II Genotype of the Angiotensin-Converting Enzyme Gene Increases the Risk for Subarachnoid Hemorrhage From Ruptured Aneurysm

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Background and Purpose—Evidence exists in support of a role of genetic factors in susceptibility to aneurysmal subarachnoid hemorrhage (SAH) in humans. Meta-analysis of 2 previous studies showed that the I allele of angiotensin-converting enzyme (ACE) gene insertion/deletion (I/D) polymorphism was a weak, but significant, risk factor for aneurysmal SAH. Moreover, a recent study has shown that the local renin-angiotensin system (RAS) is involved in the development of intracranial aneurysm. The aim of this study was to investigate the association between ACE I/D polymorphism and a risk for aneurysmal SAH in a Polish population.

Methods—Ninety patients with aneurysmal SAH (mean age: 48.9 ± 14.0 years) and 128 healthy controls matched for age and sex were genotyped for the ACE I/D polymorphism. Aneurysmal SAH was diagnosed by cranial computed tomography and/or lumbar puncture and digital subtraction angiography. ACE gene polymorphism was detected by polymerase chain reaction amplification of the intron 16-specific I/D fragments, 490-bp and 190-bp, respectively.

Results—The ACE genotype distribution in patients with aneurysmal SAH (II, 52.2%; ID, 15.6%; DD, 32.2%) differed significantly from controls (II, 23.4%; ID, 50.8%; DD, 25.8%) (P < 0.001). A logistic regression model showed that the II genotype of ACE gene was independent from female sex and smoking as a risk factor for aneurysmal SAH (OR, 4.57; 95% CI, 2.35 to 8.90).

Conclusion—Here we report that II genotype of ACE gene is a risk factor for aneurysmal SAH. (Stroke. 2004;35:000-000.)

Key Words: aneurysm ● genetics ● subarachnoid hemorrhage ● angiotensin-converting enzyme

Subarachnoid hemorrhage (SAH) from ruptured intracranial aneurysm is the most common cause of all SAHs, with a mortality rate as high as 50%. Approximately 90% of aneurysms are saccular. The pathogenesis of SAH from ruptured saccular aneurysm (aneurysmal SAH) is poorly understood. There is some evidence that hemodynamic factors and structural properties of the arterial wall are involved in the development of saccular aneurysms; however, the trigger factors remain unknown. Several modifiable risk factors for the formation of aneurysm, like hypertension, heavy alcohol consumption, and smoking, have been identified. There is also growing evidence that genetic factors may play a role in the development of aneurysms. For example, cerebral aneurysm is associated with some heritable disorders, such as polycystic kidney disease or Ehlers–Danlos syndrome. The concept of familial aggregation of cerebral aneurysms is increasingly supported. There are also data showing that a family history of hemorrhagic stroke significantly increases the risk for aneurysmal SAH. A recent study has shown that under conditions of increased hemodynamic stress, a downregulation of the local renin-angiotensin system (RAS) is observed in intracranial aneurysm disease. Moreover, meta-analysis of 2 previous studies assessing the significance of angiotensin-converting enzyme (ACE) insertion/deletion (I/D) polymorphism for the development of aneurysmal SAH, showed that the I allele of the ACE gene was a risk factor for this disease. The aim of this study was to assess the significance of ACE I/D polymorphism as a risk factor for aneurysmal SAH in a Polish population.

Materials and Methods

Study Population

The study population consisted of 90 unrelated patients with a diagnosis of SAH from ruptured saccular aneurysm out of a total of 158 patients with subarachnoid hemorrhage admitted to the Stroke Unit, Neurology Department, and the Neurosurgery Department, Jagiellonian University, Krakow, Poland, between October 2002 and...
April 2003. Patients with dissecting and fusiform aneurysms (n=9), arteriovenous malformations (n=10), unknown origin of SAH (n=12), comatose on admission (n=24), or not agreeing to participate in this study (n=13) were not included.

In each case on admission, we performed cranial computed tomography (CT) and/or lumbar puncture to confirm the diagnosis of SAH. The diagnosis of single or multiple intracranial saccular aneurysms was established by digital subtraction angiography (DSA). We also included 128 unrelated control individuals, free of clinically detectable cerebrovascular disease and without any stroke history. They were recruited from consecutive spouses of the patients (30%) and from the community. One hundred percent of the study population were whites and came from a province in southern Poland. Controls were matched for age (±2 years) and sex with patients. Before inclusion in the study, all participants gave informed consent. The study was approved by the University Ethical Committee and was performed in accordance with the Helsinki Declaration of 1975, as revised in 1983.

For all subjects from each study group, we collected demographic data and risk factors profile. Patients and controls received a detailed clinical questionnaire including information on vascular risk factors, current medication, and physical examination. The answers were verified by the analysis of the medical documentation and, for the SAH patients, by the information from their proxies.

The individual was classified as having arterial hypertension if he or she met one of the following criteria: (1) the diagnosis of hypertension in previous medical history; (2) antihypertensive treatment before the entry of the study; or (3) systolic or diastolic blood pressure ≥140 mm Hg or ≥90 mm Hg, respectively, on at least 2 different occasions (the first 3 days of hospitalization were not considered for the SAH patients).

The history of ischemic heart disease was established on past medical history, examination of previous and current electrocardiograms, and laboratory data.

Smoking habits were defined as current smokers of ≥1 cigarette per day, former smokers, or nonsmokers. For statistical analysis, “current smokers” and “former smokers” were pooled together. Excessive alcohol intake was defined as alcohol consumption of ≥300 grams per week (>3 alcoholic drinks daily).

### Laboratory Techniques

Genetic analyses were performed by laboratory personnel who were blinded for sample identity. Uncuffed venous blood samples were drawn from each subject within 2 days after stroke onset for extraction of DNA. Leukocyte DNA was extracted from 10 mL of venous blood, anticoagulated with 1.6 mg/mL EDTA using a High Pure PCR template Preparation Kit; Behringer Mannheim). The ACE I/D polymorphism was detected by polymerase chain reaction (PCR), according to the presence or absence of an insertion in intron 16 with 2 I/D flanking primers (sense primer: 5’-CTGGAGACCACTCCACATCCTTT TCT-3’ and antisense primer 5’-GATGTGGCCACTCACATTGC TAGAT-3’). DNA was denatured at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 67°C for 1 minute, and extension at 72°C for 2 minutes, with a final extension step of 5 minutes at 72°C.13 PCR products (490-bp insertion and 190-bp deletion) were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and viewed with ultraviolet (UV) light. We use a sample with master mix without DNA as a control. Because the D allele in heterozygous samples is preferentially amplified,14 each sample found to have DD genotype was subjected to a second independent PCR amplification with a primer pair that recognizes and insertion-specific sequence (sense primer: 5’TGGGACCCACAGCGGCGCCACTAC 3’; antisense primer: 5’TCGCC CAGCCCTCCATGCCAA3’), with the following PCR conditions: denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 67°C for 45 seconds, and extension at 72°C for 2 minutes, with a final extension step of 7 minutes at 72°C. The reaction yields a 335-bp amplicon only in the presence of an I allele and no product in the samples homozygous for DD.15 This procedure correctly identified 4 (6.5%) samples with ID genotype that were misclassified as DD with the first PCR.

### Table 1. Demographic Data and Risk Factor Profile in SAH Patients and Their Controls

<table>
<thead>
<tr>
<th></th>
<th>Patients (n=90)</th>
<th>Controls (n=128)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y±SD)</td>
<td>48.9±14.0</td>
<td>50.9±13.4</td>
<td>0.28*</td>
</tr>
<tr>
<td>Female (%)</td>
<td>57 (63.3)</td>
<td>70 (64.7)</td>
<td>0.20‡</td>
</tr>
<tr>
<td>Hypertension</td>
<td>36 (40.0)</td>
<td>40 (31.5)</td>
<td>0.18†</td>
</tr>
<tr>
<td>Ischemic heart disease (%)</td>
<td>13 (14.4)</td>
<td>27 (21.6)</td>
<td>0.21†</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>60 (66.6)</td>
<td>43 (33.9)</td>
<td>0.00001†</td>
</tr>
<tr>
<td>Excessive alcohol intake (%)</td>
<td>10 (11.1)</td>
<td>6 (4.7)</td>
<td>0.07†</td>
</tr>
</tbody>
</table>

*Student t test.
†x2 test.

### Table 2. ACE Genotype and Allele Frequencies in Patients With Aneurysmal SAH and in Their Controls

<table>
<thead>
<tr>
<th>Genotype Distribution</th>
<th>Cases (n=90)</th>
<th>Controls (n=128)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II (n, %)</td>
<td>47 (52.2)</td>
<td>30 (23.4)</td>
<td></td>
</tr>
<tr>
<td>ID (n, %)</td>
<td>14 (15.6)</td>
<td>65 (50.8)</td>
<td></td>
</tr>
<tr>
<td>DD (n, %)</td>
<td>29 (32.2)</td>
<td>33 (25.8)</td>
<td></td>
</tr>
</tbody>
</table>

*Statistical calculation based on the comparison of II vs ID/DD genotypes (χ2 test).
†Statistical calculation based on the comparison of I vs D allele (χ2 test).

### Statistical Analysis

Data on quantitative characteristics are expressed as means±standard deviation. Data on qualitative characteristics are expressed as percent values or absolute numbers as indicated. Comparisons between groups were made with χ2 (nominal data) or Student t test (interval data). A value of P<0.05 was considered statistically significant.

Hardy–Weinberg equilibrium was tested by the χ2 method. The sample size was calculated with a power of at least 80% at the 0.05 significance level. The expected proportion of II homozygotes among controls was 25%.

The association of the ACE genotype with risk for aneurysmal SAH was investigated by means of logistic regression analysis,
Results
We found 129 aneurysms in 90 patients with aneurysmal SAH. Their locations were as follows: anterior cerebral artery complex, 39 (30.2%); middle cerebral artery complex, 41 (31.8%); internal carotid artery complex, 33 (25.6%); and vertebrobasilar artery complex, 16 (12.4%).

The characteristics of patients with aneurysmal SAH and their controls are shown in Table 1. Patients with aneurysmal SAH presented significantly more often with current or previous smoking and greater alcohol intake (although this does not appear to be statistically significant, using the cutoff of P<0.05).

ACE genotype and allele frequencies are presented in Table 2. Allele frequency in the control group was in Hardy–Weinberg equilibrium. SAH patients deviated from Hardy–Weinberg equilibrium (P<0.01). Patients with aneurysmal SAH presented more often with II genotype (OR, 3.57; 95% CI, 1.99 to 6.41; P=0.0001) and I allele (OR, 1.57; 95% CI, 1.07 to 2.32; P=0.02) of ACE gene when compared with their controls. The difference between genotype distributions was also statistically significant when males and females were analyzed as separate groups (OR, 3.56; 95% CI, 1.43 to 8.86; P=0.0006 and OR, 3.86; 95% CI, 1.75 to 8.51; P=0.0005, respectively).

The distribution of ACE genotypes and alleles was similar in patients with single (n=64) and multiple aneurysms (n=26) [II, 33 (51.5%); ID, 20 (31.3%); DD, 11 (17.2%) versus II, 14 (53.9%); ID, 3 (11.5%); DD, 9 (34.6%); respectively, P=NS; and I, 77 (60.1%); D, 51 (39.9%) versus I, 31 (59.6%); D, 21 (40.4%); respectively, P=NS].

After adjustment for the studied risk factors, logistic regression analysis showed that II genotype of ACE gene (OR, 4.57; 95% CI, 2.35 to 8.90; P=0.00001), female sex (OR, 2.7; 95% CI, 1.12 to 4.59; P=0.02), and smoking (OR, 4.36; 95% CI, 2.27 to 8.36; P=0.00001) were independent risk factors for aneurysmal SAH. Carrying at least one I allele of ACE gene was not an independent risk factor for aneurysmal SAH in the logistic regression model (OR, 0.88; 95% CI, 0.46 to 1.68).

Discussion
This study shows that II genotype of ACE gene increases the risk for aneurysmal SAH in a Polish population. The distribution of ACE I/D polymorphism is similar in male and female patients and is not influenced by the number of aneurysms. This study confirmed the results of the 2 previously published studies showing that I allele of ACE gene is involved in the risk for aneurysmal SAH,11,12 however, the question of how this polymorphism influences the pathogenesis of saccular aneurysms remains unanswered.

In comparison to Cambridge data12 and Japanese data,11 we found a higher percentage of II carriers (Krakow, 52.2%; Cambridge, 30.2%; Japan, 45.8%) and significantly lower percentage of ID carriers (Krakow, 15.6%; Cambridge, 48.8%; Japan, 48.2%). This difference in the genotype distribution of ACE I/D polymorphism explains why we were able to reveal that the II genotype acts in a recessive fashion. The II and ID genotypes pooled together in a dominant model did not confer a statistically significant risk for aneurysmal SAH in our population. In general, we may say that our data are in agreement with the previous study of a European population, showing that aneurysmal SAH presented with an overrepresentation of the ACE I allele (Krakow, 60.0%; Cambridge, 54.7%) when compared with their controls (Krakov, 48.8%; Cambridge, 48.2%). A potential limitation of our current study is the loss of patients with SAH who died before admission to the hospital or who did not agree to participate in the study. These factors might influence our final results.

Saccular aneurysms develop from defects in the arterial walls. Some authors have postulated that aneurysms arise from congenital defects in the muscular layer of the cerebral arteries.16 Acquired degeneration in the internal elastic membrane or vascular extracellular matrix of cerebral arteries may weaken vessel walls and serve as a trigger factor for aneurysm formation.17 The combination of the 2 aforementioned processes is proposed by some authors as a pathomechanism of aneurysm formation.18 Aneurysms most frequently develop at sites of vessel bifurcation, where blood flow is more turbulent and shear forces against the arterial walls are greater. It is thought that the development of aneurysms is a result of interplay between hemodynamics and structural properties of the arterial wall.19

ACE circulates in plasma and is presented on the surface of endothelial cells, where it stimulates the conversion of inactive angiotensin I to active angiotensin II. Angiotensin II is a potent vasoconstrictor and increases vascular smooth muscle growth.20 Typically, under increased hemodynamic stress, the local RAS is activated, inducing the thickening of the arterial wall. This process is observed in several cardiovascular diseases.20 ACE is also the predominant enzyme for bradykinin metabolism in humans. It inactivates bradykinin, a vasodilator that inhibits vascular smooth muscle proliferation, and can stimulate the release of endothelial vasodilators, including nitric oxide and prostacyclin.20 The level of ACE is genetically determined by an ACE gene I/D polymorphism, with the highest levels of the enzyme in DD carriers and the lowest in II carriers.

Our results suggest that II genotype of ACE gene may contribute to vascular dilatation at the site of aneurysm formation. One possible explanation may be the increased bradykinin activity in II carriers of ACE gene. It is also possible that another polymorphism that is in linkage disequilibrium with ACE gene I/D polymorphism is responsible for the vascular dilatation and further development of aneurysm.20 A very interesting recent study elucidated for the first time that the formation of an aneurysm is the only disorder in which the vessel wall presented with a decreased expression of RAS, measured by the expression of ACE, or angiotensin type I receptor.10 The decreased expression of RAS was related to the decreased number or degeneration of endothelial cells and the decreased number of smooth muscle cells in considering potential confounding risk variables including age, sex, and other conventional risk factors. For multivariate risk predictors, the adjusted odds ratios are given with the 95% CIs.
the aneurysm wall. The authors postulated the aneurysm formation could be caused by the lack of vascular remodeling related to the decreased local RAS expression. It cannot be excluded that such a trigger for the formation of saccular aneurysms appears over the course of a lifetime in genetically predisposed people, ie, II genotype carriers of ACE gene. One can speculate that treatment with ACE inhibitors may increase the risk for aneurysm formation and/or rupture, especially in II carriers. Further studies should answer 2 questions: (1) whether wall shear stress in II carriers of ACE gene is associated with decreased local expression of RAS in the cerebral vessel walls and, in consequence, with saccular aneurysm formation; and (2) whether treatment with ACE inhibitors increases the risk for aneurysm formation.

Our study confirmed the results of previously published data showing that cigarette smoking is one of the most important modifiable risk factors for aneurysmal SAH. Smoking as a multivariate odds ratio of 4.77 is within the important modifiable risk factors for aneurysmal SAH. Our study confirmed the results of previously published studies showing that cigarette smoking is one of the most important modifiable risk factors for aneurysmal SAH.

In conclusion, this study shows that II genotype of ACE gene may play a role in the formation of saccular aneurysms.

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References
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