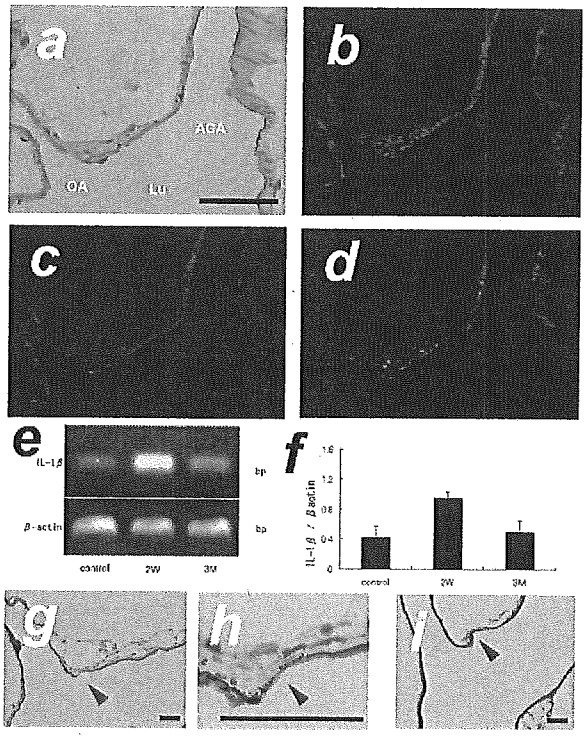
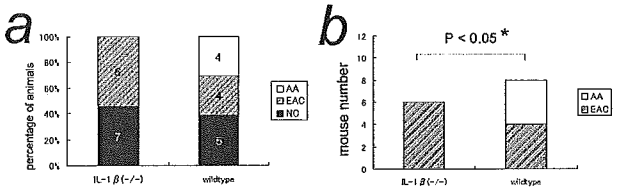
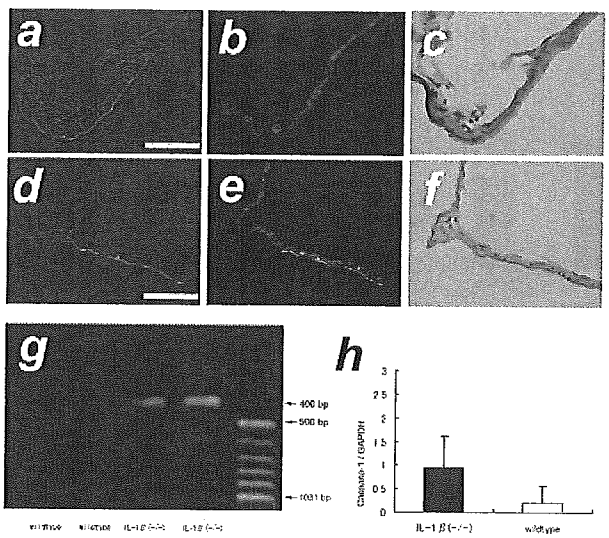
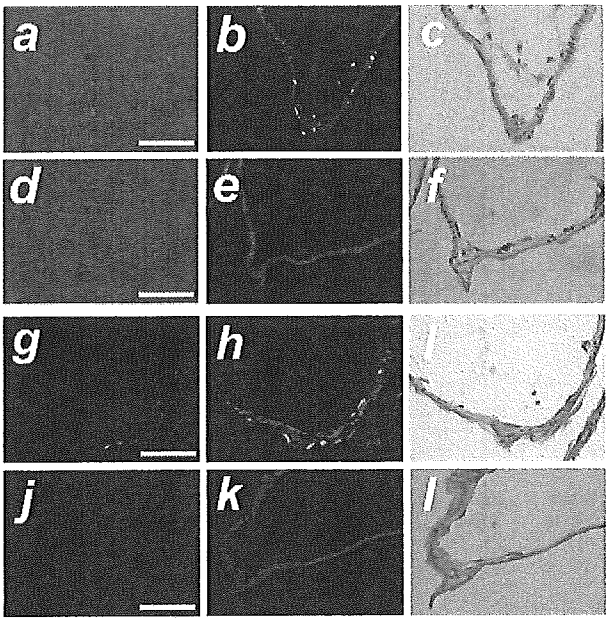
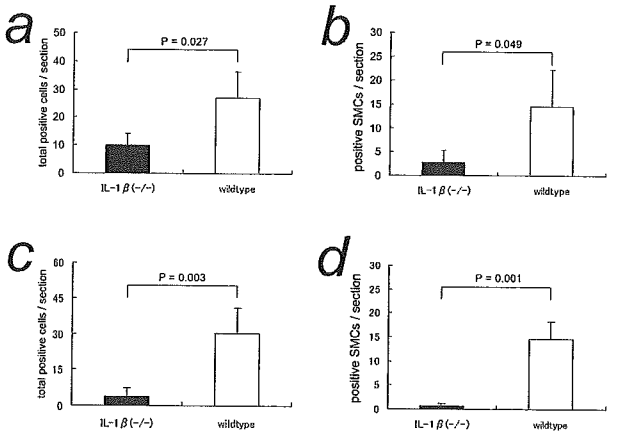
Figure 5. The number of ssDNA or TUNEL positive cells in the vascular walls of experimental aneurysms in wild-type and IL-1β deficient mice. The number of ssDNA immunoreactive cells per section in the media (a) and intima (b) was significantly smaller in IL-1β-/- mice (n=5) compared to wild-type animals (n=5). The number of TUNEL positive cells per section in the media (c) and intima (d) was significantly smaller in IL-1β-/- mice (n=5) compared to wild-type animals (n=5).











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

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Title Page (R2)

Full title: Association Analysis of Common Variants of *ELN*, *NOS2A*, *APOE*, and *ACE2* to Intracranial Aneurysm

Cover title: Association of 4 Candidate Genes with IA

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Abstract Page

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 synthetase2A (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 (htSNPs). 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.

Text

It has recently been recognized that genetic factors have an impact on the pathogenesis of IA. Genome-wide linkage analyses have revealed linkages to several chromosomal regions.[1-7] Among them, 7q11,[1,4] 17cen,[1,6] 19q13,[2,3,6] and Xp22[2,6] are potentially interesting since they have been replicated in several studies. *ELN*, *NOS2A*, *APOE* and *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 45kb of genomic DNA. The association of *ELN* haplotypes with IA or SAH was reported in Japanese and Dutch studies, albeit with genetic heterogeneity between the studies.[1, 8] However, other studies have failed to show an association.[9, 10] Besides, a Finnish group and we have demonstrated the absence of a linkage to 7q11.[2, 11]

Human *NOS2A* consists of 26 exons and 25 introns spanning 37kb of genomic DNA.[12, 13] Sadamasa et al reported knocking out the *iNOS* (*NOS2A*) gene reduced the size of cerebral aneurysms in mice, suggesting its potential role in the progression of IA.[14, 15]

The most common genetic alleles of *APOE* are ε2, ε3 and ε4. In a prospective case-control study by Kokubo et al, [16] the ε4 allele was reported to be a risk factor for subarachnoid hemorrhage (SAH) in eastern Japan.

Human *ACE2* contains 18 exons spanning approximately 40kb of genomic DNA,[17] and resides in chromosome Xp22 where many genes escape inactivation.[18, 19] The I/I genotype of *ACE* was reported as a risk factor for SAH in Poland.[21] *ACE2* is a homolog of *ACE* and they negatively regulate each other,[20] suggesting that *ACE2* could also be a risk factor for IA.

To validate these findings, we studied the association of SNPs and haplotypes in these candidate genes with IA in a West 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 (DSA) 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 DSA, 3-dimensional computed tomography, or by magnetic resonance angiography, (2) age at diagnosis of ≥40 years old, (3) no medical history of any stroke including IA and/or SAH, and (4) no family history of IA and/or SAH in first-degree relatives. The study was approved by the Ethics Committee of Kyoto University. For all subjects, we interviewed for their risk factors profile, including past medical history, family history, smoking habit, and alcohol consumption. Smoking habit was defined as current smokers of ≥1 cigarette per day, former smokers, and nonsmokers. For statistical analysis, current smokers and former smokers were dealt as smokers. Drinkers were defined as regular drinkers who drink >150 grams or more of alcohol per week.

SNP Screening in *NOS2A* and *ACE2*

To identify polymorphisms in *NOS2A* (GeneBank accession number; 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 PROLIGO (PROLIGO Primers & Probes; http://www.proligo.com). After PCR amplification, products were electrophoresed and purified using a QIAquick Gel Extraction Kit (Qiagen Inc, USA), followed by sequencing on an ABI Prism 3100 Avant DNA sequencer (Applied Biosystems, USA). 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 author on request.

SNP Selection

In *NOS2A* and *ACE2*, among all the SNPs identified by direct sequencing, we selected a minimized number of htSNPs 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, sets of htSNPs resolving the common haplotypes were selected at an R_h^2 (the squared correlation between estimated and true haplotype dosage) threshold of 0.8.[22] Exceptionally, all non-synonymous SNPs (i.e. SNPs located in coding regions and results in amino acid variation in the protein products of the gene) were forced in as a set of htSNPs. Selected htSNPs were genotyped in 362 cases and 332 controls. Non-synonymous SNPs were analyzed by bioinfomatics using PolyPhen software (http://tux.embl-heidelberg.de/ramensky/) to predict whether or not they were damaging to the structure or function of the protein products.

In *ELN* (NT_007758), we selected 8 out of 18 SNPs identified in previous reports[1, 8] in the following process: Since the significant association of *ELN* haplotypes with IA or SAH was found in intron 20 (INT20)/INT22 in a Japanese study and INT4/INT5/INT21 in a Dutch study,[1, 8] these 5 intronic SNPs were selected to be genotyped. In addition, all 3 exonic SNPs; exon 5 (EX5), EX20 and EX22 were selected and a total 8 SNPs were genotyped in *ELN*. In *APOE* (NT_011109), we genotyped ε alleles in exon4.[16]

SNP Genotyping

Genotyping of *ELN, NOS2A, APOE* and *ACE2* was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) protocol. *APOE* was genotyped as previously reported.[23] Because of the lack of a proper restriction enzyme for INT4 in *ELN*, real-time PCR TaqMan® analysis was conducted on the 7300/7500 Real Time PCR System (Applied Biosystems, USA).

Haplotype and LD analysis

We investigated haplotypes with a frequency of >5% for each gene.[24] *ELN*, INT20/INT22 and INT4/INT5/INT21 were also investigated. Considering the possibility of interchromosomal interaction, we examined pair-wise haplotypes that consisted of all the SNPs genotyped in *ELN*, *NOS2A* and *APOE*. LD calculations were conducted by means of r^2 and $|D'|$ using Genotype2LDBlockVO.2 (http://cgi.uc.edu/cgi-bin/kzhang/genotype2LDBlock.cgi).

Association Analysis

The association of alleles and haplotypes with IA was analyzed using THESIAS software (http://genecanvas.ecgene.net/)[25] by adjusting with covariates including age, sex, hypertension, smoking habit and heavy alcohol consumption. We also analyzed association of polymorphisms with SAH. Allele frequencies of control subjects in two major residential areas (Osaka and Kyoto) were compared by χ^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 (P value after correction [P_{corr}]).

Assuming an autosomal disease allele with population frequency of 0.20 that contributes to IA with a relative risk of ≥1.25, 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.

Identification and selection of SNPs

In *NOS2A*, we identified 12 SNPs (Table 2), of which 2 (INT16: IVS16+88 G>T, and EX19: Ex19 2503 A>G) were novel and 2 were non-synonymous; 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 (HomoloGene: 55473; http://www.ncbi.nlm.nih.gov), while Threonin747 was conserved in only 2 species; human and dog. Out 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 one 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 significant association of polymorphisms with either IA (Table3) or SAH (data not shown). All haplotypes also failed to show an association (Table 4). LD analysis revealed a weak LD pattern unlike that of *NOS2A* (data not shown).

In *NOS2A*, a total 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 3, Table 4) or SAH (data not shown).

In *APOE*, no association was observed between ε alleles and the occurrence of either IA (Table 4) or SAH (data not shown). In *ACE2*, analysis of the SNP demonstrated a lack of association either in males or females (Table 3). 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, P=0.0385 and P=0.0113, respectively; Table 5), while no difference was observed in either *NOS2A* or *APOE* (Supplement table 1). Even after applying Bonferroni correction, the P value of EX5 was statistically significant (P_{corr} =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*, since 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 amongst 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 al[1] and Ruigrok et al[8] but failed to show an association with either IA or SAH. One explanation for the disagreement could be haplotype heterogeneity amongst study populations. In fact, LD analysis of *ELN* showed very weak LD even in the same ethnic group, being consistent with other reports[1, 8] and HapMap LD data (http://www.hapmap.org/cgi-perl/gbrowse/gbrowse/hapmap). Considering that LD is negatively correlated 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 polymorphisms 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 and/or in study protocols. While knockout mice model a loss of function of *NOS2A*, our study investigated qualitative functional changes. In addition, minor allele frequencies of two non-synonymous SNPs (S608L and T747A) were below 7%, which made it difficult to detect positive signals due 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 interpretation because Serine608 is conserved in various species and S608L is predicted to be a deleterious mutation. Although haploinsufficiency is not likely to be associated with IA, S608L cannot be discarded as a risk factor for IA in its homozygous 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 population.[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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