Confirmation of an Association of Single-Nucleotide Polymorphism rs1333040 on 9p21 With Familial and Sporadic Intracranial Aneurysms in Japanese Patients

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Background and Purpose—Genetic factors are important determinants of intracranial aneurysm (IA). Recently, a multinational, genome-wide association study identified 3 loci associated with IA, located on 2q (rs700651), 8q (rs10958409), and 9p (rs1333040 and rs10757278). The aim of this study was to evaluate these associations.

Methods—Familial and sporadic cases were investigated. Familial cases, consisting of 96 subjects with IA, and 46 subjects of unknown status from 31 pedigrees were analyzed with the transmission disequilibrium test and linkage analysis. Associations of single-nucleotide polymorphisms (SNPs) with IA were tested in 419 sporadic IA cases and in 408 control subjects. Sequencing of CDKN2A, CDKN2B, and CDKN2BAS revealed additional SNPs, and their associations with IA were also tested.

Results—The transmission disequilibrium test revealed associations of 2 SNPs, rs700651 (P=0.036) and rs1333040 (P=0.002), with familial IA. Analysis of SNPs in sporadic cases revealed an allelic association of rs1333040 with IA (odds ratio 1.28; 95% CI, 1.04–1.57; P=0.02) but failed to show associations of rs10757278 and rs496892 with IA. We sequenced 3 candidate genes; CDKN2A, CDKN2B, and CDKN2BAS. All 6 index cases from IA families had the rs1333040-T allele and SNPs (rs10965215, rs10120688, and rs7341791) in CDKN2BAS. None of these SNPs had linkage disequilibrium with rs1333040 and was associated with IA.

Conclusions—A region between introns 7 and 15 of CDKN2BAS carrying the rs1333040-T allele may confer risk for IA.

Key Words: genetics ■ intracranial aneurysm ■ association study ■ CDKN2BAS ■ single-nucleotide polymorphisms

Subarachnoid hemorrhage (SAH) is fatal in ~50% of cases, and significant disability is caused in ~30% of cases, despite the diagnostic and therapeutic developments of the past few decades. In Japan, the total annual mortality rate for SAH was estimated to be 22.5 per 100 000 person-years. Rupture of intracranial aneurysm (IA) accounts for >90% of SAH cases. Both environmental and genetic factors are associated with IA.

To clarify the genetic component of IA, we previously conducted genetic studies by using a multiplex IA family approach. Nonparametric linkage analysis revealed 3 loci located on 17q, 19q13, and Xp22, and parametric analysis revealed a locus on 19q13. On 17q, we found TNFRSF13B to be a candidate gene for IA. Several other studies have revealed several loci or candidate genes in different populations.

In 2008, Helgadottir et al. reported that the locus tagged by rs10757278 on chromosome 9p21 is a risk factor for IA, but not for diabetes mellitus. Bilguvar et al. reported a multistage, genome-wide association study of European and Japanese populations and identified 3 common single-nucleotide polymorphisms (SNPs) associated with IA on chromosomes 2q, 8q, and 9p. The aim of the present study was to investigate whether these associations could be replicated in the multiplex IA families and in sporadic Japanese IA cases.

Subjects and Methods

Study Population
Subjects from 2 groups participated. The first group consisted of subjects from previously reported families and from 3 additional families: 96 cases (male n=33; female n=63) from 31 pedigrees (Figure 1). Among the 29 previously reported pedigrees, some of...
the affected members could not be genotyped for rs700651, rs10958409, and rs1333040 because the DNA was exhausted by use in previous studies.

Pedigrees were recruited as previously reported. In short, when cases had ≥3 living family members with IAs or ≥2 living family members with SAH, including the index cases, their families were regarded as suitable for the present study. The index cases were confirmed to have saccular aneurysms from medical records. Clinical interview and screening by magnetic resonance angiography was performed in all available relatives age 30 years or older. In subjects suspected to have IAs large enough for clinical intervention, an additional examination (digital subtraction angiography or 3-dimensional computed tomography angiography) was conducted. In addition, 46 pedigree subjects (male n = 24; female n = 22) who did not meet the original inclusion criteria for genetic analysis because of their young age or phenotypic uncertainty were genotyped for the current study.

The second group consisted of 419 sporadic unrelated cases (male n = 142; female n = 277) and 408 unrelated controls (male n = 196; female n = 212) collected from several collaborative hospitals in

Figure 1. Pedigrees of families with IA and genotypes of individuals for rs700651 and rs1333040. Genotypes of 96 affected members are shown. Some cases have died since donating DNA samples. The identification numbers of the pedigrees are the same as previously reported. Owing to depletion of DNA, pedigree 26 was eliminated from the figure.
Japan. Sporadic cases were diagnosed by digital subtraction angiography or were confirmed to have IAs during surgery. Control subjects met the following criteria: (1) confirmation of the absence of IA by digital subtraction angiography, magnetic resonance angiography, or 3-dimensional computed tomography angiography; (2) an age at examination of ≥40 years; (3) no medical history of any stroke, including IA or SAH; and (4) no family history of IA or SAH in first-degree relatives.

For all affected participants, except the 46 newly genotyped subjects, past history, lifestyle (current smoking habit and drinking habit), and comorbidity were examined from clinical records or from questionnaires conducted during interview, as previously reported. For the 46 newly genotyped subjects, only their ages and relationships within pedigrees were known, whereas their IA status, comorbidities, and lifestyles were unexplored. The participation of these individuals was expected to provide greater genotype certainty in the family studies. We excluded cases or families with autoimmune diseases (including systemic lupus erythematosus, rheumatoid arthritis, and Takayasu arteritis) or known heritable diseases (including Ehlers-Danlos syndrome type IV, Marfan syndrome, neurofibromatosis type 1, and autosomal-dominant polycystic kidney disease). This study was approved by the ethics committee of the Kyoto University institutional review board, and appropriate informed consent was obtained from all subjects.

Genotyping
We performed genotyping of 8 SNPs (rs700651, rs10958409, rs496892, rs10965215, rs10120688, rs1333040, rs7341791, and rs10757278) by using the polymerase chain reaction invader assay with TaqMan probes (Applied Biosystems TaqMan SNP genotyping assays, Foster City, Calif). The rationale for selecting the 9p SNP set, rs496892, rs1333040, and rs10757278, was the linkage disequilibrium (LD) structure of SNPs on 9p21.3. The LD block can be divided into 2 major blocks: 1 associated with vascular diseases (vascular disease block) and 1 with diabetes mellitus (diabetes block). The SNPs selected are in the vascular disease block. The physical distances between rs496892 and rs1333040 and between rs1333040 and rs10757278 are 59 and 41 kb, respectively. rs10965215, rs10120688, and rs7341791 were selected on the basis of the sequencing results of cyclin-dependent kinase inhibitor 2B antisense RNA (CDKN2BAS).

Linkage Analysis and Transmission Disequilibrium Test for IA Pedigrees
Two-point nonparametric logarithm of the odds scores were calculated with GENEHUNTER (version 2.1.6) for 3 SNPs (rs700651, rs10958409, and rs1333040) in the 96 affected members and in the 46 newly genotyped subjects with a disease frequency of 0.001, a frequency ratio of 0.3 with a relative risk of 1.5 when D' = 0.9 for a genetic marker. The statistical power of the present study was 76% for α = 0.05 when the frequency of the risk allele was 0.3 with a relative risk of 1.5 when D' = 0.9 for a genetic marker.

Association Study of SNPs With IA
An association study was performed on the 419 sporadic unrelated cases and 408 unrelated control subjects with the SNP&Variation suite v7 (Golden Helix Inc, available at http://www.goldenhelix.com/) with or without adjustment for covariates, including sex, age, current smoking habit, and hypertension. D' and r² values were also calculated for SNPs around rs1333040. Statistical power was estimated with a power calculator (available at http://hpgu.mgh.harvard.edu/~purcell/gpc/cc2.html). The statistical power of the present study was 76% for α = 0.05 when the frequency of the risk allele was 0.3 with a relative risk of 1.5 when D' = 0.9 for a genetic marker.

Table 1. Characteristics of Familial Cases, Sporadic Cases, and Controls

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Familial Cases</th>
<th>Sporadic Cases</th>
<th>Controls</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects, n, male: female</td>
<td>96 (33/63)</td>
<td>419 (142/277)</td>
<td>408 (196/212)</td>
<td>0.000086*</td>
</tr>
<tr>
<td>Cases per pedigree, mean±SD</td>
<td>3.10±1.13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, mean±SD, y</td>
<td>60.5±13.6</td>
<td>60.0±10.7</td>
<td>62.0±10.1</td>
<td>0.025†</td>
</tr>
<tr>
<td>Cases with SAH, n (%)</td>
<td>60 (62.5%)</td>
<td>241 (57.5%)</td>
<td></td>
<td>0.37*</td>
</tr>
<tr>
<td>Subjects with hypertension, n (%)</td>
<td>39 (40.6%)</td>
<td>215 (51.3%)</td>
<td>162 (39.7%)</td>
<td>0.0024*</td>
</tr>
<tr>
<td>Subjects currently smoking, n (%)</td>
<td>37 (38.5%)</td>
<td>151 (36.0%)</td>
<td>161 (39.5%)</td>
<td>0.59*</td>
</tr>
</tbody>
</table>

*Comparison among familial cases, sporadic cases, and controls was made by the χ² test. †Comparison was made by ANOVA.

Sequencing Candidate Genes
Three genes, CDKN2A, CDKN2B, and CDKN2BAS, which are in the vicinity of rs1333040, were selected for sequencing in 6 arbitrarily selected index cases from the 31 families with IA. Six index cases were selected before initiation of the study without any biases from the genotype results. For CDKN2A and CDKN2B, we conducted direct sequencing of all coding exons, putative promoter sequences (2 kb upstream from the initiation codon), and 100 bp on either side of intron-exon boundaries. For CDKN2BAS, we sequenced 19 exons and 100 bp on either side of intron-exon boundaries.


Statistical Analysis
We conducted statistical analysis with SNP&Variation suite v7 software (Golden Helix Inc). Multiple comparisons were not corrected. A nominal P<0.05 was considered significant. Proportions were compared by the χ² test, and means were compared by ANOVA with SAS software (version 8.2; SAS Institute Inc, Cary, NC).

Results
Demographic Characteristics of the Study Population
Demographic data of the study population are shown in Table 1. For familial cases, an average of 3.10±1.13 (ie, mean±SD) cases (range, 2 to 7 cases) per family was included in this study (Figure 1). The proportions of female cases and of those with hypertension were different among the 3 groups (P<0.05), whereas the proportion of cases with a current smoking habit was not different. Hypertension was more prevalent in sporadic cases, and the proportion of females was significantly greater in the control group. No significant difference between groups was found in the proportion of cases with SAH (Table 1). To avoid confounding effects from
known risk factors (female sex, age, and hypertension), we adjusted for these factors in the association study.

Transmission Disequilibrium Test for SNP Association With IA in Familial Cases

The transmission disequilibrium test revealed significant associations of rs700651 (P = 0.036) and rs1333040 (P = 0.002; Figure 1 and Table 2) with IA, whereas the association of rs10958409 with IA was not significant (P = 0.962). Nonparametric logarithm of the odds scores indicated that none of the SNPs were significantly linked with IA (Table 2).

Association of SNPs With IA in Sporadic Cases

We then investigated the association of rs700651, rs10958409, rs496892, rs1333040, and rs10757278 with IA in sporadic cases. A significant association of rs1333040 with IA was shown by allelic association (P = 0.02) and by the additive model (P = 0.04), whereas associations of the other SNPs were not significant (Table 3). SNPs rs496892, rs1333040, and rs10757278 were not in strong LD in the study population (Figure 2). The allele frequencies of the risk allele rs1333040-T in controls and in cases were 0.64 and 0.70, respectively. These values are similar to those reported in Japanese subjects (controls:cases = 0.65:0.72), but the value for cases is larger than that reported for white subjects (controls:cases = 0.47:0.52 in Finland and 0.55:0.62 in the Netherlands).

Sequence Analysis of CDKN2A, CDKN2B, and CDKN2BAS

rs1333040 is located deep in the 12th intron of CDKN2BAS; therefore, we searched for more substantial genomic variants than rs1333040. CDKN2A and CDKN2B are reported to be associated with cell proliferation, aging, senescence, and apoptosis, and the transcriptional regulation of CDKN2BAS is coordinated with that of p16/CDKN2A and p15/CDKN2B. Therefore, we investigated whether 6 index cases from IA families harbor mutations in these genes. Sequencing failed to show new polymorphic variants in the coding regions of CDKN2A and CDKN2B. In contrast, several polymorphisms were found in CDKN2BAS (Table 4). It is of interest that a common haplotype was shared by the 6 index cases, who carry the rs1333040-T allele. Because rs10965215-A, rs10120688-A, and rs7341791-G were on the same haplotype, we investigated the associations of these 3 SNPs with IA. These SNPs, however, did not show either LD with rs1333040 (Figure 2) or association with IA (Table 3), indicating a possibility that the core risk haplotype is located between introns 7 and 15 of CDKN2BAS. The LD block for cases did not differ from that for controls (data not shown).

Discussion

Many studies have been performed to identify IA susceptibility gene(s). Some loci have been confirmed but others have not. Difficulties inherent in the identification of genetic risk factors are considered to be associated with population stratification, confounding nongenetic factors, or both. To overcome such difficulties, large-scale studies

Table 2. Significant Associations of rs700651 and rs1333040 With IA in 31 Pedigrees, Assessed by the TDT

<table>
<thead>
<tr>
<th>SNP</th>
<th>Position</th>
<th>P for TDT</th>
<th>NPL Score</th>
<th>P for NPL</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs700651 (A/G)</td>
<td>2q33.1</td>
<td>0.036</td>
<td>-0.212</td>
<td>0.571</td>
</tr>
<tr>
<td>rs10958409 (G/A)</td>
<td>8q11.12–12.1</td>
<td>0.962</td>
<td>-0.605</td>
<td>0.72</td>
</tr>
<tr>
<td>rs1333040 (C/T)</td>
<td>9p21.3</td>
<td>0.002</td>
<td>-0.434</td>
<td>0.658</td>
</tr>
</tbody>
</table>

TDT indicates transmission disequilibrium test; NPL, nonparametric logarithm of the odds.

Table 3. Analysis of 8 SNPs for Association With IA in a Case-Control Study

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>(No. of Subjects)</th>
<th>Risk Allele Frequency</th>
<th>HWE, P</th>
<th>Allelic Association†</th>
<th>Additive Dominant Recessive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Allele,</td>
<td>d/d</td>
<td>d/D</td>
<td>D/D</td>
<td></td>
<td>OR (95% CI)/P</td>
</tr>
<tr>
<td>rs700651</td>
<td>A/G</td>
<td>112</td>
<td>208</td>
<td>99</td>
<td>0.48</td>
<td>0.90</td>
</tr>
<tr>
<td>rs1333040</td>
<td>C/T</td>
<td>9p21.3</td>
<td>112</td>
<td>192</td>
<td>104</td>
<td>0.49</td>
</tr>
</tbody>
</table>

†With adjustment for covariates.
have been undertaken. Helgadottir et al20 and Bilguvar et al21 reported association studies based on a multiethnic population. Helgadottir et al20 reported that rs10757278 (9p21) was associated with IA and that its association was independent of the diabetes block. On the other hand, Bilguvar et al21 revealed a set of SNPs, rs700651 (2q33), rs10958409 (8q11), and rs1333040 (9p21), associated with IA.

We confirmed an association of rs1333040 with IA in 2 independent patient groups with a high or low likelihood of genetic predisposition: the multiplex IA families and sporadic cases, respectively. Such associations of rs1333040-T with IA in 2 independent populations are firmly suggestive of a substantial association with IA. To search for the gene on 9p modifying the risk of IA, we sequenced 3 genes in the vicinity

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**Figure 2.** Six selected SNPs on 9p21.3 and LD blocks. Physical positions of 6 SNPs and related genes are illustrated, based on the NCBI database (build 37.1). LD blocks are shown by D’ and r² measures for a population including both cases and controls.
Genetic transmission suggests that a haplotype shared by 6 index cases carries the risk allele of rs1333040-T. Three SNPs (rs10965215, rs10120688, and rs7341791), however, did not demonstrate either an association or LD with rs1333040, suggesting that a region carrying the rs1333040-T allele between introns 7 and 15 of CDKN2BAS may confer a bona fide risk for IA.

The 9p21 locus is associated not only with vascular diseases but also with diabetes mellitus.20 The LD structure of this locus was investigated rigorously by Helgadottir et al20 and was found to be composed of 2 separate LD blocks: vascular disease and diabetes blocks. The vascular disease block was tagged by SNPs, rs10757278 or rs1333040, which are in strong LD. The present study, however, failed to show a strong LD for these SNPs in the Japanese population. In the current population, IA was associated with rs1333040 but not with rs4496892 or rs10757278. More important, the current study suggests the possibility of another small LD block between introns 7 and 15 of CDKN2BAS, which is tagged by rs1333040.

There are several limitations of this study. The major limitations are the size and single ethnicity of the study population. The limited statistical power, with a maximum sensitivity of 76%, may have failed to detect associations of other SNPs with IA, including rs10757278. In addition, we did not correct for multiple comparisons in the association studies. Another limitation was the inability to provide a biological explanation for the locus associated with IA. We could not evaluate, either directly or indirectly, any functional effect of CDKN2BAS with respect to IA. A major strength of the study, however, was elucidation of the fine structure of the LD block in the vicinity of rs1333040.

In conclusion, we succeeded in replicating the association of rs1333040 on 9p21 with IA in 2 independent IA patient groups and suggest that the region between introns 7 and 15 of CDKN2BAS may be a risk modifier. Further study is needed to elucidate the biological mechanism of this association.

Acknowledgments
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Disclosures

None.

References


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