Association Analysis of Common Variants of ELN, NOS2A, APOE and ACE2 to Intracranial Aneurysm

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Background and Purpose—Previous studies have shown positive evidence of linkage of the intracranial aneurysm (IA) at chromosome 7q11, 17cen, 19q13, and Xp22. These regions contain elastin (ELN), nitric oxide synthetase 2A (NOS2A), apolipoprotein E (APOE), and angiotensin-I converting enzyme 2 (ACE2), which are considered to be promising candidate genes for IA. We aimed to examine the association of single-nucleotide polymorphisms (SNPs) with IA in these candidate genes.

Methods—To identify polymorphisms in NOS2A and ACE2, all exons and exon-intron boundaries were screened by direct sequencing in 30 randomly selected controls. The program tagSNPs was used to select an optimal set of haplotype-tagging SNPs. For ELN and APOE, SNPs were selected from previous reports. These selected SNPs were then genotyped in 362 cases with IA and 332 residential area matched controls. THESIAS software was used to investigate the association of alleles and haplotypes with IA by adjusting with covariates.

Results—We genotyped 8 SNPs in ELN, 8 SNPs in NOS2A, 3 ε alleles in APOE and 1 SNP in ACE2. No alleles or haplotypes of 4 candidate genes revealed any significant association with IA.

Conclusions—Investigated polymorphisms in this study were not associated with IA. (Stroke. 2006;37:1189-1194.)

Key Words: genetics ■ intracranial aneurysm ■ single nucleotide polymorphism ■ subarachnoid hemorrhage

It has recently been recognized that genetic factors have an impact on the pathogenesis of intracranial aneurysm (IA). Genome-wide linkage analyses have revealed linkages to several chromosomal regions. Among them, 7q11, 17cen, 19q13, and Xp22 are potentially interesting because they have been replicated in several studies. Elastin (ELN), nitric oxide synthetase 2A (NOS2A), apolipoprotein E (APOE) and angiotensin-I converting enzyme 2 (ACE2) are located on 7q11, 17cen, 19q13 and Xp22, respectively, and they are considered promising candidate genes for IA.

Human ELN consists of 34 exons and spans 45 kb of genomic DNA. The association of ELN haplotypes with IA or subarachnoid hemorrhage (SAH) was reported in Japanese and Dutch studies, albeit with genetic heterogeneity between the studies. However, other studies have failed to show an association. Besides, a Finnish group and we have demonstrated the absence of a linkage to 7q11.

Human NOS2A consists of 26 exons and 25 introns spanning 37 kb of genomic DNA. The most common genetic alleles of APOE are ε2, ε3 and ε4. In a prospective case-control study by Kokubo et al., the ε4 allele was reported to be a risk factor for SAH in eastern Japan.

Human ACE2 contains 18 exons spanning ~40 kb of genomic DNA, and resides in chromosome Xp22 where many genes escape inactivation. The If/If genotype of ACE was reported as a risk factor for SAH in Poland. ACE2 is a homolog of ACE, and they negatively regulate each other, suggesting that ACE2 could also be a risk factor for IA.

To validate these findings, we studied the association of single-nucleotide polymorphisms (SNPs) and haplotypes in these candidate genes with IA in a western Japan–based population.

Materials and Methods

Study Population

The study population consisted of 362 unrelated case subjects with IA, who were diagnosed by digital subtraction angiography or by operations in collaborating hospitals in western Japan. The residential areas of cases and controls were matched to eliminate the effect of population stratification by heterogeneity. Control subjects met the following criteria: (1) confirmation that they did not harbor IA by digital subtraction angiography, 3-dimensional computed tomogra-

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1189
SNP Screening in NOS2A and ACE2
To identify polymorphisms in NOS2A (GeneBank accession No. NT_010799) and ACE2 (NT_011757), all exons, intron-exon boundaries, putative promoter sequence and the 3’UTR were analyzed by direct sequencing in 30 randomly selected controls. Primers for coding exons were designed from an intronic sequence >50 bp away from the intron-exon boundaries and commercially synthesized by PROLOGO (PROLOGO Primers & Probes; http://www.prologo.com). After polymerase chain reaction (PCR) amplification, products were electrophoresed and purified using a QIAquick Gel Extraction Kit (Qiagen Inc), followed by sequencing on an ABI Prism 3100 Avant DNA sequencer (Applied Biosystems). We checked the SNP database (dbSNP; http://www.ncbi.nlm.nih.gov/SNP/) as reference. Primers and PCR conditions of each gene are available from the request on author.

SNP Selection
In NOS2A and ACE2, among all the SNPs identified by direct sequencing, we selected a minimized number of haplotype-tagging (ht) SNPs to be genotyped using the program tagSNPs (tagSNPs version 1; http://www-rcf.usc.edu/~stram/tagSNPs.html).21 We ran the program with the following criteria: common haplotypes were defined as the minimal set of haplotypes that covers 80% of existing haplotypes with IA or SAH was found in intron ELN of 0.20 that contributes to IA with a relative risk of 1.25, sampling 80% power for a significant threshold of \( P < 0.05 \). By adjusting with covariates including age, sex, hypertension, smoking habit and heavy alcohol consumption. We also investigated association of polymorphisms with SAH. Allele frequencies of control subjects in 2 major residential areas (Osaka and Kyoto) were compared by \( \chi^2 \) test using SAS software (Version 8.2. SAS Institute, Inc). For ACE2, data for each sex were analyzed separately because it is on the X chromosome. Bonferroni correction was done as needed (probability value after correction \( P_{corr} \)).

Assuming an autosoal disease allele with population frequency of 0.20 that contributes to IA with a relative risk of \( \geq 2.5 \), sampling would require an equal number of 314 cases and controls to provide 80% power for a significant threshold of \( P = 0.05 \). (Genetic Power Calculator, http://statgen.iop.kcl.ac.uk/gpc/cc2.html).

Results
Clinical Data
As shown in Table 1, the percentage of females and hypertension was higher among cases than controls. No significant difference was found in either smoking habit or alcohol consumption.

| TABLE 1. Characteristics of Cases Versus Controls, and Controls in Osaka Versus Controls in Kyoto |
|-----------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| No.                                           | Cases           | Controls        | Osaka           | Kyoto           |
| Female, %                                     | 362             | 332             | 0.0005          | 0.4408          |
| Age at diagnosis, y                           |                  |                 | 62.9 ± 9.3      | 60.6 ± 9.8      |
| Mean ± SD                                     | 59.2 ± 10.8     | 62.2 ± 9.9      | 0.000172*       |
| Range                                         | 26–90           | 40–88           | 0.1134*         |
| Hypertension, %                               | 56.1            | 42.5            | 0.0003          |
| Current or exsmoker, %                        | 39.5            | 37.7            | 0.617           |
| Drinker, %                                    | 39.2            | 43.7            | 0.235           |
| Ruptured IA, %                                | 24.0            | 0               | 0               |
| Family history of IA or SAH, %                |                  |                 | 0               |

\( P \) values were calculated by \( \chi^2 \) test. * was calculated by Student t test. Osaka indicates control subjects in Osaka; Kyoto, control subjects in Kyoto.
Identification and Selection of SNPs
In NOS2A, we identified 12 SNPs (supplemental Table I, available online at http://stroke.ahajournals.org), of which 2 (INT16: IVS16+88 G>T, and EX19: Ex19 2503 A>G) were novel and 2 were nonsynonymous, EX16 (S608L) and EX19 (T747A). S608L was predicted to have a possible damaging structure or function of NOS2A by PolyPhen. Serine608 is conserved among 5 species including rat and mouse (Homolo Gene: 55473; http://www.ncbi.nlm.nih.gov), whereas Threonin747 was conserved in only 2 species, human and dog. Of 12 SNPs identified in NOS2A, 8 SNPs (INT7, INT7*, INT8, INT12, EX16, INT16, EX19, and EX22) were selected according to the tagSNP program. In ACE2, only 1 registered SNP (rs2285666) in INT3 was identified. In ELN and APOE, 8 SNPs and 3 ε alleles were selected as already stated.

Association Analysis
In ELN, 8 SNPs and 8 haplotypes including INT20/INT22 and INT4/INT5/INT21 were analyzed. We observed no

TABLE 2. Comparisons of Allele Frequencies Between Cases With IA and Controls by Adjusting With Covariates

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Allele</th>
<th>Cases, n (%)</th>
<th>Controls, n (%)</th>
<th>Odds Ratio (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELN</td>
<td>INT4</td>
<td>G</td>
<td>515 (81.5%)</td>
<td>488 (79.7%)</td>
<td>0.99 (0.95–0.98)</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>117 (18.5%)</td>
<td>124 (20.3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EX5</td>
<td>C</td>
<td>682 (94.2%)</td>
<td>637 (95.9%)</td>
<td>1.48 (0.92–2.37)</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>42 (5.8%)</td>
<td>27 (4.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>INT5</td>
<td>G</td>
<td>462 (63.8%)</td>
<td>446 (67.4%)</td>
<td>1.09 (0.87–1.37)</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>262 (36.2%)</td>
<td>216 (32.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EX20</td>
<td>G</td>
<td>591 (81.6%)</td>
<td>543 (81.8%)</td>
<td>1.05 (0.78–1.43)</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>133 (18.4%)</td>
<td>121 (18.2%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>INT20</td>
<td>T</td>
<td>553 (76.4%)</td>
<td>510 (76.8%)</td>
<td>1.05 (0.81–1.36)</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>171 (23.6%)</td>
<td>154 (23.2%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>INT21</td>
<td>G</td>
<td>704 (97.2%)</td>
<td>648 (97.8%)</td>
<td>1.29 (0.61–2.67)</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>20 (2.8%)</td>
<td>14 (2.2%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>INT22</td>
<td>T</td>
<td>517 (71.4%)</td>
<td>454 (68.8%)</td>
<td>0.90 (0.71–1.14)</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>207 (28.6%)</td>
<td>206 (31.2%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EX25</td>
<td>G</td>
<td>706 (97.8%)</td>
<td>653 (98.3%)</td>
<td>1.28 (0.60–2.72)</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>16 (2.2%)</td>
<td>11 (1.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOS2A</td>
<td>INT7</td>
<td>A</td>
<td>364 (50.3%)</td>
<td>346 (52.1%)</td>
<td>1.08 (0.87–1.33)</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G</td>
<td>360 (49.7%)</td>
<td>318 (47.9%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>INT7*</td>
<td>I</td>
<td>665 (91.9%)</td>
<td>607 (91.4%)</td>
<td>0.90 (0.60–1.37)</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D</td>
<td>59 (8.1%)</td>
<td>57 (8.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>INT8</td>
<td>A</td>
<td>332 (45.9%)</td>
<td>331 (50.2%)</td>
<td>0.83 (0.67–1.05)</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G</td>
<td>392 (54.1%)</td>
<td>329 (49.8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>INT12</td>
<td>C</td>
<td>535 (73.9%)</td>
<td>495 (74.8%)</td>
<td>1.07 (0.84–1.36)</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>189 (26.1%)</td>
<td>167 (25.2%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EX16</td>
<td>C</td>
<td>676 (93.6%)</td>
<td>622 (93.7%)</td>
<td>0.93 (0.59–1.48)</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>46 (6.4%)</td>
<td>42 (6.3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>INT16</td>
<td>G</td>
<td>611 (85.1%)</td>
<td>568 (86.1%)</td>
<td>1.06 (0.78–1.44)</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>107 (14.9%)</td>
<td>92 (13.9%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EX19</td>
<td>A</td>
<td>686 (97.2%)</td>
<td>644 (97.0%)</td>
<td>1.06 (0.56–2.01)</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G</td>
<td>20 (2.8%)</td>
<td>20 (3.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EX22</td>
<td>G</td>
<td>537 (74.2%)</td>
<td>493 (74.5%)</td>
<td>1.04 (0.82–1.33)</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>187 (25.8%)</td>
<td>169 (25.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APOE</td>
<td>EX4</td>
<td>e2</td>
<td>13 (3.6%)</td>
<td>13 (4.1%)</td>
<td>1.35 (0.74–2.46)</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>e3</td>
<td>44 (12.2%)</td>
<td>38 (11.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>e4</td>
<td>305 (84.2%)</td>
<td>280 (84.4%)</td>
<td>0.88 (0.63–1.22)</td>
<td>0.42</td>
</tr>
<tr>
<td>ACE2</td>
<td>INT3</td>
<td>T</td>
<td>65 (55.1%)</td>
<td>76 (50.3%)</td>
<td>0.60*</td>
<td>0.44*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>53 (44.9%)</td>
<td>75 (49.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(male)</td>
<td>INT3</td>
<td>T</td>
<td>248 (50.8%)</td>
<td>193 (53.3%)</td>
<td>1.17 (0.88–1.54)</td>
</tr>
<tr>
<td></td>
<td>(female)</td>
<td>C</td>
<td>240 (49.2%)</td>
<td>169 (46.7%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Was calculated by χ² test. No difference in genotype frequencies was detected between cases and controls.
significant association of polymorphisms with either IA (Table 2) or SAH (data not shown). All haplotypes also failed to show an association (Table 3). LD analysis revealed a weak LD pattern unlike that of NOS2A (data not shown).

In NOS2A, a total of 8 htSNPs and 4 haplotypes were analyzed, and all these SNPs were in Hardy-Weinberg equilibrium after Bonferroni correction. No SNPs or haplotypes were associated with either IA (Table 2, Table 3) or SAH (data not shown).

In APOE, no association was observed between alleles and the occurrence of either IA (Table 3) or SAH (data not shown). In ACE2, analysis of the SNP demonstrated a lack of association either in males or females (Table 2). Besides this, none of the pair-wise haplotypes consisting of all SNPs in ELN, NOS2A and APOE could have shown the association (data not shown).

In the analysis of regional differences of allele frequency, the frequency of EX5, INT20 and INT21 in ELN was significantly different between Osaka and Kyoto (P = 0.0042, 0.0385 and 0.0113, respectively; Table 4), whereas no difference was observed in either NOS2A or APOE (supplemental Table II). Even after applying Bonferroni correction, the probability value of EX5 was statistically significant (Pcorr = 0.034). Characteristics of control subjects in Osaka and Kyoto were listed in Table 1.

Discussion
In the present study, we examined the association of polymorphisms of ELN, NOS2A, APOE and ACE2 with IA. For ELN and APOE, we selected the SNPs to be analyzed based on previous association studies.1,8,16 For NOS2A and ACE2, because there were no previously published association studies, we sequenced all exons and exon-intron boundaries to search for SNPs. Considering various modes of associations, we also test the associations of polymorphisms with a related phenotype of IA, SAH by using a large number of cases and controls that promised us sufficient statistical power. Furthermore, we investigated interchromosomal interactions among these genes. Thus, within the present experimental settings,
design and quality enabled us to detect signals as weak as a
relative risk of 1.25.

We tested the association of ELN SNPs reported by Onda et al1 and Ruigrok et al8 but failed to show an association with
either IA or SAH. One explanation for the disagreement
could be haplotype heterogeneity among study populations.
In fact, LD analysis of ELN showed very weak LD even in the
same ethnic group, being consistent with other reports1,8 and
HapMap LD data (http://www.hapmap.org/ cgi-perl/gbrowse/
gbrowse/hapmap). Considering that LD is negatively corre-
lated with recombination rates,26 ELN is likely to have a
recombination hotspot; therefore, it is easy to have haplotype
heterogeneity even among adjacent populations. So, there is a
possibility that untested SNPs in this study were associated
with IA or SAH. However, the most likely explanation for the
disagreement would be that a significant association of ELN
haplotype with IA may represent LD with an unknown gene.

For APOE, Kokubo et al reported the positive association
of ε4 allele with SAH in eastern Japan.16 Our study, however,
could not confirm their findings, suggesting that polymor-
phisms of APOE may not be a major genetic risk factor for
either SAH or unruptured IA in western Japan.

For NOS2A, knockout mice were proven to have reduced
sizes of aneurysms.14,15 The present study, however, could
not show any association with either IA or SAH. The
apparent discrepancy may be attributable to differences in
species or in study protocols. Although knockout mice model
a loss of function of NOS2A, our study investigated qualita-
tive functional changes. In addition, minor allele frequencies
of 2 nonsynonymous SNPs (S608L and T747A) were below
7%, which made it difficult to detect positive signals attrib-
able to the limitation of statistical power. Indeed, our study
indicates that NOS2A is not likely to take a major role in the
pathogenesis of IA or SAH. However, the effect of a rare
polymorphism, such as S608L, needs more cautious interpre-
tation because Serine608 is conserved in various species and
S608L is predicted to be a deleterious mutation. Although
haplinsufficiency is not likely to be associated with IA,
S608L cannot be discarded as a risk factor for IA in its
homozgyous state. Further study will be needed for this rare
polymorphism.

ACE2 is a homolog of ACE, the I/I genotype of which has
been proven to be associated with SAH in Polish popula-
tion.19 In the present study, however, no association was
observed.

We examined the association of SNPs and haplotypes of 4
promising candidate genes with IA. However, investigated
polymorphisms in this study were not associated with either
IA or SAH.

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makawa (Gero-spring Hospital), Atsushi Kawarazaki (Kawarazaki Hos-
ital), Masayuki Matsuda (Shiga University of Medical Science),
Michiyasu Suzuki and Sadahito Nomura (Yamaguchi University School
of Medicine), Takaaki Kaneko, Nozomu Murai, and Susumu
Kenamoto (Hikone Municipal Hospital), Tatsuhito Yamagami and
Motoharu Fuji (Kyoto Kizugawa Hospital), Hiroku Ohishi and
Kiminnari Ohtaka (Senboku Kumiai Sougou Hospital), Kenji Kikuchi
and Yutaka Yamazaki (Yuri Kumiiai Sougou Hospital), Jun Taka-
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(Kyoto University Graduate School of Medicine).

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