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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Tables

TABLE 1. Characteristics of Cases vs Controls, and Controls in Osaka vs Controls in Kyoto

	Cases	Controls	*P* value	Osaka	Kyoto	*P* value
Number	362	332		136	66	
Female, %	67.4	54.5	0.0005	57.4	59.1	0.4408
Age at diagnosis, y						
Mean±SD	59.2 ± 10.8	62.2 ± 9.9	0.000172*	62.9± 9.3	60.6± 9.8	0.1134*
Range	26-90	40-88		40-81	43-86	
Hypertension, %	56.1	42.5	0.0003	47.8	40.9	0.0019
Current or ex smoker, %	39.5	37.7	0.617	39.7	27.3	<0.0001
Drinker, %	39.2	43.7	0.235	46.3	33.3	<0.0001
Family history of IA and/or SAH, %	24.0	0		0	0	
Ruptured IA, %	50.6	0		0	0	

P values were calculated by χ^2 test. * was calculated by Student's t test.

Osaka represents control subjects in Osaka; Kyoto, control subjects in Kyoto.

TABLE 2. Comparisons of Allele Frequencies between Cases with IA and Controls by Adjusting with Covariates

Gene	SNP	Allele	Cases, n (%)	Controls, n (%)	Odds ratio (95%CI)	*P* value
ELN	INT4	G	515 (81.5%)	488 (79.7%)	0.99 (0.95-0.98)	0.94
		A	117 (18.5%)	124 (20.3%)		
	EX 5	C	682 (94.2%)	637 (95.9%)	1.48 (0.92-2.37)	0.10
		T	42 (5.8%)	27 (4.1%)		
	INT5	G	462 (63.8%)	446 (67.4%)	1.09 (0.87-1.37)	0.46
		A	262 (36.2%)	216 (32.6%)		
	EX20	G	591 (81.6%)	543 (81.8%)	1.05 (0.78 - 1.43)	0.74
		A	133 (18.4%)	121 (18.2%)		
	INT20	T	553 (76.4%)	510 (76.8%)	1.05 (0.81-1.36)	0.70
		C	171 (23.6%)	154 (23.2%)		
	INT21	G	704 (97.2%)	648 (97.8%)	1.29 (0.61-2.67)	0.50
		A	20 (2.8%)	14 (2.2%)		
	INT22	T	517 (71.4%)	454 (68.8%)	0.90 (0.71-1.14)	0.39
		C	207 (28.6%)	206 (31.2%)		
	EX25	G	706 (97.8%)	653 (98.3%)	1.28 (0.60-2.72)	0.52
		C	16 (2.2%)	11 (1.7%)		
NOS2A	INT7	A	364 (50.3%)	346 (52.1%)	1.08 (0.87-1.33)	0.49
		G	360 (49.7%)	318 (47.9%)		
	INT7'	I	665 (91.9%)	607 (91.4%)	0.90 (0.60-1.37)	0.63
		D	59 (8.1%)	57 (8.6%)		
	INT8	A	332 (45.9%)	331 (50.2%)	0.83 (0.67-1.05)	0.12
		G	392 (54.1%)	329 (49.8%)		
	INT12	C	535 (73.9%)	495 (74.8%)	1.07 (0.84-1.36)	0.59
		T	189 (26.1%)	167 (25.2%)		
	EX16	C	676 (93.6%)	622 (93.7%)	0.93 (0.59-1.48)	0.77
		T	46 (6.4%)	42 (6.3%)		
	INT16	G	611 (85.1%)	568 (86.1%)	1.06 (0.78-1.44)	0.71
		T	107 (14.9%)	92 (13.9%)		
	EX19	A	696 (97.2%)	644 (97.0%)	1.06 (0.56-2.01)	0.85
		G	20 (2.8%)	20 (3.0%)		
	EX22	G	537 (74.2%)	493 (74.5%)	1.04 (0.82-1.33)	0.73
		A	187 (25.8%)	169 (25.5%)		
APOE	EX4	ε2	13 (3.6%)	13 (4.1%)	1.35 (0.74-2.46)	0.33
		ε3	44 (12.2%)	38 (11.5%)		
		ε4	305 (84.2%)	280 (84.4%)	0.88 (0.63-1.22)	0.42
ACE2	INT3	T	65 (55.1%)	76 (50.3%)	0.60*	0.44*
	(male)	C	53 (44.9%)	75 (49.7%)		
	INT3	T	248 (50.8%)	193 (53.3%)	1.17 (0.88-1.54)	0.28
	(female)	C	240 (49.2%)	169 (46.7%)		

CI indicates confidential interval. * was calculated by χ^2 test. No difference in genotype frequencies was detected between cases and controls.

TABLE 3. Comparisons of Haplotype Frequencies in *ELN* and *NOS2A* between Cases with IA and Controls by Adjusting with Covariates

Gene	Variables	Haplotype	Cases, %	Controls, %	Odds ratio (95%CI)	*P* value
ELN	Haplotype	GCGGTGTG	13.4	14.2	0.92 (0.63-1.36)	0.69
		GCGGTGCG	6.6	6.8	0.90 (0.52-1.56)	0.72
		GCGATGTG	7.4	6.1	1.16 (0.68-1.98)	0.59
		GCGATGCG	8.3	9.9	0.90 (0.56-1.45)	0.66
		GCAGTGTG	21.6	21.6	Reference	
		ACGGCGTC	9.7	10.2	0.90 (0.58-1.40)	0.65
	INT20/INT22	TT	52.8	53.0	Reference	
		TC	23.6	23.7	1.01 (0.75-1.36)	0.95
		CT	18.6	15.6	1.21 (0.87-1.70)	0.26
		CC	5.0	7.6	0.71 (0.39-1.33)	0.29
	INT4/INT5/INT21	GGG	43.8	45.3	Reference	
		GAG	30.9	29.8	0.96 (0.73-1.26)	0.78
		AGG	19.4	20.8	0.90 (0.64-1.25)	0.53
NOS2A	Haplotype	AIACCGAG	18.4	21.8	0.78 (0.57-1.07)	0.12
		AIATCGAA	20.0	20.7	0.95 (0.71-1.27)	0.72
		GIGCCGAG	30.0	29.1	Reference	
		GIGCCTAG	6.6	5.8	1.05 (0.64-1.72)	0.83

In haplotypes of *NOS2A*, I indicates insertion.

TABLE 4. Regional Difference of Allele Frequency of *ELN* in Controls

SNP	allele	Osaka, n	Kyoto, n	χ^2	*P* value	P_{corr}
INT4	G	176	109	0.35	0.5512	
	A	44	23			
EX5	C	267	122	8.18	**0.0042**	**0.034**
	T	5	10			
INT5	G	182	97	1.54	0.2143	
	A	88	35			
EX20	G	220	104	0.25	0.6203	
	A	52	28			
INT20	T	224	97	4.28	**0.0385**	0.308
	C	48	35			
INT21	G	267	125	6.42	**0.0113**	0.090
	A	3	7			
INT22	T	181	83	0.53	0.4649	
	C	87	47			
EX25	G	268	128	1.12	0.2912	
	C	4	4			

Significant valiables ($P<0.05$) were shown in bold. P_{corr} indicates *P* value after Bonferroni correction.

Search on Chromosome 17 Centromere Reveals *TNFRSF13B* as a Susceptibility Gene for Intracranial Aneurysm: A preliminary study

Inoue et al.: *TNFRSF13B* and IA

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Abstract

Background-Our previous studies have shown a significant linkage of intracranial aneurysms (IAs) to chromosome 17.

Methods and Results- Nine genes (*TNFRSF13B, M-RIP, COPS3, RAI1, SREBF1, GRAP, MAPK7, MFAP4 and AKAP10*) were selected from 108 genes which are located between D17S1857 and D17S1871 by excluding 99 genes that were pseudogenes, hypothetical genes, or well characterized genes but not likely associated with IA. Direct sequencing of all coding and regulatory regions in 58 cases (29 pedigree probands and 29 unrelated non-pedigree cases) was performed. Deleterious changes were found only in *TNFRSF13B*, K154X and c.585-586insA in exon4. Association of IA with *TNFRSF13B* was further studied in 304 unrelated cases and 332 control subjects. Rare non-synonymous changes, a splicing acceptor site change and a frame shift were found in unrelated cases (2.3%: 14/608) more frequently than in control subjects (0.8%: 5/664) (p=0.035). The association study using single nucleotide polymorphisms (SNPs) in an unrelated case-control cohort revealed a protective haplotype (Odds=0.69, 95%CI: 0.52-0.92, p=0.012) as compared with the major haplotype by adjusting for covariates.

Conclusion-We propose that *TNFRSF13B* is one of the susceptibility genes for IA.

Key Words: aneurysm, cerebrovascular disorders, genes, immune system

Introduction

Intracranial aneurysms (IAs) are one of the major public health problems in Japan. Mortality rate of subarachnoid hemorrhage (SAH), of which more than 90% is attributable to IA rupture, is estimated at 70 deaths per 10,000 person years and accounts for 2% of annual total deaths.[1] The consequences of SAH are catastrophic, with approximately half of IA ruptures resulting in immediate death.

In familial IAs, there is a three to five fold increase in risk for first-degree relatives of affected individuals compared with the general population.[2,3] A positive family history is a risk factor as strong as smoking, hypertension and heavily drinking alcohol.[4,5]

In an attempt to isolate susceptibility gene(s) for IA, four genome-wide linkage analyses have been reported.[6-9] In a series of studies, we have failed to identify a positive association with reported candidate genes.[9,10]

Since disease and genetic heterogeneity are postulated for IA,[11] extensive efforts are required to find the susceptibility gene(s) for IA, if the approach is limited to traditional positional cloning. On the other hand, the candidate gene approach relies serendipity.

In the present study, we hypothesize that the many rare variants contribute to a common phenotype.[12] We further assume that while deleterious changes are likely to be rare in the unaffected cohort, they may be more common in aggregate in the affected cohort. Consequently

we have assumed that variants associated with functional changes such as nonsense or non-synonymous variants should be more abundant in candidate genes that determine susceptibility for IA. On this rationale, candidate genes were searched in a primary gene set in the 17 centromere region between D17S1857 and D17S1871, where we found the maximum non-parametric LOD score (MNS) peak [MNS 3.00] at D17S2196.[9]

Methods

Study Design

Subjects from three groups participated. The first group was probands of 29 pedigrees with IA clustering.[9] The second group was consisted of 333 unrelated non-pedigree cases with IA and the third group had 332 control subjects.[10] Members of the first group and 29 unrelated cases which were selected randomly from the second group, constituted the first cohort. Remaining 304 unrelated non-pedigree cases of the second group constituted the second cohort and the third group constituted the third cohort. The response rates to our request of participation in our study were 95.1% in the second group and 94.8% in the third group, respectively.

The target region was 4.3 Mb encompassing D17S1857 and D17S1871 where we found significant linkage in families with an IA cluster.[9] 108 genes are now assigned to this region

(Supplement Table 1). We set an exclusion principle to choose the primary candidate gene set: we excluded 99 genes; 26 pseudogenes; 29 hypothetical genes; 22 enzymes and transporters; 8 developmentally regulated genes, 4 genes associated with Smith-Magenis Syndrome; 3 genes associated with neoplastic syndrome; 3 zinc finger proteins genes; 3 similar to keratin genes and one open reading frame. Finally, nine genes remained. These were *TNFRSF13B*, *M-RIP*, *COPS3*, *RAI1*, *SREBF1*, *GRAP*, *MAPK7*, *MFAP4* and *AKAP10* (Table 1). These nine genes were directly sequenced in all subjects of the first cohort. Whether or not sequence variants were functional was predicted by PolyPhen (http://tux.embl-heidelberg.de/ramensky).[13] Except for *TNFRSF13B*, none of the sequence variants in other genes were predicted to be deleterious. Further analysis was thus limited to *TNFRSF13B*. Using observed polymorphisms, an association study was conducted in the second cohort and the third cohort.

Study population

The probands of pedigrees and unrelated non-pedigree cases were diagnosed by digital subtraction angiography (DSA) or in operations throughout collaborating hospitals in western Japan. We have excluded cases with IA affected with known heritable diseases or autoimmune diseases. Control subjects were screened at the brain check-up in the same hospitals as cases and met the following criteria: (a) confirmation of absence of IA by DSA, 3-dimensional computerized tomography (3-D CT), or magnetic resonance angiography (MRA), (b) an age at