Endothelial Nitric Oxide Synthase T-786C Single Nucleotide Polymorphism

A Putative Genetic Marker Differentiating Small Versus Large Ruptured Intracranial Aneurysms

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Background and Purpose—Anecdotal evidence exists for at least 2 subpopulations of intracranial saccular aneurysms, namely, those that may form rapidly and rupture when small versus those that enlarge slowly and may rupture particularly when ≥10 mm in diameter. We sought to determine whether the endothelial nitric oxide synthase (eNOS) T-786C single nucleotide polymorphism (SNP), implicated in cardiovascular disease susceptibility, could facilitate differentiation between small (<5 mm) versus large (≥10 mm) ruptured aneurysms.

Methods—In accordance with institutional guidelines, clinical data were recorded prospectively and genomic DNA was isolated from blood samples obtained from 52 aneurysmal subarachnoid hemorrhage (SAH) patients (cases) and 90 randomly selected controls. Samples were assayed for eNOS gene promoter T-786C SNP with the use of gene microarray technology. Statistical analyses included multiple logistic regression.

Results—Although there was no difference in genotype distributions between cases and controls, all 13 patients with large aneurysms were (T/C) heterozygous for the polymorphism, while 9 of 22 patients (41%) with small aneurysms were (T/T) or (C/C) homozygous (P = 0.01). The mean (±SD) ruptured aneurysm diameter among all heterozygotes (8.5 ± 5.2 mm) was significantly greater than that for (C/C) (7.0 ± 3.3 mm) or (T/T) (4.7 ± 1.8 mm) homozygotes (P = 0.04). With the use of multivariate analysis, heterozygosity remained significantly associated with aneurysm size ≥10 mm (P = 0.03).

Conclusions—The eNOS T-786C SNP distinguishes genetically between small and large ruptured aneurysms. Although not predictive of SAH in the population at large, our data suggest that among persons with known intracranial aneurysms, eNOS T-786C genotype may be a factor influencing the size at which an aneurysm ruptures, a finding that should be taken into consideration along with other anatomic features of the aneurysm. (Stroke. 2003;34:2555-2559.)

Key Words: aneurysm, ruptured • genetics • intracranial aneurysm • nitric oxide synthase • polymorphism • subarachnoid hemorrhage

Seminal studies by Wiebers et al1,2 reported that during a mean follow-up of 8.3 years, no ruptures occurred in 102 asymptomatic intracranial aneurysms <10 mm in diameter compared with 15 ruptures among 51 aneurysms ≥10 mm in size at the time of diagnosis. This finding was further substantiated by the International Study of Unruptured Intracranial Aneurysms (ISUIA),3 which observed that among patients without prior subarachnoid hemorrhage (SAH), the rupture rate of aneurysms <10 mm at diagnosis was <0.05%/y, compared with a rate of approximately 1%/y for aneurysms ≥10 mm and 6%/y for aneurysms ≥25 mm in diameter. Therefore, ISUIA presented convincing evidence that aneurysm size at the time of diagnosis was an independent predictor of rupture risk and that aneurysm represented a critical size beyond which the rupture risk significantly increased.3 Much of the controversy pertaining to these studies4–5 has centered on the fact that the vast majority of ruptured aneurysms are <10 mm in diameter at the time of diagnosis and treatment,4–9 reinforcing the notion that aneurysm size alone should not be used as the sole criterion guiding the decision to treat an unruptured aneurysm. Hypotheses proposed to explain this discrepancy include the possibility that there are 2 distinct subpopulations of intracranial aneurysms: one comprising aneurysms that develop relatively rapidly and rupture when <10 mm in diameter (ie, the variety that tends to be seen in the emergency department setting) and the other comprising aneurysms that enlarge slowly, are amenable to being studied over months or years by serial imaging, and are more prone to rupture when ≥10 mm in diameter (ie, those seen and followed in the clinic. 

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setting. To date, evidence for 2 subpopulations has been, at best, circumstantial. One reason for this is that widespread population serial radiological screening would be required to detect aneurysms in the former subpopulation, a largely impractical task. Given the mounting evidence that genetic factors may play a role in intracranial aneurysm formation and rupture, the key questions that arise are as follows: can genetic technologies be applied to detect different subpopulations of intracranial aneurysms, and can such information be used to improve aneurysm patient care?

With the completion of the Human Genome Project, attention is now focused on the field of functional genomics, whose principal aim is to establish links between genes, the proteins they encode, and the molecular pathogenesis of human disease. With regard to the cardiovascular system, a key molecule involved in vasomotor function and structural remodeling is nitric oxide (NO). This molecule, which in normal blood vessels is derived principally from the endothelial isoform of nitric oxide synthase (eNOS), exhibits considerable cross talk with other molecular systems. The eNOS gene, located on chromosome 7q35-36, has recently been demonstrated to be functionally polymorphic, ie, subject to relatively frequent focal genetic alterations that lead to increased cardiovascular disease susceptibility.

One such polymorphism, a single nucleotide polymorphism (SNP), was identified by Nakayama et al in the eNOS promoter region. This SNP, involving a substitution of the nucleotide thymine (T) to cytosine (C) at a locus 786 base pairs upstream of the eNOS gene, is referred to as the eNOS T-786C SNP and is associated with increased susceptibility to coronary vasospasm in homozygotes (CTC) and heterozygotes (TTC), ie, individuals expressing the mutant allele (containing C).

Given the critical role played by NO in vascular function and remodeling, including aneurysm pathobiology, and the circumstantial evidence suggesting the presence of different subpopulations of intracranial aneurysms, we hypothesized that a polymorphism such as eNOS T-786C SNP, implicated in cardiovascular disease susceptibility, may be used to genetically distinguish between small versus large ruptured intracranial aneurysms.

Subjects and Methods

Human Subjects

This prospective case-control study, approved by our institutional review board, involved 142 human subjects. There were 52 patients (cases) admitted to our hospital between December 2001 and February 2003 diagnosed with aneurysmal SAH on the basis of clinical and radiological findings (including cerebral angiography in each case) and 90 controls selected randomly from a countywide registry. After receipt of informed consent, a single 20-mL sample of venous blood was obtained from all participants for subsequent DNA extraction and genetic analysis. Severity of SAH on admission CT scan was classified according to the grading system of Fisher (Fisher grades 1 to 4). Severity of SAH on admission clinical examination was classified according to the World Federation of Neurological Surgeons (WFNS) grading system (WFNS grades 1 to 5). Patients were managed in our Neurological-Neurosurgical Intensive Care Unit according to established clinical protocols.

Polymerase Chain Reaction and Gene Microarray Technology

Genomic DNA was extracted from peripheral blood lymphocytes with the QIAamp DNA Blood Minikit (Qiagen). As detailed by Sohni et al, the polymerase chain reaction (PCR) was optimized for the eNOS T-786C SNP, which was genotyped with the use of active electronic microarrays. Oligo 6.61 software was used to design PCR primers (IDT) on the basis of GenBank sequences. Primer sequences were 5′-GCATGCACCTCTGGCCTGAAGT-3′ (forward) and 5′-CAGGAAGCTGCCCTCCAGTGC-3′ (reverse). The forward primer was biotinylated to permit capture of biotinylated amplicons to streptavidin embedded in the microarray permeation layer. The thermal cycled PCR mixture consisted of 25 μL AmpliTaq Gold Master Mix (Applied Biosystems), 1 μmol/L primers, 20 ng DNA template, and water to 50 μL. PCR products were desalted with the use of MultiScreen PCR plates (Millipore) and resuspended in water to obtain a concentration within the range of 5 to 40 nmol/L. A mapping protocol was used to electronically address biotinylated amplicons to user-designated sites onto the microarray (NanoChip, Nanogen). Instrument software was used to create a map to address the amplicons to designated sites on the chip array. The reporter probe oligonucleotides were designed with the SNP at the 3′ terminal base and a fluorophore at the 5′ end. The wild-type reporter probe (5′-AGGGTCAGCCA-3′) was labeled with Cy3, and the probe for the variant allele (5′-GGTGTCAGCCG-3′) was labeled with Cy5 (IDT) in all cases. The SNP reporting mixture consisted of 500 nmol/L of each reporter probe, 250 nmol/L of anchor stabilizer oligonucleotide (5′-GCCAGGGAAGGCTGATGCCCTGAGGGTGAGC-3′), and a high-salt buffer in a final volume of 60 μL. Stabilizer and reporter oligonucleotides were complementary to the biotinylated amplicon strand. Temperature was used to discriminate between matched and mismatched reporter probes, and microarrays were imaged with the use of separate lasers for both Cy3 and Cy5 in the instrument reader. Homozygous wild-type alleles hybridized with Cy3-labeled reporter probe, while homozygous variant alleles hybridized with Cy5-labeled reporter probes. Heterozygous complexes hybridized with both labeled probes. Known heterozygotes, verified by dye-terminator sequencing performed on ABI-377 DNA sequencers in both forward and reverse directions, were used to normalize hybridization efficiency between dye-labeled reporters. Genotypes were designated on the basis of manufacturer-recommended biallelic fluorescence intensity ratio (a biallelic fluorescence intensity ratio ≥1.3 was defined as heterozygous, and a ratio ≤1.5 was defined as homoygous). No genotype designations were made for fluorescence intensity ratios between 1.3 and 1.5.

Data Analysis

Where specified, data are expressed as mean±SD or as percentage of column totals. Ruptured aneurysms were divided by size (ie, maximum diameter measured independently by neuroradiologists using digitized angiography software) into 3 groups chosen in the context of published aneurysm natural history studies: small (<5 mm), medium (6 to 9 mm), and large (≥10 mm). Comparisons between aneurysm size group and either WFNS (clinical) grade or Fisher (CT) grade were made with the use of the Fisher exact test. Genotypes of eNOS T-786C SNP were either homozygous for the nucleotide thymine (TTT) or cytosine (CTC) or heterozygous (TTC). In comparing cases and controls, the probability value for age was calculated by 1-way ANOVA, while probability values for sex, medical history variables, and eNOS T-786C SNP genotypes were determined with the χ² test. Among cases, comparisons of genotypes versus ruptured aneurysm size group were made with the Fisher exact test, while comparisons of genotypes versus mean ruptured aneurysm diameter were made with 1-way ANOVA. Hardy-Weinberg equilibrium was tested by comparing observed versus expected genotype frequencies for the controls via a χ² test. An exact multiple logistic regression analysis controlling for the variables age, sex, and smoking status was used to determine any association between eNOS T-786C genotype and aneurysm size. Probability values of <0.05 were regarded as statistically significant.

Results

Clinical Data

Compared with controls, cases were significantly younger and had a greater preponderance of women and smokers.
More than 75% of patients were admitted in good neurological grade (ie, WFNS grade 1 or 2), and 50% of patients presented with Fisher grade 3 of SAH (Table 2). No significant difference was found in either Fisher CT grade ($P=0.55$) or WFNS grade ($P=0.57$) among the 3 groups classified according to aneurysm size.

### Genetic Data

Among controls, the frequencies of eNOS T-786C SNP genotypes were in agreement with those predicted by the Hardy-Weinberg equilibrium. There was no significant difference in the distribution of eNOS T-786C SNP genotypes between cases and controls (Table 1). Of the 52 patients, 13 had large ruptured aneurysms. All 13 of these patients were heterozygous for the eNOS T-786C SNP. This was significantly different from the 22 patients with small aneurysms, 9 (41%) of whom were homozygous ($P=0.01$; Table 3). All 17 homozygotes in this study had ruptured aneurysms $\geq 10$ mm in maximum diameter. On the basis of a multiple logistic regression analysis controlling for age, sex, and smoking status, the eNOS heterozygous genotype remained significantly associated with aneurysm size $\geq 10$ mm in diameter ($P=0.03$). Of the 52 SAH patients, the ruptured aneurysm mean diameter among heterozygotes was $8.5 \pm 5.2$ mm ($n=35$). This was significantly larger than the ruptured aneurysm mean diameter among $T/T$ (4.7 $\pm$ 1.8 mm; $n=12$) and $C/C$ (6.0 $\pm$ 2.3 mm; $n=5$) homozygotes ($P=0.04$; Figure).

## Discussion

### Aneurysm Size Versus Rupture Controversy

Although there is considerable epidemiological evidence supporting a multifactorial etiology for intracranial aneurysm formation, including factors regarded as “congenital” (heritable connective tissue disorders, familial predisposition, and

### TABLE 1. Comparison of SAH Cases With Non-SAH Controls*

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=90)</th>
<th>Cases (n=52)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, y</td>
<td>69±12</td>
<td>54±13</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>White race</td>
<td>90 (100%)</td>
<td>52 (100%)</td>
<td>NA</td>
</tr>
<tr>
<td>Female sex</td>
<td>44 (49%)</td>
<td>35 (67%)</td>
<td>0.03</td>
</tr>
<tr>
<td>History of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>18 (20%)</td>
<td>6 (12%)</td>
<td>0.19</td>
</tr>
<tr>
<td>Hypertension</td>
<td>35 (39%)</td>
<td>21 (40%)</td>
<td>0.86</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>12 (13%)</td>
<td>4 (8%)</td>
<td>0.31</td>
</tr>
<tr>
<td>Previous stroke</td>
<td>4 (4%)</td>
<td>4 (8%)</td>
<td>0.46</td>
</tr>
<tr>
<td>Smoking</td>
<td>36 (40%)</td>
<td>37 (71%)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>eNOS T-786C SNP genotype</td>
<td></td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td>C/C</td>
<td>16 (18%)</td>
<td>5 (10%)</td>
<td></td>
</tr>
<tr>
<td>T/C</td>
<td>46 (51%)</td>
<td>35 (67%)</td>
<td></td>
</tr>
<tr>
<td>T/T</td>
<td>28 (31%)</td>
<td>12 (23%)</td>
<td></td>
</tr>
</tbody>
</table>

NA indicates not applicable.

*Except for age (mean±SD), all results expressed as No. (% of total controls or cases).

### TABLE 2. Clinical Characteristics of SAH Patients (n=52)*

<table>
<thead>
<tr>
<th></th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WFNS score at admission†</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>23 (44%)</td>
</tr>
<tr>
<td>2</td>
<td>18 (35%)</td>
</tr>
<tr>
<td>3</td>
<td>5 (10%)</td>
</tr>
<tr>
<td>4</td>
<td>4 (8%)</td>
</tr>
<tr>
<td>5</td>
<td>2 (4%)</td>
</tr>
<tr>
<td>Fisher CT grade of SAH†</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2 (4%)</td>
</tr>
<tr>
<td>2</td>
<td>8 (15%)</td>
</tr>
<tr>
<td>3</td>
<td>29 (56%)</td>
</tr>
<tr>
<td>4</td>
<td>13 (25%)</td>
</tr>
<tr>
<td>Aneurysm location</td>
<td></td>
</tr>
<tr>
<td>Anterior circulation</td>
<td>43 (83%)</td>
</tr>
<tr>
<td>Posterior circulation</td>
<td>9 (17%)</td>
</tr>
<tr>
<td>No. of aneurysms</td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>41 (79%)</td>
</tr>
<tr>
<td>Multiple</td>
<td>11 (21%)</td>
</tr>
<tr>
<td>Aneurysm size (maximum diameter)</td>
<td></td>
</tr>
<tr>
<td>Small ($\leq 5$ mm)</td>
<td>22 (42%)</td>
</tr>
<tr>
<td>Medium (6–9 mm)</td>
<td>17 (33%)</td>
</tr>
<tr>
<td>Large ($\geq 10$ mm)</td>
<td>13 (25%)</td>
</tr>
</tbody>
</table>

*Expressed as No. (% of all cases).
†See text for details.
female sex) and “acquired” (cigarette smoking and hypertension), there is much controversy surrounding the natural history of intracranial aneurysms. Particularly since the publication of the studies by Wiebers et al.,1,2 in order to improve overall outcome in what is otherwise a devastating disease, a multitude of investigators have sought to identify factors that may determine which aneurysms are more prone to rupture.4 In a systematic and comprehensive manner, ISUIA has shed some light on this by identifying aneurysm size ≥10 mm in diameter in patients with no previous SAH and posterior location as independent predictors of rupture.1 However, as Forget et al.4 have recently reported, the findings of ISUIA are contended by the fact that the majority of aneurysms present after rupture are <10 mm in size (>85% in their report). Explanations for this discrepancy include a much higher prevalence of smaller aneurysms, decrease in aneurysm size after rupture, or existence of a subpopulation of aneurysms that rupture at a smaller critical size soon after formation.2,4 The former 2 explanations have been substantially rejected;3 the third seems more plausible and is the basis for the hypothesis tested herein.

Does Genetics Hold the Answer?

With identification of genetic polymorphisms of the eNOS gene that increase susceptibility to cardiovascular disease21–23 and mounting evidence for an association between altered expression of NOS isoforms and aneurysm formation,10,26–29 the possibility exists that eNOS genetic aberrations may also enable identification of aneurysm subpopulations by size and propensity for rupture. Nakayama et al.24 recently reported an association between a polymorphism of the eNOS gene promoter (eNOS T-786C SNP) and susceptibility to coronary vasospasm (variant or Prinzmetal’s angina)33 in Japanese individuals. They identified the allele with thymine (T) as being normal or “wild type” and the allele with cytosine (C) as “abnormal.” The authors reported that the allele containing C was significantly overrepresented among cases with coronary vasospasm compared with controls (the rare C/C genotype was exclusively present in cases, and the T/C genotype was 4 times more frequent in controls).24 Furthermore, they found that the presence of the abnormal allele resulted in a significant reduction in eNOS gene promoter activity, thereby providing biochemical evidence for a predilection toward coronary spasm. In the context of those findings and given the pivotal role played by NO in cerebrovascular physiology16–19 and its implication in aneurysm pathobiology,26–29 the principal goal of our study was to determine whether eNOS T-786C SNP genotypes were relevant to ruptured intracranial aneurysm size.

Genetic Data

To our knowledge, this is the first report of a genetic difference between subgroups of intracranial saccular aneurysms, ruptured or unruptured. We recognize that genotype analysis of controls and cases did not reveal a difference in terms of prevalence of the eNOS T-786C polymorphism. This indicates that in the population at large, this polymorphism does not itself predict aneurysm formation or rupture. However, our data suggest that among persons with known intracranial aneurysms, the polymorphism does allow genetic distinction between large versus small ruptured aneurysms and that in such persons eNOS T-786C genotype may be a factor influencing size at which rupture occurs. Specifically, all homozygotes in our study had ruptured aneurysms <10 mm in size, ie, only heterozygotes (T/C) had aneurysms ≥10 mm in size, a finding that remained statistically significant after multiple logistic regression analysis controlling for age, sex, and smoking status (ie, factors found to be different between our controls and cases). Notably, Nakayama at al.24 found the abnormal allele (C) as a heterozygote (T/C) in 4 times as many coronary vasospasm cases compared with controls. Our study suggests that homozygosity versus heterozygosity for this polymorphic gene, rather than the allele represented, may differentiate between small- and large-diameter ruptured aneurysms, respectively. In corroboration with this finding, ruptured aneurysm mean diameters among all heterozygotes in our study were significantly larger than those among homozygotes. In regard to the eNOS T-786C polymorphism, the molecular significance of homozygosity versus heterozygosity in the context of aneurysm development and rupture remains undetermined. The difference however, enabled distinction between small- versus large-diameter ruptured aneurysms. Although speculative at present, the observation of this genetic difference suggests that in persons with intracranial aneurysms, some interaction between normal and abnormal alleles may translate to differences in expression and/or function of eNOS in the vessel wall, perhaps confirmable in future via molecular analysis of aneurysmal sac tissue obtained during surgery. This notion is supported by the additional tissue culture experiments performed by Nakayama et al.24 involving T-786C polymorphic eNOS, the findings of Kuhlenbeccd et al.,26 Johanning et al.,27,28 and Fukuda et al.,29 suggesting a role for NOS isoforms in aneurysm formation and growth, and the findings of Kotani et al.10 associating eNOS polymorphism with development of aortic aneurysms. Whether related to increased local oxidative stress leading to vessel wall damage,28,29,34 predilection toward development of systemic hypertenston,29 the presence of aberrant smooth muscle proliferation16 (all associated with NO signaling dysfunction), or some other molecular event, differential expression and function of eNOS resulting in different local levels of NO among heterozygotes compared with homozygotes may affect the capacity of the blood vessel wall to withstand aneurysmal dilatation, thereby manifesting as rupture at markedly different aneurysm sizes. In conclusion, on the basis of the findings of this study, when considering factors predictive of aneurysmal rupture, we believe that due consideration should be given to pertinent genetic data in addition to knowledge of anatomic features such as aneurysm size, location, and the presence of daughter sacs.

Acknowledgments

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References


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