in control populations. There were no racial differences in the eNOS gene polymorphism distributions in similar races, such as Korean and Japanese patients. However, there were racial differences in different races, such as Korean and American or Korean and German patients. There were even some racial differences between American (b) and German patients. This is because the American (b) group included a variety of races such as Latinos, Asians, and African Americans. These racial differences with regard to the distribution of eNOS gene polymorphisms might be related to differences in the incidence and prevalence of intracranial aneurysms. Therefore, if the genotypic distribution and allelic frequencies are studied relative to the prevalence and incidence of intracranial aneurysms, it might be possible to identify relationships between polymorphisms of the eNOS gene and the formation of an intracranial aneurysm.

Conclusions

Endothelial NOS 27VNTR, T786C, and G894T polymorphisms cannot be used as indicators of the formation of intracranial aneurysms in Korean patients. However, our study group was relatively small. Therefore, further analysis with a larger group will be needed to confirm the relationship between the genotypic distribution of the eNOS gene polymorphisms and aneurysmal formation. There were no differences in the genotypic distributions and allelic frequencies between similar races in aneurysmal patients, whereas there were some differences between different races, which were demonstrated previously in normal populations.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: TG Kim, Baek. Acquisition of data: TG Kim, NK Kim, Huh. Analysis and interpretation of data: TG Kim. Drafting the article: TG Kim. Statistical analysis: TG Kim. Administrative/technical/material support: Huh, Chung, Choi. Study supervision: Yu.

References


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