See the Editorial and the Response in this issue, p 433.

J Neurosurg 102:526–531, 2005

The presence of tandem *endothelial nitric oxide synthase* gene polymorphisms identifying brain aneurysms more prone to rupture

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Object. It is becoming apparent that the presence of certain genetic variations (polymorphisms) may increase the individual's susceptibility to cardiovascular diseases, even in the absence of a family history. We hypothesized that brain aneurysms more prone to rupture may be identified on the basis of an individual's genotype for endothelial nitric oxide synthase (eNOS), a critical vasomodulatory protein found to be increasingly relevant to the pathobiology of aneurysms.

Methods. Patients' clinical data were recorded prospectively. Genomic DNA was isolated from blood samples obtained from individuals presenting consecutively to the Mayo Clinic with ruptured (58 patients) or unruptured (49 patients) intracranial saccular aneurysms. Using polymerase chain reaction and gene microarray technology, the following *eNOS* genetic polymorphisms were studied: intron-4 27–base pair variable number of tandem repeats (27 VNTR); promoter single nucleotide polymorphism (T-786C SNP); and exon-7 SNP (G894T SNP).

Both groups of patients had similar demographic and clinical characteristics. For all three polymorphisms, variant alleles ($p \leq 0.003$) and their corresponding genotypes ($p \leq 0.006$) were found two to four times more frequently in patients with ruptured aneurysms than in patients with unruptured aneurysms. Strikingly, the odds ratio for presenting with a ruptured brain aneurysm among individuals demonstrating the copresence of all three variant alleles was 11.4 (95% confidence interval 1.7–75.9, $p = 0.004$).

Conclusions. The authors have uniquely identified a set of tandem *eNOS* gene variations whose presence can be used to identify patients with aneurysms likely to rupture. We believe that if this finding is reproducible in a large multicenter study, in addition to known anatomical factors a rapid and cost-effective screening tool will become available to clinicians as a genetic aid to predict the risks of rupture in patients presenting with unruptured intracranial aneurysms.

KEY WORDS • functional genomics • genetic polymorphism • intracranial aneurysm • nitric oxide synthase • subarachnoid hemorrhage

THE discrepancy between the prevalence of brain aneurysms (as high as 5% or 10–15 million in the US population alone) and the incidence of aneurysmal rupture (~ 30,000 cases identified annually in this country) leads investigators to infer that some brain aneurysms are more prone to rupture than others.[11,32] Despite diagnostic and therapeutic advances, it is currently estimated that one half of persons afflicted with aneurysmal rupture—SAH—will die or become markedly disabled as a result of the original hemorrhage or a major complication such as rebleeding or vasospasm.[14–16,36] The typically sudden and unexpected onset of this condition and its staggering human and economic costs have catalyzed the search for factors that may aid in the prediction of which persons may be susceptible to intracranial aneurysm rupture. In this context, investigators from ISUIA[12] have reported that larger brain aneurysms and those located in the posterior circulation have a higher tendency to rupture. Nevertheless, there is considerable disparity between the physical characteristics of aneurysms predicted by ISUIA investigators to be more prone to rupture and those of ruptured aneurysms actually seen in emergency departments worldwide.[6,20,37] Therefore, in this study we ask the following question: Can brain aneurysms that are more prone to rupture be identified genetically?

There is an increasing body of evidence showing that variant (polymorphic) alleles[29] of the gene encoding eNOS,[26] a critical vasomodulatory molecule,[4,27] are significantly overrepresented in individuals in whom a variety of cardiovascular disorders have been diagnosed (Fig. 1).[9,10,21,24,28,38,39] Furthermore, polymorphic *eNOS* genotypes have recently been implicated as markers for aneurysmal rupture and vasospasm.[19,21,25,28] In this light, our main goal was to compare the frequency of certain *eNOS* gene polymorphisms between patients who harbor ruptured brain aneurysms and those with unruptured brain aneurysms, to determine if the *eNOS* genotype can be used to predict an increased susceptibility to aneurysm rupture.

Abbreviations used in this paper: CI = confidence interval; eNOS = endothelial nitric oxide synthase; HWE = Hardy–Weinberg equilibrium; ISUIA = International Study of Unruptured Intracranial Aneurysms; OR = odds ratio; PCR = polymerase chain reaction; RA = ruptured aneurysm; SAH = subarachnoid hemorrhage; SNP = single nucleotide polymorphism; UA = unruptured aneurysm; VNTR = variable number of tandem repeats.

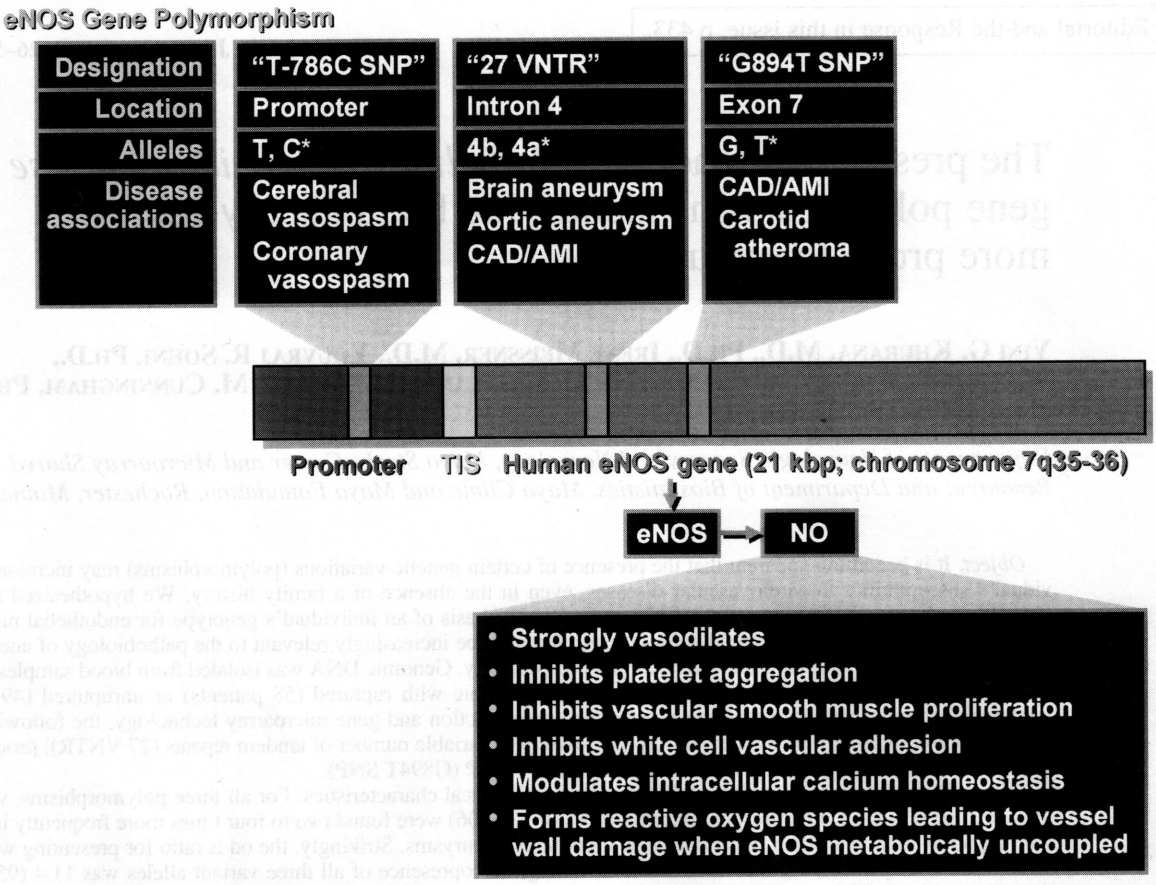

FIG. 1. Schematic representation in which the three *eNOS* gene polymorphisms investigated in this study are listed along with their respective locations in the *eNOS* gene. The eNOS protein encoded by this gene is a constitutive enzyme that catalyzes the conversion of L-arginine into L-citrulline, producing a rapidly diffusing signaling molecule, NO, as the major byproduct. Some important biological functions of NO are listed. For each polymorphism, the putative abnormal allele is indicated by an asterisk. AMI = acute myocardial infarction; C = cytosine; CAD = coronary artery disease; G = guanine; kbp = kilo–base pairs; T = thymine; TIS = transcription initiation sequence; 4a = four 27–base pair tandem repeats; 4b = five 27–base pair tandem repeats.

Materials and Methods

Study Participants

This prospective case–control study, approved by our institutional review board, involved 107 patients, each of whom gave informed consent for participation. Our control group consisted of 49 persons who consecutively presented to our institution with the diagnosis of an unruptured intracranial saccular aneurysm. Our case group was composed of 58 persons who were consecutively admitted to our hospital with the diagnosis of aneurysmal SAH based on each patient's history and findings of radiological studies, including both computerized tomography scanning of the head and four-vessel cerebral angiography obtained at hospital admission.

Genetic Analysis

On the basis of recent studies,[10,19–21,28,38] we investigated three particular eNOS polymorphisms (Fig. 1). A single 20-ml sample of peripheral vein blood was obtained from all participants for subsequent DNA extraction and genetic analysis. Genomic DNA was extracted from peripheral blood lymphocytes by using a QIAamp DNA Blood Minikit (Qiagen, Germantown, MD). The SNPs were genotyped using Nanochip active electronic arrays (Nanogen, San Diego, CA), as described elsewhere.[34] Oligo software was used to design PCR primers (version 6.61; IDT, Coralville, IA) based on GenBank sequences. The PCR mixtures consisted of 25 μl AmpliTaq Gold Master Mix (Applied Biosystems, Foster City, CA), 1 μM primers, 20 ng DNA template, and enough water to provide a 50-μl mixture. All oligonucleotides were synthesized by IDT. The primer sequences were 5′-biotin-GCATGCACTCTGGCCTGAAGT-3′ (forward) and 5′-CAGGAAGCTGCCTTCCAGTGC-3′ (reverse) for eNOS T-786C SNP, and 5′-biotin-CTGGAGATGAAGGCAGGAGAC-3′ (forward) and 5′-CTCCATCCCACCCAGTCAATC (reverse) for eNOS G894T SNP. The thermal cycling conditions for each primer were 95°C for 10 minutes, 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 45 seconds, with a final extension at 72°C for 7 minutes. For eNOS T-786C SNP, the reporter probes were 5′-Cy3-AGGGTCAGCCA-3′ and 5′-Cy5-GGGTCAGCCG-3′ with the stabilizer oligonucleotide 5′-GCCAGGGAAGAGCTTGATGCCCTGGTGGGAGC-3′, and for eNOS G894T SNP, the reporter probes were 5′-Cy3-GTTCTGGGGGC-3′ and 5′-Cy5-AGTTCTGGGGGA-3′ with the stabilizer oligonucleotide 5′-TCATCTGGGGCCTGCAGCAGCAGGGGCAGCA-3′. Known heterozygotes, verified by dye-terminator sequencing performed on ABI Prism 377 DNA sequencers (Applied Biosystems) in both forward and reverse directions, were used as controls to normalize hybridization efficiency between dye-labeled reporters. The PCR conditions and methods for analyzing the eNOS 27 VNTR polymorphism are detailed elsewhere.[33] The PCR products were analyzed using a DNA 500 LabChip kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE) following the manufacturer's instructions. Sizes of the DNA fragments were determined for each sample from

TABLE 1

Summary of demographic and clinical data for persons with unruptured and ruptured aneurysms in the brain

Variable	No. of Patients (%)		p Value
	UA Group	RA Group	
no. of patients	49	58	—
age (yrs)*	57.1 ± 11.5	53.2 ± 12.7	0.10
female sex	36 (73)	39 (67)	0.48
caucasian race	49 (100)	58 (100)	—
cardiovascular comorbidity			
diabetes mellitus	4 (8)	4 (7)	0.80
hypertension	25 (51)	24 (41)	0.32
coronary artery disease	4 (8)	7 (12)	0.51
ischemic stroke	10 (20)	5 (9)	0.08
history of smoking	28 (57)	41 (71)	0.14
family history of brain aneurysm or SAH	8 (16)	4 (7)	0.12
aneurysm size (mm)*	9.6 ± 5.8	7.5 ± 4.7	0.037
multiple aneurysms	16 (33)	15 (26)	0.44
aneurysm location			<0.001
anterior communicating artery	3 (6)	16 (28)	
anterior cerebral artery	2 (4)	5 (9)	
middle cerebral artery	17 (35)	5 (9)	
internal carotid artery	17 (35)	7 (12)	
posterior communicating artery	1 (2)	13 (22)	
posterior cerebral artery	1 (2)	2 (3)	
basilar artery	6 (12)	7 (12)	
vertebral artery	2 (4)	3 (5)	
aneurysm treatment			<0.001
clip placement	22 (45)	24 (41)	
coil embolization	10 (20)	30 (52)	
coil embolization followed by clip placement	0 (0)	1 (2)	
none	17 (35)	3 (5)	

* Values are represented as the means ± standard deviations. — = not applicable.

TABLE 2

Allele and genotype data for persons with unruptured and ruptured aneurysms in the brain

Locus	No. of Patients (%)		p Value
	UA Group	RA Group	
no. of patients	49	58	—
allele frequency			
eNOS 27 VNTR			0.003
allele 4a*	10 (10)	30 (26)	
allele 4b	88 (90)	86 (74)	
eNOS T-786C SNP			0.003
allele C*	21 (21)	47 (41)	
allele T	77 (79)	69 (59)	
eNOS G894T SNP			<0.001
allele T*	10 (10)	38 (33)	
allele G	88 (90)	78 (67)	
genotype frequency			
eNOS 27 VNTR			0.006
4a/4a	0 (0)	1 (2)	
4a/4b	10 (20)	28 (48)	
4b/4b	39 (80)	29 (50)	
eNOS T-786C SNP			<0.001
C/C	5 (10)	6 (10)	
C/T	11 (22)	35 (60)	
T/T	33 (67)	17 (29)	
eNOS G894T SNP			<0.001
T/T	2 (4)	6 (10)	
T/G	6 (12)	26 (45)	
G/G	41 (84)	26 (45)	

* Variant allele.

the calibration curve in conjunction with markers and a sizing ladder. Genotypes were designated based on fragment sizes obtained at the end of the run. For each polymorphism, amplicons were randomly sequenced to determine concordance with microarray genotyping as described earlier.

Statistical Analysis

To evaluate the association between the demographic and genetic markers of interest and aneurysmal disease, comparisons were made between patients with ruptured aneurysms and those with unruptured aneurysms. Demographics are presented as means ± standard deviations for continuous variables and as percentages of column totals for categorical variables. Univariate associations between demographic variables and disease were assessed using a two-sample t-test for continuous variables and the Pearson chi-square or Fisher exact test (when sample sizes were limited) for categorical variables. Before any statistical analysis of disease-marker associations, the allele frequency distribution at each polymorphism locus was tested against the HWE under the Mendelian biallelic expectation by performing the chi-square test. Univariate associations of allele (which treats each chromosome as a unit) and genotype (which treats a person as a unit) with the disease were evaluated using contingency table methods provided by SAS software (version 8.2; SAS Institute Inc., Cary, NC). Allele associations were assessed by applying the Pearson chi-square or Fisher exact test (when sample sizes were limited), and genotype associations were assessed by performing the Cochran–Armitage trend test. The multiple polymorphism marker–disease association with haplotype was evaluated using the Haplo.score (S-PLUS, version 6.0; Insightful Corp., Seattle, WA), which accounts for the ambiguous linkage phase.[23,31] Haplotype ORs and 95% CIs were calculated using Haplo.glm (S-PLUS, version 6.0). The haplotype composed of three wild-type alleles (4b-T-G) was used as a comparison to calculate the haplotype's specific OR and CI. Linkage disequilibrium was assessed using the Graphical Overview of Linkage Disequilibrium software package.[1,2] All tests were two-sided and probability values less than 0.05 were considered statistically significant.

Results

Clinical Data

When the RA group was compared with the UA group, there was no significant difference in mean age, sex, race, history of cardiovascular diseases or smoking, or family history of brain aneurysms or SAH (Table 1). Although the multiplicity of aneurysms was similar between the two groups, patients in the RA group presented with significantly smaller aneurysms than those in the UA (control) group (7.5 ± 4.7 mm and 9.6 ± 5.8 mm, respectively; $p = 0.037$). The distribution and treatment of aneurysms also differed significantly between the two groups (both $p < 0.001$; Table 1).

Genetic Data

Hardy–Weinberg Equilibrium. Among control patients (UA group), the genotype frequencies for eNOS 27 VNTR ($p = 1.00$) and eNOS G894T SNP ($p = 0.06$) were in agreement with those predicted by the HWE. The genotype frequencies for eNOS T-786C SNP ($p = 0.03$) were not in agreement with those predicted by the HWE. We therefore applied the Cochran–Armitage trend test (which is unaffected by a departure from the HWE) and showed consistent results. We found that for eNOS T-786C SNP, the de-

TABLE 3

*Haplotype data for persons with unruptured and ruptured aneurysms in the brain**

eNOS 27 VNTR	eNOS T-786C SNP	eNOS G894T SNP	Simulated p Value	UA Group Haplotype Frequency	RA Group Haplotype Frequency	OR (95% CI)
4a	C	T	0.004	0.02	0.08	11.4 (1.7–75.9)
4a	T	T	—	0.01	<0.001	—
4a	C	G	0.02	0.03	0.14	8.6 (1.8–41.3)
4a	T	G	0.87	0.04	0.03	2.2 (0.4–13.1)
4b	C	T	0.008	0.03	0.13	9.3 (1.7–49.9)
4b	T	T	0.07	0.04	0.11	4.4 (0.9–22.4)
4b	C	G	0.10	0.13	0.05	0.5 (0.1–1.8)
4b	T	G	<0.001	0.70	0.45	1.0 (—)

* Tested in 20,000 simulations.

parture from the HWE was due to a homozygote favoring, which has been shown to have a minimal effect on haplotype estimation.[23] Furthermore, to exclude the possibility of a genotyping error, 37 T-786C SNP amplicons were randomly sequenced and the results were fully concordant with those of the microarray genotyping.

Allele and Genotype Frequencies. For each of the three polymorphisms, significant differences in allele and genotype frequencies were found between the RA and UA groups, with variant alleles and their corresponding genotypes being present two to four times more often in patients in the RA group (Table 2). A linkage disequilibrium analysis was performed using both D' and R2 to detect pair-wise linkage disequilibrium among the three polymorphisms.[2] No significant linkage disequilibrium was detected (data not shown).

Haplotype Frequencies. A haplotype analysis consisting of 20,000 simulations was conducted to assess the multiple polymorphism marker–disease associations. The observed results were summarized using the simulation probability value, RA and UA groups' haplotype frequencies, the OR, and the 95% CI for each of the eight possible haplotypes (Table 3). Haplotype 4a-C-T, which includes the variant allele for all three polymorphisms, was found in 8.4% of patients in the RA group and 2.3% of those in the UA (control) group (simulated p = 0.0038); patients with this haplotype had an 11.4-fold (95% CI 1.7–75.9-fold) increased odds of being in the RA group. The second identified risk haplotype, 4a-C-G, which includes the variant allele for eNOS 27 VNTR and eNOS T-786C SNP, was found in 14.1% of patients in the RA group and 3.1% of those in the UA group (simulated p = 0.0196); patients with this haplotype had an 8.6-fold (95% CI 1.8–41.3-fold) increased odds of being in the RA group. The third risk haplotype, 4b-C-T, which includes the variant allele for eNOS T-786C SNP and eNOS G894T SNP, was found in 13.2% of patients in the RA group and 2.7% of those in the UA group (simulated p = 0.0077); patients with this haplotype had a 9.3-fold (95% CI 1.7–49.9-fold) increased odds of being in the RA group.

Discussion

Key Findings

The unique and most important finding of this study is that the presence of two or three (that is, tandem or multiple) variant *eNOS* alleles in a patient with a brain aneurysm is associated with an approximately 10-fold increased odds of presenting with aneurysmal rupture. Based on our data we can also suggest that there are two distinct subpopulations of intracranial aneurysms, distinguishable by anatomical and genetic features, with one being more prone to rupture than the other.

Role of eNOS in the Pathobiology of Aneurysms

The selection of the *eNOS* gene as a candidate for this study was deliberate and based on findings of numerous studies, which support a pivotal role for NO in the cerebral vasculature.[4,7,15,17–19,30] Pertaining specifically to aneurysm pathobiology, variant alleles and corresponding genotypes of the eNOS 27 VNTR have been associated with rupture-prone aortic aneurysms[20] and are overrepresented in patients with ruptured brain aneurysms compared with normal population controls.[21] In a recent study from our group in which the frequency of certain *eNOS* gene polymorphisms were examined in patients with SAH (cases) compared with healthy volunteers (controls), we observed a significant difference only in the distribution of genotypes for the eNOS 27 VNTR polymorphism.[19] Heterozygosity for this polymorphism was almost three times as prevalent among patients with ruptured aneurysms than controls (p = 0.002), whereas the putative variant "4a" allele was found in 26 (51%) of 51 cases compared with 20 (22%) of 90 controls; this difference was also significant (p = 0.007).[19] In addition, variant alleles and corresponding genotypes of the eNOS T-786C SNP have been implicated as genetic markers for vasospasm,[25,28] including post-SAH cerebral vasospasm.[19] It is important to note that a polymorphic NOS dysfunction may result from the transcription and translation of a putatively abnormal allele in either the homozygous or heterozygous form.[28] The precise molecular effects of these polymorphisms have not been elucidated, although there is biochemical evidence for decreased *eNOS* gene promoter activation associated with the T-786C SNP variant and reduced eNOS protein expression and enzymatic activity associated both with the eNOS 27 VNTR and T-786C polymorphism variants.[28,35] It is certainly conceivable that such variants may contribute toward the pathobiology of aneurysms and cerebral vasospasm through increased local oxidative stress, leading to vessel wall damage, a predilection toward development of atherogenic intimal hyperplasia and systemic hypertension, the presence of aberrant vascu-

lar smooth-muscle proliferation, and increased platelet aggregation and proinflammatory monocyte adhesion—all of which are associated with an NO signaling dysfunction.[5,8,13,15,22,27] Such mechanisms may also account for the impaired vasorelaxation and heightened vascular wall inflammation characteristic of post-SAH vasospasm.[5,15,17,18]

Evidence Supporting Rupture-Prone and Rupture-Resistant Brain Aneurysm Subpopulations

Our case and control groups (RA and UA groups, respectively) were similar in demographic and clinical variables such as age, sex, race, cardiovascular comorbidities, smoking, and family history of brain aneurysm or SAH. Additionally, patient demographics and aneurysm features (such as multiplicity, average size, and location) in our UA group were consistent with those reported in Group 1 in the ISUIA (patients with unruptured aneurysms and no preceding SAH from another aneurysm).[12] In the RA and UA groups in the present study the lesions differed significantly with regard to anatomical and genetic factors. Consistent with findings in other series of ruptured brain aneurysms,[6,37,40] in our study aneurysms in the RA group were smaller (mean size 7.5 mm) than those in the UA group (mean size 9.6 mm), and tended to be located much more frequently in the anterior and posterior communicating arteries, and much less frequently in the posterior circulation. These findings, however, are at odds with those provided by the ISUIA. In that study, unruptured aneurysms that were larger than 10 mm or were located in the vertebrobasilar circulation or along the posterior cerebral artery were identified as being more prone to rupture, whereas aneurysms that were located along the anterior communicating artery were found to rupture relatively infrequently.[12] This disparity underscores arguments put forth by investigators that ISUIA-type aneurysms may in fact represent only one type of intracranial saccular aneurysm, that is, the type that is not prone to rupture. These arguments have been articulated elsewhere.[6,20]

The notion of rupture-prone and rupture-resistant subpopulations of brain aneurysms is further reinforced by the novel genetic data reported in the present article. Despite the similarities of demographic and clinical characteristics between our two groups of patients, the genetic differences between the two groups were striking. We found polymorphic variant alleles and their corresponding genotypes between two and four times more frequently in the RA group than in the UA (control) group, and our haplotype analysis indicated that the presence of two or three variant alleles was associated with an 8.6 to 11.4 increased odds of being in the RA group. Taken together, our anatomical and genetic data indicate that there are distinct differences between ruptured and unruptured aneurysms: the former are smaller, have a greater predilection for the anterior and posterior communicating arteries, and have a tendency to occur more commonly in persons with two or three variant eNOS polymorphic alleles.

Clinical Implications of this Study

Among the estimated 5 to 15% of aneurysm-harboring individuals with a relatively strong family history of brain aneurysms or with a heritable connective tissue disorder, such as Ehlers–Danlos, Marfan, or autosomal dominant polycystic kidney disease,[32] noninvasive radiological screening for brain aneurysms is accepted as being worthwhile.[3] For the remaining persons at this time referred to as having sporadic unruptured brain aneurysms, however, there is currently no adequate screening tool. To identify such individuals via population-wide serial radiological screening seems largely impractical and no aneurysm gene has been identified thus far. Therefore, perhaps one of the most important aspects of brain aneurysm management at this time relates to how to counsel a patient with a newly diagnosed brain aneurysm about observation and treatment. Investigators from the ISUIA have suggested that certain aneurysms are more prone to rupture; however, for reasons outlined earlier and elaborated in detail elsewhere,[6,20] counsel based on the ISUIA data alone may not cover the gamut of rupture-prone aneurysms. We therefore believe that the findings of our study shed an important new light on the genetics of what are otherwise recognized as sporadic brain aneurysms (found in 85–95% of all aneurysm-harboring individuals). For example, a person harboring a lesion diagnosed incidentally or otherwise as an unruptured intracranial aneurysm (especially one located in a higher-risk cerebrovascular territory identified by ourselves and others in ruptured aneurysm cohorts) in whom two or three variant *eNOS* polymorphic alleles are found by gene microarray technology (now becoming more readily available) would be counseled toward earlier treatment rather than observation based on the data reported herein. We believe that if our findings are reproducible in the setting of a large multicenter study then, in addition to known anatomical factors, a rapid and cost-effective screening tool will become available to clinicians as a genetic aid to predict rupture risks in patients presenting with unruptured intracranial aneurysms.

Acknowledgment

We thank Ms. Mary Soper for assistance in the preparation of the manuscript.

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Manuscript received August 25, 2004.
Accepted in final form November 22, 2004.
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