Endoglin Is Not a Major Susceptibility Gene for Intracranial Aneurysm Among Japanese

Hideaki Onda, MD; Hidetoshi Kasuya, MD; Taku Yoneyama, MD; Tomokatsu Hori, MD; Toshiaki Nakajima, MD; Ituro Inoue, MD

Background and Purpose—A 6-base insertion (6bINS) polymorphism in intron 7 of the endoglin gene (ENG), which codes for a component of the transforming growth factor-β receptor complex, was reported to be associated with intracranial aneurysm (IA) in a Japanese population. A recent report using a white population could not replicate the association. We tested for this association with high statistical power in our independent Japanese subjects and evaluated the linkage between markers on chromosome 9, which contains ENG, and IA.

Methods—The sample for the linkage study comprised 179 individuals with IA in 85 nuclear families, with 104 possible affected sibpairs. For the association study of the 6bINS polymorphism and 4 single nucleotide polymorphisms (SNPs) in ENG, 172 Japanese patients with IA and 192 control subjects were examined.

Results—There was no evidence of linkage in the vicinity of ENG by analysis of affected sibpairs. The allele frequency of the 6bINS polymorphism was 104 of 344 (30.2%) in the total IA group and 122 of 382 (31.9%) in the control group. The statistical difference in allele frequency between the 2 groups was not significant (χ²=0.245, df=1, P=0.620). The power of the present association study was 98.3% at a significance level of 0.05 on the basis of the allele frequencies in the previous study. In addition, no associations between the 4 SNPs in ENG and IA were detected.

Conclusions—We provide evidence that there is no association between the 6bINS polymorphism or 4 SNPs in ENG and IA and that there is no linkage between the ENG locus and IA, indicating that ENG is not a major susceptibility gene for IA in Japanese.

Key Words: association ■ genes ■ intracranial aneurysm ■ linkage (genetics)

Rupture of intracranial aneurysms (IAs [MIM105800]) causes subarachnoid hemorrhage with high morbidity and mortality1–4 and continues to be a major public health problem. Although genetic and environmental factors are thought to play important roles in the pathogenesis of IA, recent advances in molecular genetics make it possible to investigate the genetic determinants directly. We previously reported a genome-wide linkage study of IA in affected Japanese sibpairs in whom positive evidence of linkage on chromosome 5q22-31, 7q11, and 14q22 was detected.5

In a recent study by Takenaka and colleagues,6 a 6-base insertion (6bINS) in intron 7 of the endoglin gene (ENG [MIM131195]) was reported to be genetically associated with IA in a Japanese population. In addition, Krex et al7 reported that although 6bINS was not significantly associated with IA in a white population, there were ethnicity-related differences in allele frequencies between white and Japanese control subjects. We have carefully tested for allelic associations between 6bINS and 4 single nucleotide polymorphisms (SNPs) located in ENG with IA in 172 affected individuals; we also tested for linkage between chromosome 9, which contains ENG, and the disease in 104 affected sibpairs. We found that there is no discernible association in the Japanese population.

Subjects and Methods

Affected subjects had at least 1 IA >5 mm in diameter, as diagnosed by conventional angiography, 3-dimensional CT angiography, MR angiography, or surgical findings. The sample for the linkage study comprised 179 individuals in 85 nuclear families enrolled through neurosurgical services certified by the Japan Neurosurgical Society; the number of possible affected sibpairs was 104.8 The collected sample comprised 77 pairs, 7 trios, and 1 quartet of siblings with IA. The detailed clinical features of these families have been reported elsewhere.8 For the association study, 172 Japanese patients with IA (total IAs: 70 male, 102 female; mean±SD age, 59.8±10.5 years) were enrolled. Of the 172 patients, 87 had first-degree relatives with IA (familial IAs; 27 male, 60 female; mean±SD age, 60.7±9.7 years), and the other 85 patients had no family history of IA (sporadic IAs; 43 male, 42 female; mean±SD age, 58.9±11.1 years). Characterizations of patients with IA are summarized in Table 1. In women, unruptured IA and IA at the middle cerebral artery were more predominant in familial IAs than in sporadic IAs. One hundred ninety-two control subjects (91 male, 101 female;
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Annealing at 55°C for 15 seconds, and extension at 72°C for 30°C followed by 35 cycles of denaturation at 94°C for 15 seconds, were as follows. Initial denaturation was at 95°C for 12 minutes, followed by 35 cycles of denaturation at 94°C for 15 seconds, and extension at 72°C for 30°C, and was completed by a final extension at 72°C for 10 minutes with a thermal cycler (GeneAmp PCR System, model 9700, Applied Biosystems). Polymerase chain reaction (PCR) conditions in the reaction mixture containing 1.5 mmol/L MgCl2, and 0.2 U AmpliTaq Gold DNA Polymerase (Applied Biosystems), 300 nM of each primer, 200 nM dNTP mix, 1.5 mmol/L MgCl2, and 0.2 U AmpliTaq Gold DNA Polymerase (Applied Biosystems). Polymerase chain reaction (PCR) conditions were as follows. Initial denaturation was at 95°C for 12 minutes, followed by 35 cycles of denaturation at 94°C for 15 seconds, annealing at 55°C for 15 seconds, and extension at 72°C for 30 seconds, and was completed by a final extension at 72°C for 10 minutes with a thermal cycler (GeneAmp PCR System, model 9700, Applied Biosystems).

Twenty microsatellite markers spanning chromosome 9 were used for the linkage analysis. Of the 20 markers, 17 included in Linkage Mapping Set version 2 (Applied Biosystems) were used as described before.5 The other 3 markers (D9S265, D9S1678, D9S166) obtained from online information (http://www-genome.wi.mit.edu) were added to the original set to fill in gaps >20 cM.6 The average interval between neighboring markers was 8.1 cM; the maximum interval, between D9S175 and D9S167, was 13.6 cM. The average heterozygosity of the 20 markers was 0.744 in 64 unrelated healthy Japanese.

PCR amplification was performed for genotyping of the 6bINS polymorphism in intron 7 of ENG with the following primers: 6FAM-6-carboxy-fluorescein labeled 5'-GAGGCGCTGACATAACCCT-3' and 5'-GTTTCTCTTGGGCACTCATCCAAAGG-3'. By placing the GTATTCTT sequence on the 5' end of the primer, the PCR product resulted in nearly 100% adenylation at the 3' end of the antistrand, which facilitates accurate genotyping.10

Verification and Genotyping of SNPs in ENG

Direct sequencing was performed with a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems) on PCR-amplified segments. Fifteen SNPs in ENG had been previously registered on the NCBI SNP database (http://www.ncbi.nlm.nih.gov/SNP). Of the 15 SNPs, 4 had allele frequencies >5% and were not in linkage disequilibrium (LD) with each other. The 4 SNPs were genotyped in 192 control subjects and 172 IA patients by direct sequencing, and allele frequencies were compared between groups. Sequences of primers used in this study and PCR conditions are shown in Table 2.

Statistical Analysis and Calculation

Multipoint nonparametric linkage analysis was performed by a maximum-likelihood method implemented in the GENEHUNTER program.11 Maximum limit of detection score was calculated by the method of possible triangle constrains. All sibpairs from sibships containing >2 affected individuals were counted, and the unweighted option was used.

Genotype and allele frequencies were compared between groups, and allelic association with IA was evaluated by χ2 test statistic. The power of the present analysis was calculated as follows13: n = 2nAnBn/(na + nb), where na and nb are the number of alleles tested in patients with IA and control subjects, respectively; h = θA − θb, θb = 2 arcsin(pA1/2) and θb = 2 arcsin(pB1/2), where pA and pB are the frequency of alleles with 6bINS in patients with IA and control subjects, respectively; Z1−β = h(α/2)1/2 − Zα/2 (formula 1), where α and β are type I and II errors, respectively; and the quantity of Z1−β gives the probability of normal distribution with zero mean.

Results

Linkage Study

We tested for linkage across chromosome 9 in a maximum of 104 Japanese affected sibpairs. ENG lies at ~140 cM from the petit-arm-terminal end (pter) of chromosome 9. Although there was marginal evidence of linkage near the marker of D9S288 (maximum limit of detection score, 1.19), located 8.8 cM from pter, we could not find any evidence for linkage in the vicinity of ENG (Figure 1).

### TABLE 1. Characterization of Patients With IAs for Association Study

<table>
<thead>
<tr>
<th>Group</th>
<th>Familial</th>
<th>Sporadic</th>
<th>P (df=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Familial history of IA</td>
<td>Positive</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>87</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>Mean (SD) age, y</td>
<td>60.7 (9.7)</td>
<td>58.9 (11.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (M/F), n</td>
<td>27/60</td>
<td>43/42</td>
<td>0.0091*</td>
</tr>
<tr>
<td>Ruptured IA, n</td>
<td>61</td>
<td>77</td>
<td>0.0007*</td>
</tr>
<tr>
<td>Multiple IA, n</td>
<td>15</td>
<td>13</td>
<td>NS</td>
</tr>
<tr>
<td>Location, n</td>
<td>38</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>ACoA</td>
<td>18</td>
<td>27</td>
<td>NS</td>
</tr>
<tr>
<td>MCA</td>
<td>40</td>
<td>22</td>
<td>0.0061*</td>
</tr>
<tr>
<td>ICA</td>
<td>16</td>
<td>22</td>
<td>NS</td>
</tr>
<tr>
<td>Other</td>
<td>12</td>
<td>24</td>
<td></td>
</tr>
</tbody>
</table>

ACoA indicates anterior communicating artery; MCA, middle cerebral artery; and ICA, internal carotid artery.

*Female, unruptured IA, and IA at MCA were predominant in familial IAs.

Mean±SD age, 59.0±16.5 years were enrolled from outpatients of Tokyo Women’s Medical University Hospital who presented with headache or other neurological complaints. Selected control subjects had no history of subarachnoid hemorrhage and showed no evidence of IA on conventional brain CT examination. Mean age and distribution of sexes in control subjects were matched to those in total IAs. All affected and control subjects were of Japanese ethnicity. The ethics committee at Tokyo Women’s Medical University approved the study protocol, and all participants or family members gave written, informed consent.

Genotyping

Microsatellite alleles were determined by a fluorescence-based technique on a DNA Sequencer, model 377 (Applied Biosystems). The microsatellite alleles were assigned by GENESCAN and GENOTYPER software (Applied Biosystems). Genomic DNA was extracted from peripheral whole blood with a Genomix kit (TALEN, Trieste) according to the supplier’s manual. Then, 5 to 10 ng genomic DNA was amplified in a total of 6 μL reaction mixture containing 1× AmpliTaq Gold Buffer (Applied Biosystems), 300 nM of each primer, 200 nM dNTP mix, 1.5 mmol/L MgCl2, and 0.2 U AmpliTaq Gold DNA Polymerase (Applied Biosystems). Polymerase chain reaction (PCR) conditions were as follows. Initial denaturation was at 95°C for 12 minutes, followed by 35 cycles of denaturation at 94°C for 15 seconds, annealing at 55°C for 15 seconds, and extension at 72°C for 30 seconds, and was completed by a final extension at 72°C for 10 minutes with a thermal cycler (GeneAmp PCR System, model 9700, Applied Biosystems).

### TABLE 2. Sequences of Primers and PCR Conditions for Genotyping SNPs in Endoglin

<table>
<thead>
<tr>
<th>SNP</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Product Size, bp</th>
<th>AT, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>INT1</td>
<td>GGCACCTAGCGCTTTCCTCTG</td>
<td>ACAGCATTCATGGCCTACAAT</td>
<td>931</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>CTGGTGCCAGTGCCGCA*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INT2</td>
<td>CTGGGCACTGGACACACTAC</td>
<td>GTGCCTTCAGCAATTCTTC</td>
<td>335</td>
<td>64</td>
</tr>
<tr>
<td>EX8</td>
<td>GGCCACACAGTGACACACAGA</td>
<td>CCAACGTCAAGGAGATTAG</td>
<td>496</td>
<td>64</td>
</tr>
<tr>
<td>INT11</td>
<td>CCCCATCCTCAGGGCAATCTA</td>
<td>AGCAGATAGTGGCCACCTTTG</td>
<td>456</td>
<td>64</td>
</tr>
</tbody>
</table>

AT indicates annealing temperature.

*Internal primer for sequence.
Association Study With SNPs in ENG

Sequencing in Japanese subjects revealed 5 SNPs, registered on the GenBank database as rs1998923, rs2417056, rs1800956, rs1330683, and rs1330684, to have relatively high allele frequencies. Evaluation of degrees of pairwise LD between combinations of the 5 SNPs indicated that 4 SNPs were not in LD with each other. Because allele frequencies of SNPs in strong degrees of pairwise LD show similar consequences in general, these 4 SNPs were selected for association studies. The 4 SNPs are located in intron 1, 2, and 11 and exon 8 of ENG and are designated INT1 (rs1998923), INT2 (rs2417056), INT11 (rs1330683), and EX8 (rs1800956), respectively (Figure 2). Allele frequencies of the 4 SNPs were compared in 192 control subjects versus 172 total IAs or 87 familial IAs (Table 3). Although a weak allelic frequency difference between total IAs and control subjects was detected for INT1 ($\chi^2=4.022, df=1, P=0.045$), the statistical differences of the other 3 SNPs in allele frequency between total IAs and control subjects were not significant (INT2: $\chi^2=1.129, df=1, P=0.288$; EX8: $\chi^2=0.902, df=1, P=0.342$; INT11: $\chi^2=0.342, df=1, P=0.559$). Similar results in allele frequencies between control subjects and familial IAs were observed for the 4 SNPs.

Association Study for Replication

The genotype frequency of the 6bINS polymorphism was compared between IAs and control subjects (Table 4). We detected the homozygous and heterozygous insertion in 12.8% and 34.9% of total IAs and in 13.1% and 37.7% of control subjects, respectively. Distributions of the genotype frequency in all groups were in Hardy-Weinberg equilibrium. Statistical differences in genotype frequency between the total IAs and control subjects ($\chi^2=0.376, df=2, P=0.829$) and even between familial IAs and control subjects ($\chi^2=0.553, df=2, P=0.758$) were not significant. The allele frequency of 6bINS was 104 of 344 total IAs (30.2%), 58 of 174 familial IAs (33.3%), and 122 of 382 control subjects (31.9%). Differences in allele frequency between total IAs and control subjects ($\chi^2=0.245, df=1, P=0.620$) and between familial IAs and controls ($\chi^2=0.106, df=1, P=0.744$) were not significant.

Figure 1. Multipoint linkage analysis on chromosome 9 in 104 Japanese sibpairs with IA. Twenty microsatellite markers covering chromosome 9 were tested for multipoint linkage on the GENEHUNTER program with 104 IA sibpairs. There was only marginal evidence of linkage near the marker of D9S288 (maximum limit of detection score, 1.19), located 8.8 cM from the pter of chromosome 9. There was no evidence for linkage in the vicinity of the endoglin gene at -140 cM from pter (indicated by arrow).

Figure 2. Exon-intron organization of ENG and positions of 6bINS and SNPs. ENG consists of 15 exons spanning 39.7 kb. Localization of 6bINS and the 4 SNPs (INT1, INT2, EX8, and INT11) within ENG are indicated by arrows.
**Table 3. Allelic Association Study of Patients With IA and Control Subjects With SNPs in Endoglin**

<table>
<thead>
<tr>
<th>SNP</th>
<th>Accession Number</th>
<th>Location (Position*)</th>
<th>Change</th>
<th>Allele Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>INT1</td>
<td>rs1998923</td>
<td>Intron 1 (+3663)</td>
<td>C→T</td>
<td>0.253 (92/364)</td>
</tr>
<tr>
<td>INT2</td>
<td>rs2417054</td>
<td>Intron 2 (+6449)</td>
<td>C→T</td>
<td>0.049 (18/368)</td>
</tr>
<tr>
<td>EX8</td>
<td>rs1800956</td>
<td>Exon 8 (+105)</td>
<td>G→C</td>
<td>0.048 (17/354)</td>
</tr>
<tr>
<td>INT11</td>
<td>rs1330683</td>
<td>Intron 11 (+589)</td>
<td>C→G</td>
<td>0.342 (128/374)</td>
</tr>
</tbody>
</table>

*Number of nucleotides from start of intron or exon.*
†\( P<0.05.\

**Power Calculation**

The power required in the present study to detect a specified difference in the frequency of the 6bINS allele (Table 5) was calculated as follows. Let \( p_{IA} = 0.408 \) and \( p_{C} = 0.276 \) be the frequency of the 6bINS allele in patients with IA and control subjects, respectively, as illustrated in the study of Takenaka et al.\(^6\) The numbers of alleles tested in the present study were \( n_{IA} = 344 \) and \( n_{C} = 382 \) in patients with IA and control subjects, respectively. We computed as follows: \( \theta_{IA} = 2 \times \text{arsin}(0.408^{1/2}) = 1.387, \theta_{C} = 2 \times \text{arsin}(0.276^{1/2}) = 1.107, h = \theta_{IA} - \theta_{C} = 0.280, \) \( n = 2 \times 344 \times 382/(344 + 382) = 362, (n/2)^{1/2} = 13.45. \) To calculate power at a significance level of \( \alpha = 0.05, Z_{1-\alpha} \) was obtained from a table of the normal distribution: \( Z_{1-\alpha} = 1.65. \) Using formula 1 presented previously, we obtained \( Z_{1-\beta} = 2.12. \) This gives the value of \( t \) to be referred to the table of normal distribution to obtain the power \( 1 - \beta = 0.983. \)

**Discussion**

Endoglin, a homodimeric membrane glycoprotein, is highly expressed in human vascular endothelium and is a component of the transforming growth factor-β receptor complex.\(^13,14\) Li et al.\(^15\) reported that mice lacking \( ENG \) died of defective vascular development, the loss of \( ENG \) causing poor vascular smooth muscle development and arresting endothelial remodeling. Because many disease-related mutations in \( ENG \) have been identified for hereditary hemorrhagic telangiectasia type I, an autosomal dominant disorder characterized by multisystemic vascular dysplasia and recurrent hemorrhage,\(^16–18\) \( ENG \) is a possible candidate gene underlying IA. First, we tested for evidence of linkage in the vicinity of \( ENG \) on chromosome 9 with a maximum of 104 Japanese affected sibpairs by multipoint nonparametric linkage analysis. Our data showed no evidence of linkage in the region of \( ENG \) (Figure 1), indicating that \( ENG \) is not a major susceptibility locus for IA in Japanese. Because our genetic linkage study might lack the power to detect certain genetic causalities, we performed an allelic association study with the polymorphisms in \( ENG \) as already performed by Takenaka et al.\(^6\) but with a larger sample. Takenaka et al reported that the 6bINS polymorphism in \( ENG \) was associated with IA on the basis of an allelic association study in 82 IA patients and 114 control subjects of Japanese origin. Krex et al\(^7\) could not replicate the association between the polymorphism and IA in a white population and claimed that \( ENG \) might be a susceptibility gene only in people of Japanese ethnicity. However, using our larger sample size, we were able to confirm that there is no association between 6bINS in \( ENG \) and IA in the Japanese population (Table 4). Indeed, we extended the screening of the exons and their flanking regions in \( ENG \), testing 4 SNPs, and again found no association with IA. Because the promoter, 5' untranslated region, long residuals of the introns, and 3' untranslated region of \( ENG \) were not analyzed in the present study, we cannot exclude the possibility of other variants or genetic markers in the vicinity of \( ENG \) that are associated with the development of some types of IA.

Association-based methods in which the joint distribution of phenotypes and genotypes in a population are examined may well be more powerful than linkage-based approaches for the identification of the genes (loci) underlying common diseases.\(^19\) However, evidence from association studies must be considered with caution, especially because of ascertainment bias and population stratification. When the allelic association evidence of Takenaka et al\(^6\) is evaluated, 2 types of error, type I (\( \alpha \)) and type II (\( \beta \)), must be considered. Type I error occurs when a significant difference on the basis of sample estimates does not actually appear in the whole population. Type II error occurs when a difference cannot be detected in a sample of the population although the difference appears in the whole population. Accordingly, type II error becomes the dominant concern if the test statistic does not reach significance. Type II error complement \( 1 - \beta \), or “power,” is defined as the probability of rejecting the null hypothesis (\( H_0 \); a genetic marker occurs at equal frequency in cases and controls) when it is false.\(^12\) With our sample size, the power of the present study was 98.3% at a significance level of 0.05 on the basis of the allele frequencies in the study of Takenaka et al.\(^6\) Strictly speaking, the Takenaka et al study achieved a significance level of 0.006, so negative evidence might require a power of 99.4%. However, because of the greater sample size, this difference in power should be a very small factor.

Although the positive association between IA and \( ENG \) observed by Takenaka et al\(^6\) was not found in our sample, our data are not incompatible with the role of some \( ENG \)
polymorphisms in susceptibility to some types of IA. However, the present study clearly shows that there is no association between the 6bINS polymorphism of ENG and IA or between 4 SNPs in ENG and IA and that ENG is not a major susceptibility gene for IA among Japanese.

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