Lack of Association Between Endoglin Intron 7 Insertion Polymorphism and Intracranial Aneurysms in a White Population

Evidence of Racial/Ethnic Differences

Dietmar Krex, MD; Andreas Ziegler, PhD; Hans Konrad Schackert, MD; Gabriele Schackert, MD

Background and Purpose—Endoglin is a component of the transforming growth factor-β receptor complex and is predominantly expressed on cell surfaces of endothelial cells. A polymorphism of the endoglin gene has previously been found to be associated with the occurrence of intracranial aneurysms in a Japanese population. In our study, we investigated whether this polymorphism is associated with the development of cerebral aneurysm in a white population.

Methods—The study population consisted of 121 white patients who had been treated for intracranial aneurysms, 124 healthy white blood donors, and 15 Japanese volunteers. Exon 7 of the endoglin gene and adjacent intronic sequences were amplified by polymerase chain reaction and analyzed by using an automated laser fluorescence detection system.

Results—A well-known insertion polymorphism (5′-TCCCCC-3′, starting 23 bp distal from the 3′ end of exon 7) was identified. The allele frequencies of the polymorphism were 35 (14.5%) of 242 alleles in the aneurysm group and 35 (14.1%) of 248 alleles in the white control group, which does not represent a statistically significant difference (P=0.85). The sequence of the polymorphism is complementary to that reported in the previously mentioned Japanese study. However, the 2 polymorphisms are identical. Under this assumption, the allele frequencies differ significantly among the Japanese controls in that particular study and the white controls in our study (27.8% versus 14.1%, respectively; P=0.0003).

Conclusions—The genetic polymorphism in the vicinity of 3′ end of exon 7 in the endoglin gene was not significantly associated with the occurrence of intracranial aneurysms in the white population. There are ethnic-related differences of allele frequencies between our white controls and the previously reported Japanese controls. (Stroke. 2001;32:2689-2694.)

Key Words: aneurysm ■ cerebral vessels ■ extracellular matrix ■ polymorphism (genetics)

Subarachnoidal hemorrhage (SAH) continues to represent a serious disease with a 30-day mortality rate ranging between 32% and 59%1-4 and management morbidity ranging between 9.1% and 46%.5-8 In addition, this disease constitutes a major public health problem causing estimated lifetime costs of almost $2 billion for hospitalized patients with SAH in the United States.9 The main reason for spontaneous SAH is ruptured cerebral aneurysm.10-11 The mean incidence in Western Europe and North America is 10 per 100 000 per year and has been constant over the last few decades.1,2,3

In addition to hemodynamic factors, elevated arterial blood pressure and environmental and genetic factors are thought to play a role in the pathogenesis of cerebral aneurysms.14,15 Recent studies point to the importance of remodeling processes in the extracellular matrix of cerebral arteries and aneurysm walls.16,17 Elevated circulating levels of members of the matrix metalloproteinase family17 and an increased focal activity of proteolytic enzymes in the aneurysm tissue16,18 may contribute to structural weakness of the arterial vessel wall, which, in turn, may be related to aneurysm formation and growth. Dysregulation of enzymatic activity can be caused by genetic variations. Therefore, it is reasonable to consider a role for genetic variants of genes influencing the network of the extracellular matrix in the pathogenesis of intracranial aneurysms.

Endoglin is a transforming growth factor-β-binding protein expressed on cell surfaces of endothelial cells. The importance of endoglin for vascular development and structural maintenance of the vessel wall has recently been demonstrated by a knockout mouse model. Mice lacking an intact endoglin gene die from defective vascular development at gestational day 11.5.19 These findings support the results of previous studies in which loss-of-function mutations in the
human endoglin gene were found to cause hereditary hemorrhagic telangiectasia (HHT1), a dominantly inherited vascular disorder.\textsuperscript{20,21}

In a recent study by Takenaka et al.,\textsuperscript{22} an insertion polymorphism in intron 7 of the endoglin gene was found to be significantly associated with the occurrence of cerebral aneurysms in a Japanese population.

To investigate whether this insertion polymorphism of the endoglin gene is associated with the occurrence of cerebral aneurysms in white individuals, we analyzed 121 white patients who had been treated for cerebral aneurysms, 124 healthy white blood donors, and 15 Japanese volunteers by use of a polymerase chain reaction (PCR)-based and automated laser fluorescence sequencer evaluation.

Subjects and Methods

Study Population

The study population consisted of 260 aneurysm patients and 2 control groups. The patients consisted of 121 unrelated consecutively recruited patients with intracranial aneurysms (49 men and 72 women, mean age 51 [range 23 to 75] and 53 [range 29 to 73] years, respectively). All patients presented with at least 1 aneurysm, which was confirmed by cerebral angiography, and they were all operated on or treated by an endovascular approach between 1997 and 1998 at the Department of Neurosurgery or Department of Neuroradiology, respectively, University of Technology, Dresden. Patients were all residents of the Dresden urban area. HHT1 was excluded by clinical signs and taking the patients' histories. Further details are listed in Table 1.

One control group consisted of 124 (62 male and 62 female) anonymous, healthy, blood donors enrolled from the same urban area. Because of its historical background, the city of Dresden is populated by a homogenous white population. In general, blood donation is performed by all social classes in Germany. All potential blood donors are routinely screened for disease by use of a questionnaire, red blood cell and white blood cell counts, liver function parameters, and viral titers (details on request).

Another group consisted of 15 (7 male and 8 female) randomly selected anonymous volunteers of Japanese origin, who were employees of a Japanese company in various German cities.

The present study was approved by the local ethics committee. Informed written consent for genetic analysis was obtained from all nonanonymous individuals.

Amplifying the Endoglin Locus

Peripheral venous blood from aneurysm patients and 2 mL of buffy coat from anonymous blood donors were used for isolation of genomic DNA according to instructions accompanying the isolation kit purchased (Nucleo Spin C&T extraction kit, Macherey-Nagel). PCR was used for amplifying exon 7 and adjacent intron 7 sequences, including the insertion polymorphism. The following primers were used (sense primer according to previous reports\textsuperscript{22}):

\begin{verbatim}
5’-GAAAGCTGGCATAACGCTT-3’ and 5’-GGCTCAAGAGA-GCTGTAGTT-3’ (Amersham Pharmacia Biotech). One micromole per liter of each primer (both were cyanine 5-end-labeled) was mixed with 100 ng genomic DNA, 200 \mu M deoxynucleotide triphosphates (Promega), 2.0 \mu M MgCl\textsubscript{2}, 10× PCR buffer [50 \mu M KCl, 500 \mu M Tris buffer, 160 \mu M (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, pH 8.8, and 0.1% Tween 20], and 0.5 U Taq polymerase (all from InviTek GmbH) to a final volume of 25 \mu L.
\end{verbatim}

Amplifying conditions were as follows: initial denaturation at 94°C for 4 minutes was followed by 35 cycles of denaturation at 94° for 30 seconds, annealing at 60° C for 1 minute, and extension at 72°C for 1 minute and was completed by a final extension step at 72°C for 5 minutes with the use of a thermal cycler (Perkin Elmer, Applied Biosystems GmbH). For a negative control, water was used instead of genomic DNA in PCR.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>49</td>
<td>72</td>
</tr>
<tr>
<td>Mean age (range), y</td>
<td>51.2 (23–75)</td>
<td>52.7 (29–73)</td>
</tr>
<tr>
<td>SAH, n</td>
<td>109</td>
<td>12</td>
</tr>
<tr>
<td>Location</td>
<td>ACA, n</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>MCA, n</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>ICA, n</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>PICA, n</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>BA, n</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Others, n</td>
<td>3</td>
</tr>
<tr>
<td>Multiple, n</td>
<td>7*</td>
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<tr>
<td>Risk factors</td>
<td>Former history of SAH, n</td>
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<td></td>
<td>Familial history of SAH, n</td>
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<tr>
<td></td>
<td>Current or former smoker, n</td>
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</tr>
<tr>
<td></td>
<td>Hypertension, n</td>
<td>46</td>
</tr>
<tr>
<td>Treatment</td>
<td>Clipping, n</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>Endovascular procedure, n</td>
<td>32</td>
</tr>
</tbody>
</table>

Analysis of PCR products was performed on an automated laser fluorescence sequencer (ALFexpress, Pharmacia Biotech AB). Denaturing 6.5% Long Ranger gels were used and prepared according to the manufacturer’s instructions (FMC Bioproducts). Gels were run at 40 W (1000 V, 38 mA) for 4 hours. A size, ranging from 50 to 500 bp, was used (Amersham Pharmacia Biotech UK Ltd). Runs were analyzed by Fragment Manager (Pharmacia Biotech AB) software. Genotypes were read independently by 2 experienced molecular geneticists who were blinded to phenotype.

DNA Sequencing

PCR products, amplified under the aforementioned conditions, were electrophoresed on a 0.8% agarose gel, and bands were cut out and eluted. Three microliters of the eluted PCR product were used in the reaction mix, to which 1 \mu L Thermo Sequenase Mix (Amersham Pharmacia Biotech UK Ltd) and 1 \mu M cyanine 5-end-labeled antisense primer were added. Cycling reaction was performed under the following conditions with use of a thermocycler: 94°C for 4 minutes, followed by 25 cycles at 94°C for 30 seconds and at 58°C for 30 seconds and then at 72°C for 5 minutes. Cycling products were run on ALFexpress by using denaturing 6.5% Long Ranger gels. Runs were analyzed by using ALF Evaluation (Pharmacia Biotech AB) software.

Statistical Analysis

Deviations from Hardy-Weinberg equilibrium were analyzed by using the \chi^2 goodness-of-fit test for both study groups. We
applied the exact 2-sided Cochran-Armitage trend test for genotypes and the asymptotic 2-sided Yates-corrected $\chi^2$ test for alleles to investigate association. The power of the present study was analyzed at the 5% test level by the asymptotic 2-sided Yates-corrected $\chi^2$ test with use of the allele frequencies reported by Takenaka et al22 for their insertion polymorphism; the wt/wt genotype (wild-type) reflecting the PCR product including the 5 bp of intronic sequences adjacent to the 3’ end of exon 7 has been published by 28,29 In intron 7 of the endoglin gene, a 6-bp insert, 5'-TCCCCC-3’, starting 23 bases distal from the 3’ end of exon 7 (Figure), which was found in 2 patients with the homozygous insertion and 2 white control individuals with the homozygous insertion. This polymorphism has already been described by others (GenBank, accession No. AH006911).21,23 Takenaka et al22 reported a 6-bp insert, 5’-GGGGGA-3’, which was previously described by Alberts et al,20 who identified the polymorphism in the antisense strand.

In concordance with the Japanese group, there were no mutations detected in exon 7.

An additional control group consisting of 15 unrelated, healthy, anonymous Japanese volunteers was analyzed. In this group, the wild-type genotype was present in 7 (46.7%) samples, the heterozygous genotype was present in 6 (40.0%) samples, and the homozygous insertion was found in 2 (13.3%) samples. Therefore, the allele harboring the polymorphism was found in 10 (33.3%) of 30 alleles. Sequence analysis of sense and antisense strand revealed the same polymorphism found in our white patients and controls.

### Discussion

Recently, Takenaka et al22 described a 6-bp insertion polymorphism of the endoglin gene located in the intron 7, starting 26 bp distal from the 3’ end of exon 7, which was found significantly more frequently in a Japanese population with intracranial aneurysms than in a control group of patients and volunteers without cerebral aneurysms.

We studied the intronic polymorphism for several reasons. One reason was that the genetic polymorphism might vary between different ethnic groups.24,25 Furthermore, the endoglin gene has been analyzed by several investigators, and various mutations that were all related to the occurrence of HHT1 have been found.21,26,27 However, the polymorphism in intron 7 was found to be associated with the occurrence of intracranial diseases, such as intracerebral hemorrhages and cerebral aneurysms.20,22 Genetic variants located in introns might influence a phenotype via regulation of the transcription.28,29 In intron 7 of the endoglin gene, a 6-bp insert, 5’-TCCCCC-3’, starting 23 bp distal from the 3’ end of exon 7 has been published by others (GenBank accession No. AH006911). The sequence of that polymorphism was reported to initiate binding sites for activator protein-2, a frequent transcription factor in the human genome.30

In the present study, we used a control population consisting of anonymous blood donors and not a population-based control group or specifically selected controls as described by Morton and Collins.31 Major diseases have been excluded in the control group by taking the history of the individual and by physical and biochemical testing. The prevalence of unruptured cerebral aneurysms is $\approx 2\%$ in Western Europe (except Finland) and the United States32; ie, in our 124 white controls, there are

### Results

Clinical characteristics of the 121 patients with intracranial aneurysms are listed in Table 1. The power of the present study was 85% at the 5% test level, based on the allele frequencies reported by the Japanese group.22

<table>
<thead>
<tr>
<th>Group</th>
<th>wt/wt</th>
<th>wt/I</th>
<th>I/I</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>White</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aneurysm, n (%)</td>
<td>88 (72.7)</td>
<td>31 (25.6)</td>
<td>2 (1.7)</td>
<td>121</td>
</tr>
<tr>
<td>Control, n (%)</td>
<td>91 (73.4)</td>
<td>31 (25.0)</td>
<td>2 (1.6)</td>
<td>124</td>
</tr>
<tr>
<td>Japanese*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aneurysm, n (%)</td>
<td>32 (39)</td>
<td>33 (40)</td>
<td>17 (21)</td>
<td>82</td>
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<tr>
<td>Control, n (%)</td>
<td>58 (51)</td>
<td>49 (43)</td>
<td>7 (6)</td>
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*Takenaka et al.22

No deviations from Hardy-Weinberg equilibrium were found ($P=0.70$ for the aneurysm group; $P=0.74$ for the white control group).

Sequence analysis revealed a 6-bp insertion polymorphism, 5’-TCCCCC-3’, starting 23 bases distal from the 3’ end of exon 7 (Figure), which was found in 2 patients with the homozygous insertion and 2 white control individuals with the homozygous insertion. This polymorphism has already been described by others (GenBank, accession No. AH006911).21,23 Takenaka et al22 reported a 6-bp insert, 5’-GGGGGA-3’, which was previously described by Alberts et al,20 who identified the polymorphism in the antisense strand.

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### Nomenclature

We refer to GenBank accession Nos. U37445, U37444, U17156, and U37443 regarding the homo sapiens endoglin gene, exons 5 through 8, and GenBank accession No. AH006911 regarding the 6-bp DNA insert. Results were interpreted while the sense strand was read in the 5’ to 3’ direction.

### TABLE 2. Endoglin Genotype in Our White Aneurysm Patients and Control Population Compared With a Japanese Population

<table>
<thead>
<tr>
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</table>

*Takenaka et al.22

No deviations from Hardy-Weinberg equilibrium were found ($P=0.70$ for the aneurysm group; $P=0.74$ for the white control group).
only 2 or 3 individuals expected to carry an intracranial aneurysm. As discussed by Owen et al., because of the low population prevalence, it is not necessary to use a sex- and age-matched control population proven to be free of intracranial aneurysms. Regarding these aspects, it does not seem warranted to apply an invasive diagnostic tool such as the intra-arterial digital subtraction angiography to exclude a cerebral aneurysm for certain in the healthy control subjects.

Furthermore, we might consider the ethnic and social distribution of cases and controls equal, inasmuch as the urban area of Dresden is populated by a homogeneous white population because of the historical background of the city, and blood is donated by people in all social classes.

Our group of aneurysm patients and white controls demonstrated a statistical power of 85% at the 5% test level for detecting an association between the intron 7 insertion polymorphism and cerebral aneurysms, and the study group would have been large enough to accurately determine such an association, considering the lower allele frequency of the insertion polymorphism in the white population.

In our population of white patients and controls, we also found the polymorphism 5'-TCCCCC-3', which is well known (GenBank accession No. AH006911).

However, the allele frequencies were equally distributed between white patients and controls (Table 2), suggesting that this polymorphism is not associated with the occurrence of cerebral aneurysms in that ethnic group.

The insertion polymorphism of the endoglin gene described by Takenaka et al. was previously reported by Alberts et al. to be associated with the occurrence of sporadic intracerebral hemorrhage in a North American population. In that study, 208 of 285 participants were white. In their study, Alberts et al describe the antisense sequence of the polymorphism AH006911, which we confirmed in our white and Japanese populations by sense and antisense sequencing.

The allele frequencies of the insertion polymorphism in our white control population and in the controls in the study of Alberts et al. are almost identical (35 [14.1%] of 248 and 80 [19.8%] of 404, respectively). Furthermore, 20 patients of the control population in the study by Alberts et al presenting with aneurysmal SAH did not show a higher prevalence for the insertion polymorphism, which is in agreement with our findings.

The study by Takenaka et al. in Japanese individuals described the same polymorphism as mentioned by Alberts et
al., indicating that the polymorphism that we reported is also identical to that identified by the Japanese group. In spite of the higher prevalence of cerebral aneurysms in the Japanese study population (≈4%–8%), Takenaka et al showed that allele frequencies for the insertion polymorphism are significantly different between aneurysm patients and controls in that Japanese population \(P<0.01\). It should be emphasized that the allele frequency of the insertion polymorphism in Japanese controls is much higher than in our white controls (63 \([27.6\%]\) of 228 and 35 \([14.1\%]\) of 248, respectively), which represents a significant difference \(P=0.0003\).

Obviously, there is an ethnic-related difference of the endoglin intron 7 insertion polymorphism between Japanese and white controls.

This could be explained either by the fact that the function of a polymorphic sequence might be influenced by a different genetic and environmental background in different ethnic groups or by the possibility that this intron 7 insertion polymorphism might not be related to the pathogenesis of intracranial aneurysms. Ethnic-related differences of genetic polymorphisms are reported for various genes, and this aspect should be considered in the interpretation of results from future case-control studies of candidate genes. In agreement with the Japanese study by Takenaka et al., we did not find any mutation in exon 7. Nevertheless, these results cannot yet exclude the possibility that there is a mutation in another region of the endoglin gene contributing to a functional change of the endoglin protein. For a multifunctional protein such as endoglin, it is difficult to prove an influence on a single biochemical pathway, because even an altered protein function might remain undiscovered, counterbalanced by another enzyme in the pathway. It would still be of interest to look at other genomic regions of the endoglin gene for further polymorphisms, in particular in different ethnic groups.

In conclusion, we have shown that there is no evidence of an association between an intron 7 insertion polymorphism of the endoglin gene and aneurysm development in our white population. Therefore, this genetic variant might not play a decisive role in the pathogenesis of cerebral aneurysms in white individuals. It is remarkable that the allele frequencies for that particular genetic polymorphism are different between the white and Japanese control populations. This is an important aspect that should be considered in future investigations of candidate genes for aneurysm development.

Acknowledgment

We are grateful to Shirley Faatz for her careful editorial assistance.

References


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