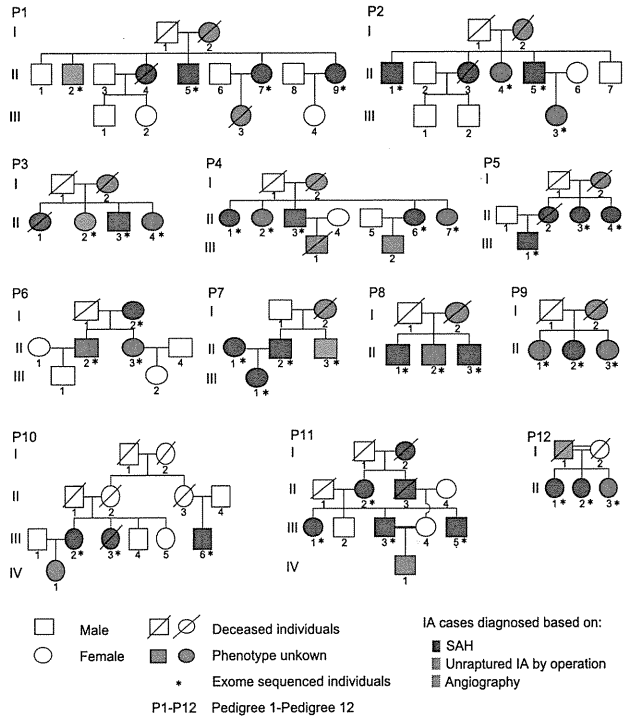

Figure 1. Pedigrees of 12 Japanese intracranial aneurysm (IA) families. SAH indicates subarachnoid hemorrhage.

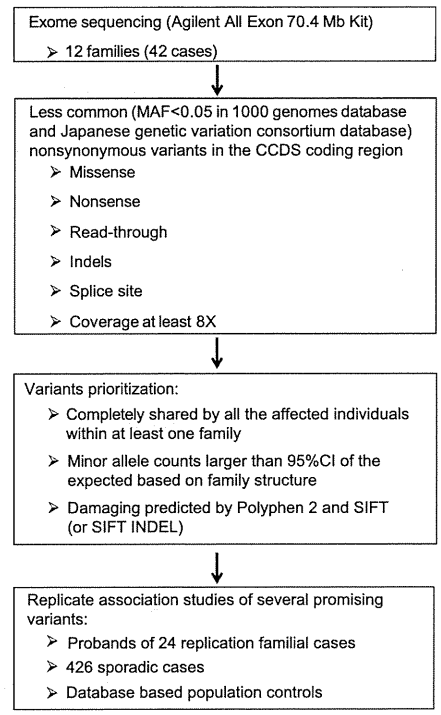

Figure 2. Overview of the study design. CCDS indicates consensus coding sequence; CI, confidence interval; and MAF, minor allele frequency.

individuals and genotyping data of common variations obtained from a cohort of 3248 individuals [http://www.genome.med.kyoto-u.ac.jp/SnpDB/]). This study was approved by the Institutional Review Board and Ethics Committee of Kyoto University School of Medicine, Japan.

Exome Sequencing, Mapping, Variant Calling, and Prioritization

Genomic DNA was extracted from peripheral blood lymphocytes using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Whole exome sequencing was performed by Riken Genesis Co, Ltd in Japan. Exon capture was performed using the SureSelect 70.4 Mb Human All Exon V4+UTR Kit (Agilent Technologies, Santa Clara, CA), and sequencing was done with using the Illumina HiSeq 2000 platform (Illumina Inc, San Diego, CA). Sequence mapping and variant detection were performed using the Burrows-Wheeler Aligner 0.6.2 and Genome Analysis Toolkit software. Details of sequencing and mapping are available in the Methods in the online-only Data Supplement.

A series of filters were used to prioritize variants. Variants were given higher priority if they were (1) predicted to affect protein-coding sequences (including missense, nonsense, read through, splice site variants and indels in the consensus coding sequence region); (2) less common in reference databases (MAF<0.05 in 1000 genome databases [ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/release/20110521/] and the Japanese genetic variation consortium database [JGVCD]); (3) completely shared by all the affected individuals within ≥1 family; (4) observed minor allele count in 42 familial cases larger than the 95% confidence interval of the expected values calculated based on family structure (expected value is calculated by simulation; detailed Methods are available in the online-only Data Supplement); (5) damaging, as predicted by protein prediction programs (Polymorphism Phenotyping V2 [PolyPhen-2; http://genetics.bwh.harvard.edu/pph2] and Sorting Intolerant From Tolerant [SIFT; http://sift.bii.a-star.edu.sg/]) or cause nonsense mediated decay, predicted by SIFT indel (http://sift.bii.a-star.edu.sg/www/SIFT_indels2.html; Figure 2).

Replicate Association Study in Additional Familial and Sporadic IA Cases

The selection of candidate variants for association study was based on the commonness and functional relevance of IA or other known diseases. In terms of functional relevance, we chose a key word, which describes the pathological process of IA, from published meta-analysis. Candidate variants were directly sequenced using the Sanger method. Further genotyping of these selected variants in additional IA cases was performed using TaqMan or restriction fragment length polymorphism methods. Direct sequencing was performed for unclear genotyping results. Primers and detailed polymerase chain reaction conditions are shown in Table I in the online-only Data Supplement.

Human Umbilical Venous Endothelial Cell Culture, RNA Interference Experiment, Transfection of *ADAMTS15* p.E133Q and Western Blot Analysis

Human umbilical venous endothelial cells (HUVECs) were obtained from Lonza (C2517A; Walkersville, MD) and cultured following a published method.[15] Transfection of small interfering RNA (siRNA) was conducted as previously reported.[16] We obtained *ADAMTS15* siRNA (sc-37491; Santa Cruz Biotechnology, Santa Cruz, CA) and *FILIP1L* siRNA (sc-94184; Santa Cruz Biotechnology) with control siRNA-A (sc-37007; Santa Cruz Biotechnology). After 24 hours transfection, HUVECs were washed with PBS and lysed.

Expression ready, untagged *ADAMTS15* wild-type expression plasmid (Human *ADAMTS15* Gene cDNA Clone [full-length open reading frame Clone]; Cat. No. HG11912-G-N) and empty vector (pCMV/hygro-negative control vector, untagged; Cat. No. CV001) were purchased from Sino Biological Inc (Beijing, P.R. China). The E133Q mutation was introduced using a QuickChange kit (Agilent Technologies). All vector constructs were verified by sequencing. Plasmids were transfected into cells using an Amaxa Nucleofector Device (Lonza), following the manufacturer recommendations as previously reported.[16]

Western blots were detected using anti-*ADAMTS15* (sc-68425; Santa Cruz Biotechnology), anti-*FILIP1L* (sc-102493; Santa Cruz Biotechnology), and anti–β-tubulin (sc-9104; Santa Cruz Biotechnology) antibodies, following a previously reported method.[16]

Table 1. Participant Characteristics

	Exome Sequencing of 12 Families	Probands of 24 Replication Families	Sporadic IA Cases
n	42	24	426
Women, %	61.9	91.7	66.2
Age of diagnosis, y			
Mean±SD	58.3±11.0	60.7±10.3	59.4±10.8
Range	27–79	41–78	30–90
Ruptured IA, %	66.7	58.3	58.2
Hypertension, %	47.6	37.5	50.9
Smokers, %	38.1	25.0	35.7
Drinkers, %	47.6	29.2	35.9

IA indicates intracranial aneurysm.

HUVECs were cultured in endothelial cell basal growth medium 2 with supplements containing vascular endothelial growth factor (Cat. No. CC-4147; Lonza, Cambridge, MA). Cell migration assay was assessed after 8 hours by scraping the cell monolayer in a 24-well plate following a previously reported method.[15] Tube formation was assessed as described previously.[16] HUVECs (40 000 cells per well) were seeded onto 96-well plates coated with Matrigel (BD Biosciences, San Jose, CA). After incubation for 12 hours at 37°C, digital images were captured of tubes that had formed. For quantification, the tube area, total tube length, and number of tube branches were calculated using ImageJ software. Parameters for assessing the tube formation function were obtained from 3 independent tube formation assays.

Statistical Power Calculations

Given the prevalence of IA in the general population of 0.03 and the significance level of 0.05, the statistical power under an arbitrarily assumed sample size for further association analysis was calculated using the CaTS Power Calculator across a range of relative risks and MAFs (http://www.sph.umich.edu/csg/abecasis/CaTS/; Figure I in the online-only Data Supplement.[17]

Statistical Analysis

For selected candidate variants, the association study with IA was analyzed using STATISTICA 64-bit (StatSoft, Tulsa, OK). The corresponding genotype count information in the Japanese genetic variation consortium database was used as the population control. By assuming an autosomal-dominant model of inheritance, logistic regression analysis was used to assess the association between candidate variants and IA phenotype. The *P* value and odds ratio with 95% confidence interval were calculated. The odds ratio was calculated with respect to the risk allele. The Bonferroni correction was used to assess the significance level of the association. We applied the Bonferroni correction on the basis of 78 independent effective tests, and the Bonferroni threshold significance level was $P=0.05/78=0.0006$. A $P<0.001$ was considered suggestive of an association.

Results

Characteristics of the Study Participants

Characteristics of the study participants are shown in Table 1. There was no significant difference in the age at diagnosis between familial and sporadic IA.

Whole Exome Sequencing Analysis

Whole exome sequencing generated 8.1 to 20.0 billion bases for 42 affected individuals. After mapping to the human reference genome (UCSC Genome Browser hg19), we obtained 6.9 to 16.5 Gb effective bases mapped to the genome, with a mean sequencing depth between 64- and 135-fold. The mean percentage of exomes covered to a read depth of ≥8-fold was >94.8%. After alignment and a series of quality controls steps, we identified 557 188 to 1 660 842 single nucleotide variants and 28 014 to 88 129 indels in the 42 individuals (Table II in the online-only Data Supplement and JGVCD).

This study focused on the analysis of less common deleterious variants in the consensus coding sequence region. After filtering against 2 reference databases with MAF<0.05, there were 7338 nonsynonymous single nucleotide variants and indels with a read depth ≥8. There were 975 variants that were completely shared by all patients with IA within ≥1 family. After simulation as described in the Methods in the online-only Data Supplement, there were 452 variants retained. Furthermore, we removed benign or tolerated variants as predicted by PolyPhen-2 and SIFT (or SIFT indel). Finally, 78 candidate variants were retained (part of the result is shown in Table 2, and the complete list of single nucleotide variants is presented in Table III in the online-only Data Supplement). Of these variants, none, except p.Y193F in *GPR63* and p.R142H in *C10orf122,* were completely shared by patients with IA in >1 family. *C10orf122* is a chromosome 6 open reading frame gene; it has not been assigned a function to date. Thus, we did not perform the subsequent replication study.

Replicate Association Study of Several Candidate Variants

In the candidate list, variants in genes that are common in patients with IA (p.Y193F in *GPR63*) are known as disease genes, or that have relevance to IA biology were selected. For a key word related to functional relevance, we searched for ontology based on functional relevance from meta-analysis articles[18–20] and found angiogenesis. Thus, we chose genes in ontologies related to angiogenesis, including extracellular matrix integrity, inflammatory mediators, blood coagulation, and vascular endothelium maintenance. Finally, we selected genes important for extracellular matrix integrity (p.E133Q in *ADAMTS15* and p.E631D in *FILIP1L*), inflammatory mediators (p.G105S in *IL10RA* and p.R261H in *IL11RA*), blood coagulation–related genes (p.R85C in *PAFAH2* and p.R473H in *ZNF222*), and vascular endothelium maintenance (p.D486Y in *THBD*), and those that are deleterious or relate to certain diseases (p.V401M, p.R5224H in *MLL2*). All selected genes were given higher priority for further association study. Replicate association study of 10 variants was performed, and Results are presented in Table 3.

We found that p.E133Q in *ADAMTS15* was aggregated significantly in the familial IA cases ($P=0.0001<0.0006$) after the Bonferroni correction, whereas *p.E631D* in *FILIP1L* was associated with familial IA with suggestive significance ($0.0006<P=0.0007<0.001$). However, p.D486Y in *THBD*, p.R261H in *IL11RA*, *p.R85C* in *PAPAH2*, and p.R473H in *ZNF222* showed higher MAF in sporadic cases when compared with the general population (all nominal, $P<0.05$) but did not reach the suggestive significance level ($P=0.001$).

Effects of Silencing *ADAMTS15* and *FILIP1L* on EC Function

Although *ADAMTS15* p.E133Q and *FILIP1L* p.E631D are deleterious by bioinformatics prediction and they were classified as genes in the ontology of angiogenesis, it is uncertain whether *ADAMTS15* and *FILIP1L* have an angiogenic function. Thus, we investigated whether they have angiogenic activity by silencing *ADAMTS15* or *FILIP1L*. As shown in Figure 3A and 3B, silencing these genes accelerated EC migration, suggesting that they indeed have antiangiogenic activity.

Table 2. Part of SNVs Present in Familial Intracranial Aneurysm Cases After Filtering (the Complete List of SNVs Is Present in Table III in the Online-Only Data Supplement)

Allele Count in 42 Cases	hg19 Chr_ Position	Gene	Function	rs Number (dbSNP135)	RefSeq mRNA	Nucleotide Change	Amino Acid Change	MAF in Japanese Variants Database	Segregated Family	Partially Carried Families*
16	6_97247030	*GPR63*	MS	rs118106616	NM_030784.2	c.578T>A	p.Y193F	0.032	P11, P12	P1(II-5,7,9); P4(II-1,3,6,7); P9(II-1,3)
13	2_219225378	*C2orf62*	MS	rs148345660	NM_198559.1	c.458G>A	p.G153D	0.044	P8	P1(II-7,9); P2(II-4); P3(II-2); P4(II-2,3,7); P10(III-3); P12(II-1,3)
11	3_42577733	*VIPR1*	MS	rs3733055	NM_004624.3	c.1334G>T	p.R445L	0.036	P11	P1(II-5,9); P2(II-5, III-3); P7(II-3), P9(II-1,3)
11	11_130319265	*ADAMTS15*	MS	rs185269810	NM_139055.2	c.397G>C	p.E133Q	0.033	P4	P1(II-5,7,9); P5(II-4); P8(II-1,2)
10	9_119249734	*ASTN2*	MS	rs142855762	NM_014010.4	c.3248G>A	p.T1083I	0.024	P12	P4(II-1,3,6); P6(II-3); P7(II-2,3, III-1)
10	20_239804	*DEFB132*	MS	rs79298157	NM_207469.2	c.145T>C	p.C49R	0.033	P4	P2(II-5); P5(II-3)
10	4_41687843	*LIMCH1*	MS	rs76461603	NM_014988.2	c.2932C>T	p.R978W	0.022	P6	P5(II-3); P7(II-1, III-1); P10(III-3); P11(II-2, III-1), P12(II-2)
9	17_48606194	*MYCBPAP*	MS	rs2290863	NM_032133.4	c.2675G>A	p.G892E	0.028	P12	P4(II-3,7); P5(II-3,4); P9(II-1,2)
8	12_49420078	*MLL2*	MS	rs3782356	NM_003482.3	c.15671C>T	p.R5224H	0.018	P9	P2(II-1, II-4); P6(II-3), P10(III-2,3)
4	12_49446404	*MLL2*	MS	...	NM_003482.3	c.1201C>T	p.V401M	0.003	P1	...
8	11_117860281	*IL10RA*	MS	rs188378450	NM_001558.3	c.313G>A	p.G105S	0.005	P2	P8(II-1,2); P12(II-1,3)
6	1_26314810	*PAFAH2*	MS	rs79851686	NM_000437.3	c.253G>A	p.R85C	0.004	P12	P5(II-3,4); P6(I-2)
6	3_99568627	*FILIP1L*	MS	rs150956085	NM_182909.2	c.1893T>G	p.E631D	0.002	P7	P11(II-2, III-3,5)
6	9_34658652	*IL11RA*	MS	rs117149170	NM_001142784.1	c.782G>A	p.R261H	0.013	P10	...
6	19_44537125	*ZNF222*	MS	rs117318348	NM_001129996.1	c.1418G>A	p.R473H	0.001	P1	P5(II-2,3)
5	2_160982996	*ITGB6*	MS	...	NM_000888.3	c.1777C>T	p.G593R	0.007	P10	P3(II-3,4)
4	20_23028686	*THBD*	MS	rs41348347	NM_000361.2	c.1456C>A	p.D486Y	0.007	P8	P5(II-3)

MAF indicates minor allele frequency; MS, missense; and SNV, single nucleotide variation.
*Data presented as family ID (the individuals carrying the variant).

Table 3. Replication and Association Study of Additional Familial and Sporadic Intracranial Aneurysms

Gene	rs Number (dbSNP135)	RefSeq mRNA	Nucleotide Change	Amino Acid Change	JGVCD				Additional 24 Familial Cases					Sporadic Cases				
					Ref/Ref	Ref/Alt	Alt/Alt	MAF	Ref/Ref	Ref/Alt	Alt/Alt	MAF	OR (95% CI)	*P* Value	Ref/Ref	Ref/Alt	Alt/Alt	MAF	OR (95% CI)	*P* Value
GPR63	rs118106616	NM_030784.2	c.578T>A	p.Y193F	1080	73	1	0.032	21	3	0	0.048	2.09 (0.61–7.15)	0.243	388	28	0	0.033	1.03 (0.66–1.77)	0.906
ADAMTS15	rs185269810	NM_139055.2	c.397G>C	p.E133Q	1071	72	2	0.033	17	7	0	0.146	5.96 (2.40–14.82)	0.00013	400	26	1	0.033	1.02 (0.65–1.59)	0.937
MLL2	rs3782356	NM_003482.3	c.15671C>T	p.R5224H	1101	40	1	0.018	24	0	0	0	...	...	416	10	0	0.012	0.65 (0.32–1.30)	0.221
MLL2	rs200102669	NM_003482.3	c.1201C>T	p.V401M	370	2	0	0.003	24	0	0	0	...	...	425	1	0	0.001	0.44 (0.04–4.84)	0.498
IL10RA	rs188378450	NM_001558.3	c.313G>A	p.G105S	1121	12	0	0.005	23	1	0	0.021	4.06 (0.51–32.56)	0.187	424	2	0	0.002	0.44 (0.10–1.98)	0.285
PAFAH2	rs79851686	NM_000437.3	c.253G>A	p.R85C	931	8	0	0.004	23	1	0	0.021	5.06 (0.61–42.25)	0.134	414	12	0	0.014	3.37 (1.37–8.32)	0.0058
THBD	rs41348347	NM_000361.2	c.1456C>A	p.D486Y	1120	17	0	0.007	24	0	0	0	...	...	413	13	0	0.015	2.07 (1.00–4.31)	0.05
IL11RA	rs117149170	NM_001142784.1	c.782G>A	p.R261H	1064	27	1	0.013	24	0	0	0	...	...	406	20	0	0.024	1.87 (1.04–3.36)	0.036
FILIP1L	rs150956085	NM_182909.2	c.1893T>G	p.E631D	799	3	0	0.002	22	2	0	0.042	24.21 (3.85–152.25)	0.0007	424	2	0	0.0024	1.26 (0.21–7.57)	0.803
ZNF222	rs117318348	NM_001129996.1	c.1418G>A	p.R473H	727	2	0	0.001	24	0	0	0	...	...	420	6	0	0.007	5.19 (1.04–25.81)	0.044

Alt indicates alternative allele; CI, confidence interval; JGVCD, Japanese genetic variation consortium database; MAF, minor allele frequency; OR, odds ratio; and Ref, reference allele.

Effects of p.E133Q in *ADAMTS15* on Tube Formation and Migration

We further investigated the effects of p.E133Q in *ADAMTS15* on endothelial function. The mutation significantly accelerated endothelial migration (Figure 3C). However, the mutation did not have an effect on tube formation (Figure 3D).

Discussion

Multiple less common deleterious variants were identified in the familial IA cases after exome sequencing and filtering (Table III in the online-only Data Supplement). Meta-analysis of >116 000 individuals identified 19 single nucleotide polymorphisms associated with IA that were mainly related to the vascular endothelium and extracellular matrix.[19] In this study, we identified several less common or rare variants of extracellular matrix genes (*ADAMTS15*, *FILIP1L*, *TTN*, *ITGB6*, *CRELD1*, *LRP4*, and *LRP5*; Table 2; Table III in the online-only Data Supplement) shared by all patients with IA within ≥1 family. A replication study of p.E133Q in *ADAMTS15* demonstrated that it was aggregated in familial IA cases significantly, even with Bonferroni correction (odds ratio, 5.96; 95% confidence interval, 2.40–14.82; P=0.00013). *ADAMTS15* is a disintegrin and metalloproteinase with thrombospondin motifs 15, and previous gene expression studies of IAs demonstrated abnormal transcription of matrix metalloproteinases, indicating a possible role in predisposition to IA development.[20] Another variant, p.E631D in *FILIP1L*, was also suggested to be involved in angiogenesis as previously predicted although it had weak evidence on association with IA.[21,22]

Because of the low allele frequencies for *ADAMTS15* p.E133Q and *FILIP1L* p.E631D, it is difficult to prove that the sporadic cases would have the same variant as the familial IA cases. Alternatively, biological investigation was conducted to test whether these genes, and not individual variants, are involved in angiogenesis. The experiments clearly demonstrated that silencing these genes enhanced migration of HUVECs, indicating that the genes have antiangiogenic activity. Given that enhanced EC migration is associated with increased surface integrin expression when stimulated by vascular endothelial growth factor[23] in endothelial cell basal growth medium 2, *ADAMTS15* and *FILIP1L* are postulated to downregulate surface integrin. Furthermore, investigation into the effects of p.E133Q in *ADAMTS15* revealed that overexpression of the mutation accelerated endothelial migration, suggesting that this is a loss-of-function mutation because it has the same effect as silencing *ADAMTS15*. Taken together, we considered that IA may be mediated by modifying the expression of integrin on ECs.

The limitations of this study should be considered. First, although the exome sequencing results might represent several candidate genes, we performed replicate association studies for only 10 of 78 candidate variants; the other candidate variants have not yet been validated. Given such a biased selection, we adhered to stringent statistical criteria, to minimize the chance of false-positives. Although proving pathogenic association of IA with rare family-specific variants (MAF<0.001) is difficult, even when using large sample sizes, gene-based association studies (such as a burden test) or multiple family replication

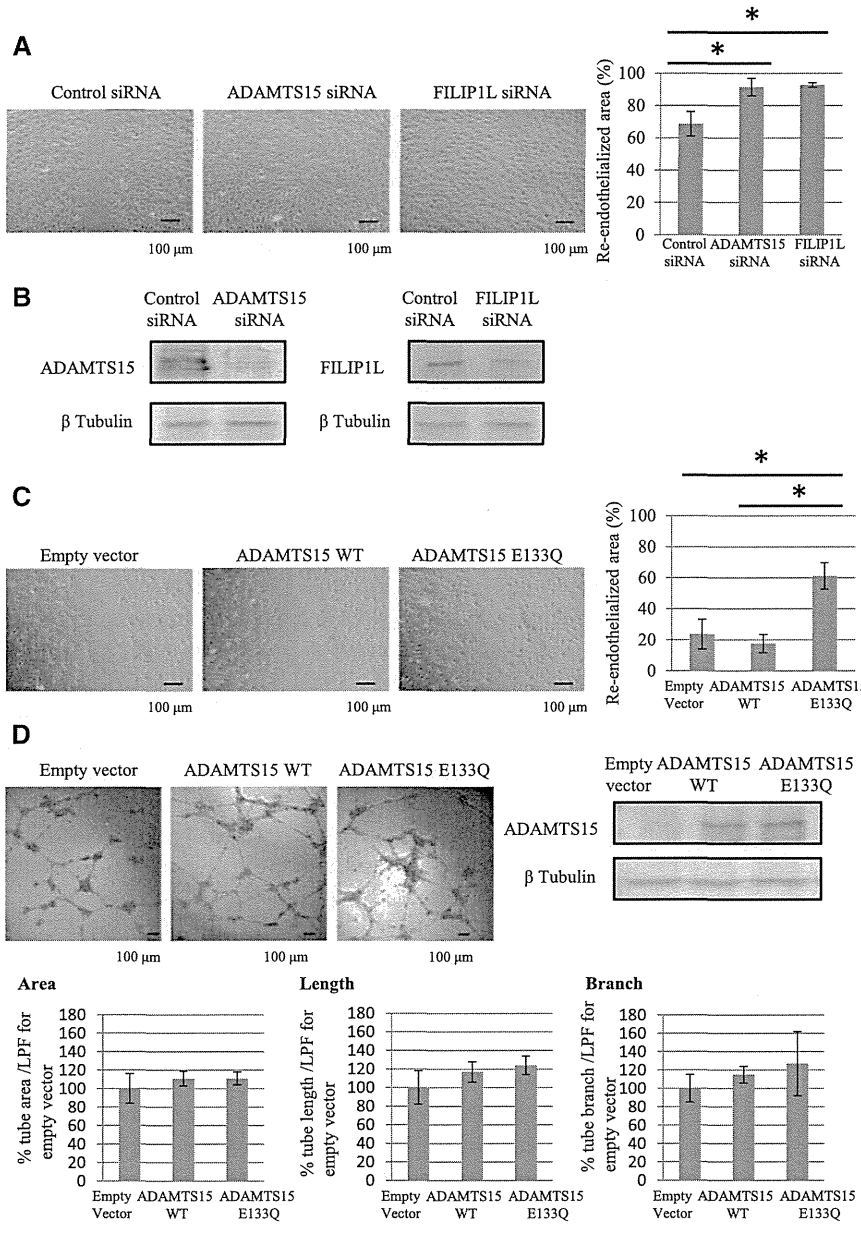

Figure 3. Effect of depletion of *ADAMTS15* or *FILIP1L* by siRNA on human umbilical venous endothelial cell (HUVEC) migration and effect of *ADAMTS 15* E133Q overexpression on HUVEC migration and tube formation. **A**, Cell migration was assessed in HUVECs with siRNA. Results of 3 independent experiments performed in triplicate are represented. The re-endothelialized areas (**right**) were quantified. Data are mean±SD *P<0.05. **B**, Western blot analysis to confirm effects of siRNA. **C**, Cell migration was assessed in HUVECs with *ADAMTS15* wild-type (WT) and E133Q overexpression. Results of 3 independent experiments performed in triplicate are represented. The re-endothelialized areas (**right**) were quantified. Data are mean±SD *P<0.05. **D**, Tube formation assay was assessed in HUVECs with *ADAMTS15* WT and E133Q overexpression. Tube area, total tube length, and number of branches per low-power field (LPF) were determined by Matrigel assay and imaging analysis. Results of 3 independent experiments performed in triplicate are represented. Data are mean±SD. There was no significant difference among groups. Western blot analysis to confirm effects of *ADAMTS15* WT and E133Q overexpression.

studies might be useful. Second, exome sequencing has its own limitations. It cannot capture structural or noncoding variants, such as copy number variations, promoters, or enhancers, which may predispose to IA susceptibility. Third, because large definitely diagnosed IA-free controls were not available, we used the general population as the control in the replicate association analysis. This may limit the independence of the replication from the discovery study and perturb the associate results. However, such an approach could be justifiable for rare variants that cannot be detected without large samples and may not perturb the results significantly for familial IA if at all. Furthermore, given that founder variants are identified in the Japanese population, it is not known whether these variants are also associated with IAs in different ethnic populations. This should be explored in future studies.

There was good analytic strength in this study. We conducted genetic analysis for 42 selected IA-definite members in 12 IA-aggregated families. The sharing of a rare variant by all patients within IA families gave an indication of causality. In addition, although we selected 10 genes subjectively based on review of the literature, we obtained a significant candidate, *ADAMTS15* p.E133Q, which showed significant association with IA even after the Bonferroni correction and was proven to be loss-of-function biologically. In conclusion, *ADAMTS15* may warrant further replication studies and biological investigation in relation to IA.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Supplemental methods

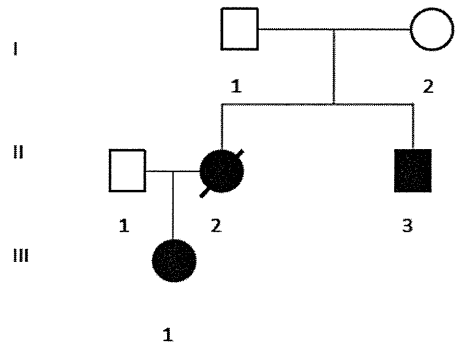
Exome sequencing, mapping and variant calling

Whole exome sequencing was performed at Riken Genesis Co., Ltd in Japan. Exon capture was performed using the SureSelect 70.4Mb Human All Exon V4+UTR Kit (Agilent Technologies, CA, USA) and sequencing was performed by the Illumina HiSeq 2000 platform (Illumina, San Diego, CA, USA). Adapter sequences were removed by cutadapt (v1.2.1). After quality control, reads were mapped to the reference human genome (hg19) using the Burrows-Wheeler Aligner (BWA ver.0.6.2; http://bio-bwa.sourceforge.net/index.shtml). Mapping results were corrected using Picard (ver.1.73) for removing duplicates and Genome Analysis Toolkit (GATK ver.1.6-13; http://www.broadinstitute.org/gsa/wiki/index.php/The_Genome_Analysis_Toolkit) for local alignment and quality score recalibration. Single nucleotide variant (SNV) and indel calls were performed with multi-sample calling using GATK, and filtered to coordinates with VQSR passing and variant call quality score ≥ 30. Annotations of SNVs and indels were based on dbSNP135, CCDS (NCBI, Nov 2011), RefSeq (UCSC Genome Browser, Nov 2011), Encode (UCSC Genome Browser, ver. 7), and 1000 Genomes (Oct 2011). Variants were further filtered according to the following criteria: with predicted functions of frameshift, nonsense, read-through, missense, deletion, insertion, or insertion-deletion.

Simulation

Considering the high possibility of segregation within one family by chance, we simulated and calculated the expected allele frequency among 42 cases in these families according to the MAF in the general population and family structure. The top 95% CI of the simulation results was used to compare the minor allele counts observed in the study families. If the observed value was larger than the expected 95% CI value, higher priority was given for further replication study.

The expected allele frequency and 95% CI of expected allele frequency among 42 members in 12 pedigrees was calculated by simulation as follows. First, for each family, we generated genotypes of unrelated founders independently for given alleles based on their frequencies in the Japanese genetic variation consortium database using the generation of random numbers. Next, genotypes of sons or daughters were constructed by random transmission of alleles from founder parents by computer. This simulation for the 12 pedigrees was conducted 10,000 times and the largest 9,500 rank among 10,000 simulations was used for the 95% CI.



Example figure. Example of genotype construction.
An illustrative example is shown. If we genotype a family in the example figure, in which II2 is known to be affected by IA but did not join this study, we first genotype founders (I1, I2 and II1) using allele frequencies for wild-type allele and single-nucleotide polymorphism (V) reported by the database. The genotypes of II2 and II3 were constructed from founder genotypes I1 and I2, based on mendelian law. The genotype of III1 is constructed from the genotype II2 and III1, by mendelian law. We counted the total number of V alleles in the IA patients in this family, i.e., II3 and III1.

Supplemental Table I. Primer sets and PCR condition of Sanger sequencing and genotyping by TaqMan/RFLP methods

A, Sanger sequncing primer set

Genes	Variants (Nucleotide change)	Forward Primer (5′ > 3′)	Reverse Primer (5′ > 3′)	Annealing temperature (°C)
GPR63	p.Y193F (c.578T>A)	GAAGGGTGTTGAGTATGCCCA	AGTGCTGAACATGCCCTTTG	56
ADAMTS15	p.E133Q (c.397G>C)	AGGTAGTCGTTCCCATCCGA	GCCGTGGAACTTGACCATT	56
IL10RA	p.G105S (c.313G>A)	TCCCAGTTTCTCCCAATGTG	CAACTAGTTCTCCGGCCAG	56
MLL2	p.R5224H (c.15671C>T)	AGAGGCGTCAGTGAAGACCA	TGAGCTCTTTTGCTGTCTTCC	56
MLL2	p.V401M (c.1201C>T)	TCCACAGAAAGTGTGGGGTCT	ATCCACAGCATTCGTGCCT	56
THBD	p.D486Y (c.1456C>A)	CAACCAGACTGCCTGTCCA	TGCAGCACTACCTCCTTGGAA	56
IL11RA	p.R261H (c.782G>A)	TGACTTTGTGTCTTGATGCCC	AACCCAGCACCCAGTACAAA	56
FILIP1L	p.E631D (c.1893T>G)	TCGTTCATTAGCATACCTTCG	CAGCGTAACCAAGGAGAGAGA	56
PAFAH2	p.R85C (c.253G>A)	GCTAAGACTCAGCAGCTGTCA	TCCAGAGGGGAGAAACACAT	56
ZNF222	p.R473H (c.1418G>A)	CTGCCAAAGAAAGCCATTGA	CAAGATCACGCCACTGCACT	56

B, TaqMan primer set and reaction condition

Gene	NCBI reference	Nucleotide change	Amino acid change	VIC-MGB	FAM-MGB	Forward Primer (5′ > 3′)	Reverse Primer (5′ > 3′)	Taqman reaction mix	Taqman program
GPR63	NM_030784.2	c.578T>A	p.Y193F	Set#-1 rs118106616-T: AACCTTAGCTCTATATGGGTTTA	Set#-1 rs118106616-A: AACCTTAGCTCTAAATGGGTTTA	ACGGCTAAAGGAAAAGCTACACA	GAGTAGCCATCCTGCTC ATCATT	DNA: 1 μl; 2×TaqMan Universal PCR Master Mix: 6.2 5μl; 40×SNP Genotyping Assay Mix: 0.16 μl; ddH_2O: 5.09 μl	50°C, 2 min; 95°C, 10 min; (92°C, 15 second; 60°C, 1 min; 40 cycles). Postread: 25°C hold
ADAMTS15	NM_139055.2	c.397G>C	p.E133Q	Set#-1 rs185269810-C: AGGCGCCCAGTATG	Set#-1 rs185269810-G:AGGCGCCGAGTAT	CCTGCGACGCTGCTTCTATT	GGGCAGCGGGCTAATGA		

C, Enzymes of RFLP methods and primer set

Gene	NCBI reference	Nucleotide change	Amino acid change	Restriction enzyme and buffer	Forward Primer (5′ > 3′)	Reverse Primer (5′ > 3′)	Conditions (temperature, time)	Gel for Electrophoresis (30 minutes)	WT fragments (bp)	Heterozygous fragments (bp)
IL10RA	NM_001558.3	c.313G>A	p.G105S	*Pvu* II-HF (CutSmart buffer)	AACTGTAGCCAGACCCTGTCCTATGA	ACGGTCCAGTTGGAGTGCCGGCAGC	37°C, 3h	4% Metaphor agarose gel	119	95; 24
MLL2	NM_003482.3	c.15671C>T	p.R5224H	*Bcc* I (CutSmart buffer)	CAGTGAAGACCAGGTCCTCCA	AGCCTCCGCACCAACAACC	37°C, 3h	4% Metaphor agarose gel	118	91; 27
MLL2	NM_003482.3	c.1201C>T	p.V401M	*Nla* III (CutSmart buffer)	TCCACAGAAAGTGTGGGGTCT	CTCTGTACGTTGCTTGCCAA	37°C, 3h	2% agarose gel	194	154; 40
THBD	NM_000361.2	c.1456C>A	p.D486Y	*Cvi* QI (NEBuffer 3.5)	TACCTTCGAGTGCATCTGCG	ATGGAGATGCCTATGAGCAA	25°C, 3h	2% agarose gel	189	113; 76
IL11RA	NM_001142784.1	c.782G>A	p.R261H	*Bcc* I (CutSmart buffer)	TGACTTTGTGTCTTGATGCCC	AACCCAGCACCCAGTACAAA	37°C, 3h	2% agarose gel	338	171; 167
FILIP1L	NM_182909.2	c.1893T>G	p.E631D	*Bmg* BI (NEBuffer 3)	TCGTTCATTAGCATACCTTCG	CAGCGTAACCAAGGAGAGAGA	37°C, 3h	2% agarose gel	328	220; 108
PAFAH2	NM_000437.3	c.253G>A	p.R85C	*Taq* I (NEBuffer)	ATTCCAGCTAACAGGCAGTC	AAACACATGTCCCCACCCATA	65°C, 3h	4% Metaphor agarose gel	103; 19	122; 103; 19
ZNF222	NM_001129996.1	c.1418G>A	p.R473H	*Bsi* HKAI (NEBuffer 4)	AAATGTGAGGACTGTGGGGA	TGTATCAAATTTCACACTCCA	65°C, 3h	4% Metaphor agarose gel	116	93; 23

Abbreviation: bp, base pairs

Supplemental Table II. Summary of exome sequencing results

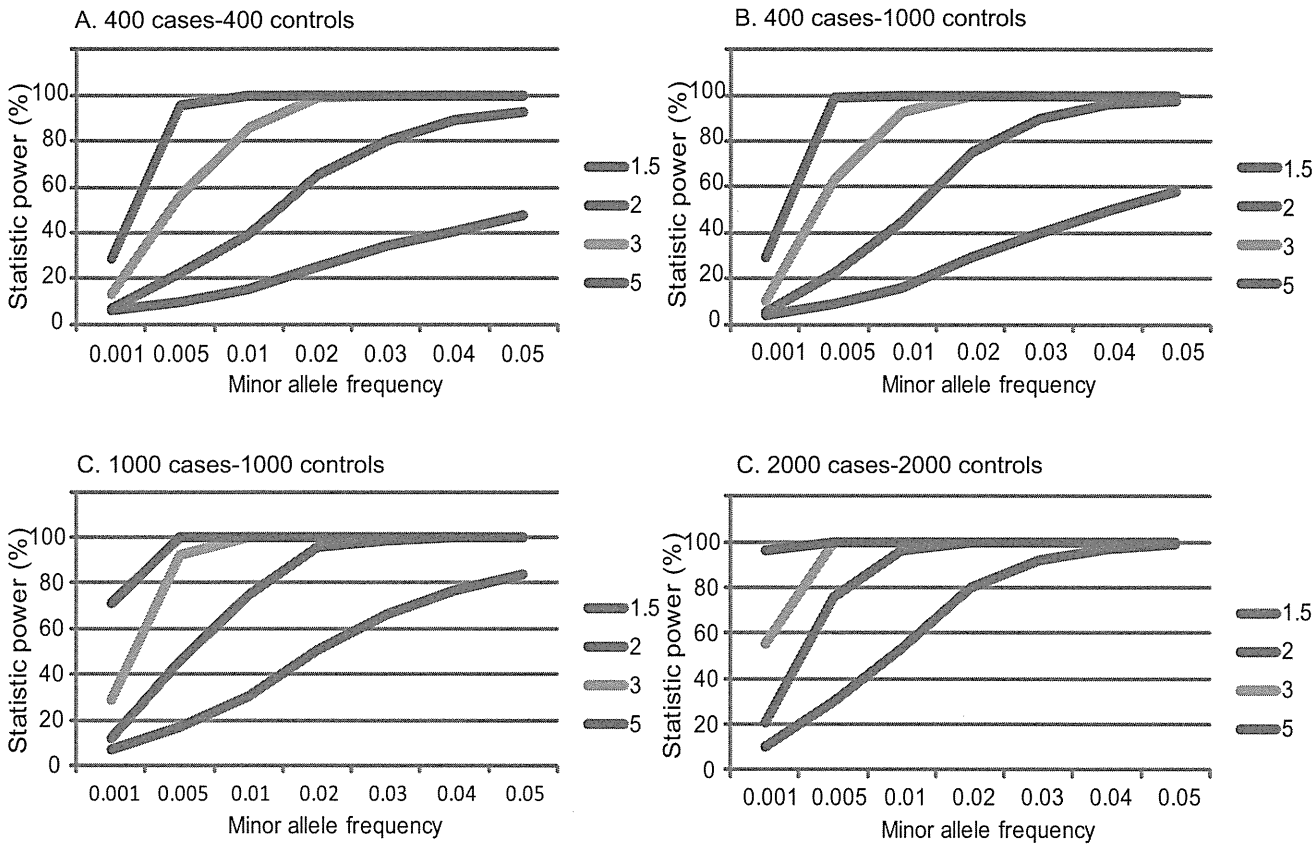
Data	Total (42)
Sequencing and mapping data	
Raw data yield (Gb)	8.1–20.0
Number of effective bases (Gb) mapped to genome	6.9–16.5
Exome capture	
Effective bases on target region (Mb)[a]	4452–9514
Mean sequencing depth on target region	63.77–135.20
Coverage of target region (%)	99.72–99.87
Fraction of target region covered with at least 8X (%)	94.79–98.36
SNV and Indel annotation	
Number of total SNVs	557,188–1,660,842
Number of novel SNVs	4,496–15,247
Missense	9,357–9,846
Nonsense	65–89
Readthrough	19–30
Splice donor site	29–46
Splice acceptor site	35–47
Number of total Indels	28,014–88,129
Number of novel Indels	2,719–7,216
The transition/transversion ratio	
Known	2.06–2.13
Novel	1.70–2.16
All	2.06–2.12
Percentage of variants identified that reside in dbSNP	
SNV	0.96–0.97
Indel	0.82–0.85
All	0.95–0.96

Abbreviation: SNV, single nucleotide variation
Known: Registered in dbSNP
Novel: Not registered in db SNP

Supplemental Table III. List of 78 SNVs present in familial IA cases after filtering

Allele count in 42 cases	95% CI of expected value by simulation	hg19 Chr_position	Gene	Function	rs number (dbSNP135)	RefSeq mRNA	Nucleotide change	Amino acid change	MAF in Japanese variants database	Segregated family	Partially carried families and carried individuals*
16	8	6_97247030	*GPR63*	MS	rs118106616	NM_030784.2	c.578T>A	p.Y193F	0.032	P11,P12	P1(II-5,7,9); P4(II-1,3,6,7); P9(II-1,3)
13	10	2_219225378	*C2orf62*	MS	rs148345660	NM_198559.1	c.458G>A	p.G153D	0.044	P8	P1(II-7,9); P2(II-4); P3(II-2); P4(II-2,3,7); P10(III-3); P12(II-1,3)
11	9	3_42577733	*VIPR1*	MS	rs3733055	NM_004624.3	c.1334G>T	p.R445L	0.036	P11	P1(II-5,9); P2(II-5, III-3); P7(II-3), P9(II-1,3)
11	9	11_130319265	*ADAMTS15*	MS	rs185269810	NM_139055.2	c.397G>C	p.E133Q	0.033	P4	P1(II-5,7,9); P5(II-4); P8(II-1,2)
10	7	9_119249734	*ASTN2*	MS	rs142855762	NM_014010.4	c.3248G>A	p.T1083I	0.024	P12	P4(II-1,3,6); P6(II-3); P7(II-2,3, III-1)
10	9	20_239804	*DEFB132*	MS	rs79298157	NM_207469.2	c.145T>C	p.C49R	0.033	P4	P2(II-5); P5(II-3)
10	7	4_41687843	*LIMCH1*	MS	rs76461603	NM_014988.2	c.2932C>T	p.R978W	0.022	P6	P5(II-3); P7(II-1, III-1); P10(III-3); P11(II-2, III-1), P12(II-2)
9	8	17_48606194	*MYCBPAP*	MS	rs2290863	NM_032133.4	c.2675G>A	p.G892E	0.028	P12	P4(II-3,7); P5(II-3,4); P9(II-1,2)
8	6	12_49420078	*MLL2*	MS	rs3782356	NM_003482.3	c.15671C>T	p.R5224H	0.018	P9	P2(II-1, II-4); P6(II-3), P10(III-2,3)
4	1	12_49446404	*MLL2*	MS	-	NM_003482.3	c.1201C>T	p.V401M	0.003	P1	-
8	7	2_128370111	*MYO7B*	MS	rs148715226	NM_001080527.1	c.3253C>T	p.R1085W	0.022	P9	P1(II-2,5,7); P2(II-1); P5(III-1)
8	7	2_152347020	*NEB*	MS	rs139333406	NM_001164507.1	c.25163C>T	p.R8388H	0.023	P10	P9(II-2,3); P11(II-2, III-3,5)
8	6	3_126180577	*ZXDC*	MS	rs138387718	NM_025112.4	c.1928G>A	p.S643L	0.019	P11	P7(II-2, III-1)
8	3	11_117860281	*IL10RA*	MS	rs188378450	NM_001558.3	c.313G>A	p.G105S	0.005	P2	P8(II-1,2); P12(II-1,3)
7	5	2_178704994	*PDE11A*	MS	-	NM_016953.3	c.1484G>A	p.P495L	0.015	P1	P2(II-4, II-5)
7	5	5_137517132	*KIF20A*	MS	rs3734116	NM_005733.2	c.187G>A	p.E63K	0.015	P8	P4(II-1,3,6)
6	1	7_107393856	*CBLL1*	MS	-	NM_024814.2	c.182A>G	p.E61G	0.003	P6	P11(II-2, III-3,5)
6	3	2_215901688	*ABCA12*	MS	rs138402017	NM_173076.2	c.974G>A	p.S325F	0.004	P4	P10(III-2)
6	3	1_26314810	*PAFAH2*	MS	rs79851686	NM_000437.3	c.253G>A	p.R85C	0.004	P12	P5(II-3,4); P6(I-2)
6	5	1_157773646	*FCRL1*	MS	rs149740001	NM_052938.4	c.308A>T	p.I103K	0.014	P8	P6(II-3)
6	0	3_99568627	*FILIP1L*	MS	rs150956085	NM_182909.2	c.1893T>G	p.E631D	0.002	P7	P11(II-2, III-3,5)
6	5	8_100454797	*VPS13B*	MS	rs117357319	NM_017890.3	c.3379G>T	p.G1127W	0.015	P8	P4(II-2,3,7)
6	5	9_34658652	*IL11RA*	MS	rs117149170	NM_001142784.1	c.782G>A	p.R261H	0.013	P10	-
6	4	10_127344605	*C10orf122*	MS	rs192657555	NM_001128202.1	c.425C>T	p.R142H	0.010	P3, P9	-
6	1	12_120995232	*RNF10*	MS	-	NM_014868.4	c.793A>G	p.I265V	0.002	P9	-
6	0	19_44537125	*ZNF222*	MS	rs117318348	NM_001129996.1	c.1418G>A	p.R473H	0.001	P1	P5(II-2,3)
6	1	19_44778738	*ZNF233*	MS	rs76164141	NM_001207005.1	c.1925A>G	p.H642R	0.003	P1	P5(II-2,3)
5	3	4_159836467	*C4orf45*	MS	rs10517695	NM_152543.2	c.401C>T	p.R134H	0.005	P7	P10(III-6)
5	3	2_179549988	*TTN*	MS	rs146400809	NM_133378.4	c.28730G>A	p.P9577L	0.004	P12	-
5	4	19_5751803	*TMEM146*	MS	rs139062634	NM_152784.3	c.1133T>C	p.M378T	0.010	P1	P2(III-3)
5	4	20_31671244	*BPIFB4*	MS	rs140765002	NM_182519.2	c.241C>T	p.L81F	0.009	P3	P4(II-2,6)
5	4	20_31678541	*BPIFB4*	MS	rs150652053	NM_182519.2	c.1079G>A	p.S360N	0.009	P3	P4(II-2,6)
5	4	1_118584643	*SPAG17*	MS	rs140661130	NM_206996.2	c.2837C>A	p.R946L	0.010	P8	P7(II-2,3)
5	3	2_160982996	*ITGB6*	MS	-	NM_000888.3	c.1777C>T	p.G593R	0.007	P10	P3(II-3,4)
5	4	3_196865191	*DLG1*	MS	rs2271822	NM_004087.2	c.884G>A	p.A295V	0.011	P6	P12(II-1,2)
5	4	6_138583857	*KIAA1244*	MS	rs149573553	NM_020340.4	c.1237G>A	p.E413K	0.008	P7	P12(II-1,2)
5	3	8_134107412	*TG*	MS	rs2272707	NM_003235.4	c.7364G>A	p.R2455H	0.005	P3	P8(II-1,3)
5	4	17_11795157	*DNAH9*	MS	rs3760436	NM_001372.3	c.11176C>T	p.R3726W	0.011	P4	-
5	4	19_44611829	*ZNF224*	MS	rs3746323	NM_013398.2	c.1516C>G	p.H506D	0.011	P4	-
5	3	20_31644401	*BPIFB3*	MS	rs138897849	NM_182658.1	c.178G>T	p.V60F	0.008	P3	P4(II-2, 6)
4	2	11_119548363	*PVRL1*	MS	rs142930935	NM_002855.4	c.635C>T	p.R212H	0.004	P12	P5(II-4)
4	3	17_59556060	*TBX4*	MS	rs78640841	NM_018488.2	c.622G>A	p.G208S	0.005	P12	P1(II-2)
4	2	17_42225328	*C17orf53*	MS	-	NM_024032.3	c.157G>A	p.V53M	0.003	P9	P5(II-4)
4	3	2_42936042	*MTA3*	MS	rs76558479	NM_020744.2	c.1331G>A	p.R444H	0.005	P6	P5(II-4)
4	2	2_219209648	*PNKD*	MS	rs185906233	NM_015488.4	c.1102C>T	p.R368W	0.005	P2	-
4	3	5_37226961	*C5orf42*	MS	rs191239995	NM_023073.3	c.1736G>A	p.A579V	0.006	P2	-
4	0	7_117199578	*CFTR*	MS	rs138427145	NM_000492.3	c.1453A>T	p.S485C	0.001	P1	-
4	1	8_145152023	*CYC1*	MS	rs74494160	NM_001916.3	c.859C>T	p.R287C	0.003	P2	-
4	2	12_123082455	*KNTC1*	MS	rs186936079	NM_014708.4	c.4533G>C	p.L1511F	0.003	P2	-
4	3	20_23028686	*THBD*	MS	rs41348347	NM_000361.2	c.1456C>A	p.D486Y	0.007	P8	P5(II-3)
4	1	12_49949686	*KCNH3*	MS	-	NM_012284.1	c.2420T>C	p.L807P	0.003	P1	-
3	1	3_9986171	*CRELD1*	MS	rs186846833	NM_015513.4	c.1171G>A	p.A391T	0.002	P12	-
3	1	10_75671451	*PLAU*	MS	rs149516796	NM_001001791.2	c.448C>A	p.A150S	0.002	P6	-
3	2	8_8235180	*SGK223*	MS	rs34019705	NM_001080826.1	c.739C>A	p.D247Y	0.003	P3	-
3	2	1_115220086	*AMPD1*	MS	rs121912682	NM_000036.2	c.1373C>T	p.R458H	0.005	P5	-
3	2	1_156536300	*IQGAP3*	MS	rs149238952	NM_178229.4	c.164G>A	p.P55L	0.004	P8	-
3	2	2_38301574	*CYP1B1*	MS	rs72549382	NM_000104.3	c.958C>A	p.V320L	0.004	P7	-
3	2	2_48809568	*STON1*	MS	-	NM_172311.2	c.1796G>A	p.R599H	0.002	P8	-
3	0	3_49362424	*USP4*	MS	rs138851995	NM_003363.3	c.536C>T	p.R179H	0.001	P8	-
3	2	9_2719083	*KCNV2*	MS	rs143382624	NM_133497.3	c.1344G>C	p.W448C	0.003	P3	-
3	0	9_131864200	*CRAT*	MS	-	NM_000755.3	c.767C>T	p.R256H	0.001	P12	-
3	2	10_16794564	*RSU1*	MS	-	NM_012425.3	c.572C>A	p.R191L	0.004	P3	-
3	1	10_64966705	*JMJD1C*	MS	rs185530790	NM_032776.1	c.4724A>G	p.V1575A	0.002	P5	-
3	0	11_46884177	*LRP4*	MS	rs138291903	NM_002334.3	c.5365G>C	p.Q1789E	0.002	P12	-
3	2	11_64331790	*SLC22A11*	MS	rs147522958	NM_018484.2	c.832G>A	p.E278K	0.003	P6	-
3	2	11_134238603	*GLB1L2*	MS	rs138130138	NM_138342.3	c.955G>A	p.G319R	0.003	P6	-
3	2	12_57351020	*RDH16*	MS	rs141054345	NM_003708.3	c.227C>A	p.R76M	0.004	P3	-
3	2	17_4458516	*MYBBP1A*	MS	rs181906424	NM_001105538.1	c.104A>G	p.F35S	0.003	P7	-
3	2	17_26862138	*FOXN1*	MS	-	NM_003593.2	c.1549G>T	p.D517Y	0.003	P7	-
3	2	18_28934293	*DSG1*	MS	rs149191001	NM_001942.2	c.2134C>T	p.R712C	0.004	P9	-
3	2	18_29867517	*FAM59A*	MS	rs188733917	NM_001242409.1	c.1043G>A	p.T348I	0.003	P12	-
3	2	19_7685310	*XAB2*	MS	rs117645581	NM_020196.2	c.2117G>A	p.T706M	0.004	P6	-
3	1	19_8368795	*CD320*	MS	-	NM_016579.3	c.446G>A	p.T149M	0.002	P9	-
3	2	19_16024709	*CYP4F11*	MS	rs146897508	NM_021187.3	c.1408C>T	p.G470R	0.003	P12	-
3	2	19_57326839	*PEG3*	MS	rs145003090	NM_006210.2	c.2971G>C	p.H991D	0.005	P12	-
3	2	22_40804844	*SGSM3*	MS	-	NM_015705.4	c.1900C>T	p.R634W	0.003	P7	-
3	0	6_87967028	*ZNF292*	MS	rs146052403	NM_015021.1	c.3681A>C	p.L1227F	0.001	P10	-
3	2	11_68216333	*LRP5*	MS	rs147618989	NM_002335.2	c.4643G>T	p.C1548F	0.004	P6	-

Abbreviations: MS, missense; CI, confidence interval; MAF, minor allele frequency; IA; intracranial aneurysm; SNV; single nucleotide variation.
*Data presented as family ID (the individuals carrying the variant).



Supplemental Figure I. Statistical power calculation under an arbitrarily assumed sample size across a range of relative risks and MAFs in association studies. Given a replication sample size of 400 cases and 1000 controls, we had 93% power to detect a putative functional coding SNV with a frequency of 0.01 and a relative risk of 3. The power decreases as the variants become rarer or carry less relative risk. A large sample size (>1000 cases and 1000 controls) is required to obtain definite evidence of an association of variants with MAFs <0.005 and relative risks <2.

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Genetic Study of Intracranial Aneurysms

Junxia Yan, Toshiaki Hitomi, Katsunobu Takenaka, Masayasu Kato, Hatasu Kobayashi, Hiroko Okuda, Kouji H. Harada and Akio Koizumi

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RESEARCH NOTE

Importance of molecular diagnosis in the accurate diagnosis of systemic carnitine deficiency

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Introduction

Systemic carnitine deficiency (SCD: MIM212140) is a rare autosomal recessive disease resulting from defects in the *SLC22A5* gene. In this study, five Japanese SCD probands with low serum free carnitine levels were analysed of whom four were identified during newborn screening (NBS). Direct sequencing of *SLC22A5* revealed six different mutations: p.S467C ($n = 3$), p.R254X ($n = 2$), p.N32S ($n = 2$), p.W132X ($n = 1$), p.W283C ($n = 1$), and p.A451P ($n = 1$). Molecular diagnosis unmasked new SCD cases in two of five pedigrees. In another pedigree, a two-year and two-month-old child, who had not undergone NBS, was previously diagnosed with Reye's syndrome was found to be affected by SCD. Although serum free carnitine levels are informative, we suggest that confirmatory diagnostic sequencing procedures that include other family members are desirable to detect masked SCD subjects.

SCD is often fatal (Eriksson *et al.* 1988; Treem *et al.* 1988). In such cases its clinical profile is characterized by progressive cardiomyopathy, skeletal myopathy, hypoketotic hypoglycaemic encephalopathy, and hyperammonaemia (Chapoy *et al.* 1980; Magoulas and El-Hattab 2012). SCD is caused by mutations in the *SLC22A5* gene, and genetic epidemiology demonstrates that the incidence of SCD carriers is about 1% in Japan (Koizumi *et al.* 1999). Currently NBS is conducted in various countries for an early diagnosis of disorders of fatty-acid oxidation, including SCD.

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Toshiaki Hitomi and Norio Matsuura contributed equally to this work.

Here we report the mutation analysis of five Japanese probands with low serum free carnitine levels, some of whom were identified in NBS trials. Direct sequencing of *SLC22A5* revealed six different mutations.

Materials and methods

Pedigrees and cases

This study was approved by the Ethics Committee of the Kyoto University Institutional Review Board and appropriate informed consent was obtained from all subjects. Molecular diagnoses were carried out at Kyoto University between 2008 and 2011.

Sequencing, restriction fragment length polymorphism (RFLP) analysis and bioinformatics analysis

Genomic DNA was isolated from 2 mL peripheral blood sample donated by patients and their blood relatives. We carried out PCR amplification of whole nine exons with 100 bp splicing donor and acceptor sites, 3′-UTR, and the potential promoter region of *SLC22A5* to detect functional variants. The PCR products were directly sequenced on an ABI Prism 3100 sequence analyser (Applied Biosystems, Foster City, USA). Presence of p.A451P RFLP was tested using the restriction enzyme *CviKI*-1 (New England Biolabs, Ipswich, USA). Primers are available on request. Two computer-based algorithms, PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/) were used to predict the pathogenicity of unpublished missense variants.

Keywords. newborn screening; *SLC22A5* gene; systemic carnitine deficiency.

Results and discussion

The mutational analysis of five unrelated pedigrees with SCD is reported in the current study (figure 1). In pedigree A, free carnitine levels in the dried blood spot (DBS) of the proband (A12) were measured as part of NBS, and found to be low at 5.6 μM (reference value >10 μM). Renal fractional excretion of free carnitine was elevated at 16.7% (reference value <2.0%). Proband's elder sister (A11) donated blood samples for molecular diagnosis, and her serum free carnitine level was found to be 4.6 μM. Genomic DNA sequencing of A11 and A12 revealed that both sisters harboured two mutations: p.W132X and p.S467C (figure 2). Mutation p.W132X was transmitted from the mother and mutation p.S467C from the father.

The proband (B13) of pedigree B had low DBS free carnitine levels in NBS: 9.4 μM and 6.3 μM on days six and 14, respectively (figure 1). Renal fractional excretion of free carnitine was elevated at 17.6%. Carnitine administration (600 mg, twice a day) was therefore commenced on day 31. This was stopped at 10 months old for a period of two-weeks for differential diagnosis. Serum free carnitine levels were 56 μM immediately after the last administration; however, two weeks after cessation the level had decreased to 9 μM, confirming that the observed lowered serum free carnitine levels were consistent. Sequence analysis revealed the existence of two variants: p.R254X and p.A451P (figure 2). p.R254X was inherited from the mother and p.A451P from the father. The p.A451P allele was not found in 250 Japanese controls by RFLP using the restriction enzyme *CviKI*-1 (figure 2). Functional prediction by bioinformatics suggested that p.A451P is 'probably damaging' by Polyphen-2 and 'affecting protein function' by SIFT. Sequencing revealed no mutation of *SLC22A5* other than p.R254X and p.A451P in B13. His father (B1), who has an allele of p.A451P, had a low serum free carnitine level (34 μM) (reference value, >38 μM; Koizumi *et al.* 1999). These pieces of evidence consistently suggest that p.A451P is likely to be the causal mutation. Pathogenicity of p.A451P, however, needs to be confirmed experimentally in future.

In pedigree C (figure 1), NBS revealed a low DBS free carnitine level in the fourth child (C14; 8.3 μM) and levels were shown to be very low in the mother (4.1 μM). Molecular diagnosis revealed that the mother unexpectedly carried two known variants: p.R254X and p.S467C. Her renal fractional excretion of free carnitine was elevated at 17.7%. All four children including the proband (C14) (figure 2) were found to be heterozygous, while the father had two wild-type alleles. This is similar to reports of other maternal cases in which SCD was identified in mothers following the NBS of neonates (El-Hattab *et al.* 2010; Lee *et al.* 2010).

The proband of pedigree D (figure 1) was a two-year and two-month-old child (D13). His medical history had been uneventful until the age of 2 years 2 months when he was admitted to hospital with symptoms of hypoglycaemia, elevated liver enzymes and hyperammonaemia; Reye's syndrome was suspected. His nasal discharge was positive for respiratory syncytial virus. After admission, he developed encephalopathy and became comatose with a flat electromyography and no spontaneous respiration. Because

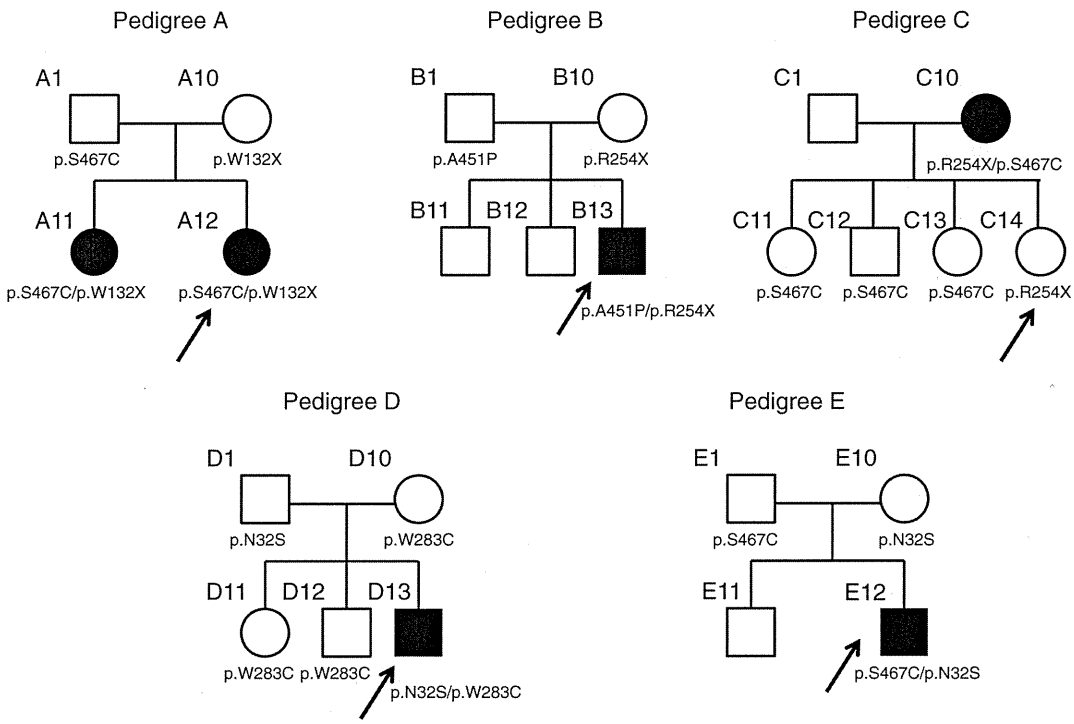

Figure 1. Pedigree and genetic diagnosis of five Japanese families with SCD. SCD patients are shown as black circles or squares. Arrows indicate probands.

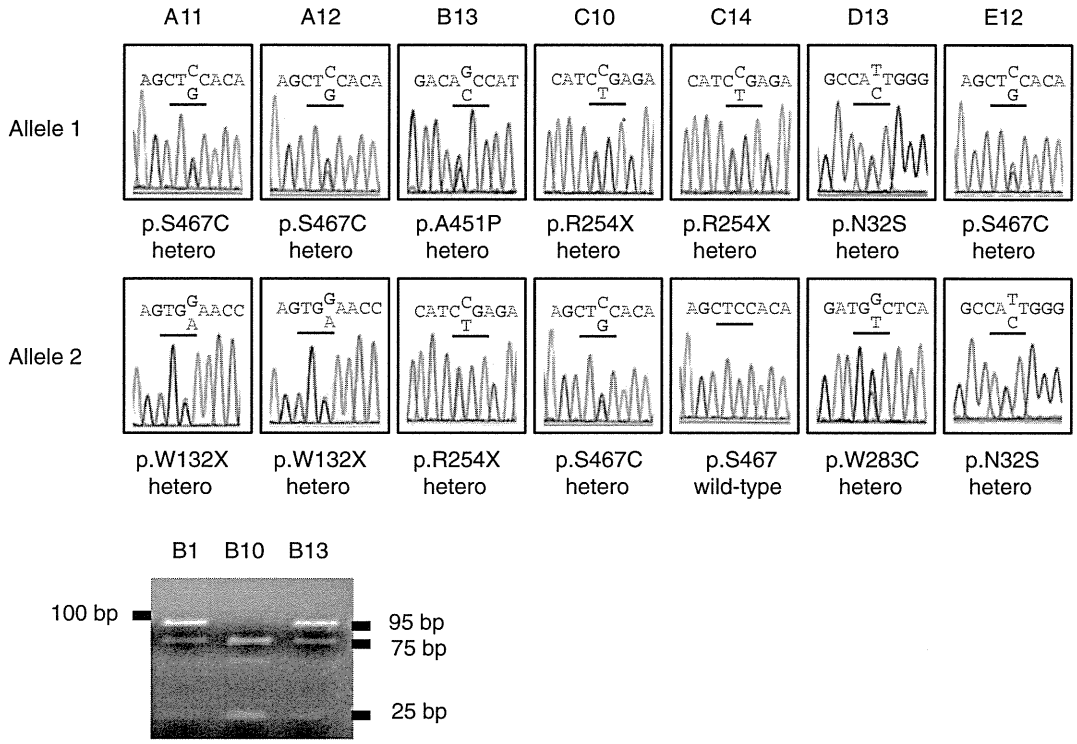

Figure 2. Sequencing analysis of *SLC22A5* mutations in SCD patients and NBS neonates (upper) and RFLP analysis for p.A451P in pedigree B (lower). The p.A451P mutation abolishes the *CviKI*-1 restriction site thus the 95bp PCR product remains undigested (4% metaphor gel).

the Reye's syndrome was suspected, his serum free carnitine level was determined and found to be very low (2.2 μM). Sequencing revealed that he is a compound heterozygote of p.N32S and p.W283C, while his two other siblings are carriers of p.W283C (figure 2). p.N32S was inherited from his father and p.W283C from his mother. If NBS had been conducted in this case, low serum free carnitine levels might have been detected during the early neonatal period and encephalopathy would have been prevented.

In pedigree E (figure 1), NBS revealed low DBS free carnitine levels (9.1 μM) in proband E12. Renal fractional excretion of free carnitine was elevated at 12.6%. Direct sequencing revealed that E12 is a compound heterozygote of p.S467C and p.N32S variants (figure 2). p.S467C was inherited from his father and p.N32S from his mother.

In the present study, molecular diagnosis unveiled two new masked cases (A11 and C10) and corrected a diagnosis of SCD with a carrier for one proband (C14) screened in NBS (figure 1). The new masked cases represent asymptomatic individuals. We also confirmed that a serious case (D13) who was suspected to have Reye's syndrome was instead affected by SCD. It has previously been reported that mild viral infection precipitates as Reye's-like syndrome in SCD subjects (Chapoy *et al.* 1980).

Most of the variants detected in this study are well known, including p.S467C, p.W132X, p.W283C, p.R254X and p.N32S. These have previously been reported in SCD cases in various countries, including Japan, suggesting the existence of founder mutations (Koizumi *et al.* 1999; Lamhonwah *et al.* 2002; Lamhonwah *et al.* 2004; Nezu *et al.* 1999; Tang *et al.* 1999). The only novel variant in the present study is p.A451P. We therefore suggest that known founder mutations should be searched initially to ensure that mutation screening is cost-effective and not time-consuming.

In conclusion, albeit high clinical specificity of measuring serum carnitine levels, confirmatory diagnostic sequencing procedures including other family members are desirable for suspected SCD cases following NBS.

Acknowledgements

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Effects of Extracranial–Intracranial Bypass for Patients With Hemorrhagic Moyamoya Disease

Results of the Japan Adult Moyamoya Trial

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Background and Purpose—About one half of those who develop adult-onset moyamoya disease experience intracranial hemorrhage. Despite the extremely high frequency of rebleeding attacks and poor prognosis, measures to prevent rebleeding have not been established. The purpose of this study is to determine whether extracranial–intracranial bypass can reduce incidence of rebleeding and improve patient prognosis.

Methods—This study was a multicentered, prospective, randomized, controlled trial conducted by 22 institutes in Japan. Adult patients with moyamoya disease who had experienced intracranial hemorrhage within the preceding year were given either conservative care or bilateral extracranial–intracranial direct bypass and were observed for 5 years. Primary and secondary end points were defined as all adverse events and rebleeding attacks, respectively.

Results—Eighty patients were enrolled (surgical, 42; nonsurgical, 38). Adverse events causing significant morbidity were observed in 6 patients in the surgical group (14.3%) and 13 patients in the nonsurgical group (34.2%). Kaplan–Meier survival analysis revealed significant differences between the 2 groups (3.2%/y versus 8.2%/y; P=0.048). The hazard ratio of the surgical group calculated by Cox regression analysis was 0.391 (95% confidence interval, 0.148–1.029). Rebleeding attacks were observed in 5 patients in the surgical group (11.9%) and 12 in the nonsurgical group (31.6%), significantly different in the Kaplan–Meier survival analysis (2.7%/y versus 7.6%/y; P=0.042). The hazard ratio of the surgical group was 0.355 (95% confidence interval, 0.125–1.009).

Conclusions—Although statistically marginal, Kaplan–Meier analysis revealed the significant difference between surgical and nonsurgical group, suggesting the preventive effect of direct bypass against rebleeding.

Clinical Trial Registration—URL: http://www.umin.ac.jp/ctr/index.htm. Unique identifier: C000000166.

(*Stroke*. 2014;45:1415-1421.)

Key Words: cerebral revascularization ■ hemorrhage ■ moyamoya disease

See related article, p 1245.

Moyamoya disease is a unique cerebrovascular disease characterized by progressive occlusion of the bilateral internal carotid arteries at their terminal portions and unusual secondarily formed vascular networks (moyamoya vessels) that act as collateral pathways.[1,2] Unlike pediatric patients, who usually present with transient ischemic attacks (TIAs) or cerebral infarction, about one half of adult patients have intracranial hemorrhage that seriously affects their prognosis.[1–3] Long-term hemodynamic stress to the collateral vessels is thought to induce vascular pathologies leading to hemorrhage.[1,2] Although the rate of recurrent bleeding is known to be extremely high, no therapeutic method of preventing rebleeding attacks has yet been established. Extracranial–intracranial bypass surgery is often used for ischemic moyamoya disease, and angiographic diminishment of moyamoya vessels can be observed after surgery, which is regarded as decreased hemodynamic stress to these vessels.[4] A hypothesis has therefore emerged that bypass surgery can also reduce this stress, even in hemorrhagic moyamoya disease, and prevent rebleeding attacks. In fact, many cases of bypass surgery for hemorrhagic moyamoya disease have been reported, but all are retrospective studies and the benefit of bypass surgery has not yet been scientifically clarified. To resolve this, the Japan Adult Moyamoya (JAM) Trial was

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planned in 1999.[5] The study started in 2001, and follow-up of all enrolled patients was completed in 2013.

Methods

Patient Eligibility

This study is a multicentered, prospective, randomized, controlled trial to clarify the effect of bypass surgery on moyamoya disease with hemorrhagic onset. Twenty-two institutes with sufficient surgical experience with moyamoya disease participated in the study. The target was adult patients who had experienced episodes of intracranial bleeding within the preceding year and could be observed for 5 years after enrollment. The diagnosis of moyamoya disease was made according to the diagnostic guidelines proposed by the Ministry of Health and Welfare of Japan.[3] Table 1 lists all the inclusion and exclusion criteria. The study office carefully checked the radiological data and determined eligibility for the trial in each case.

Table 1. Patient Eligibility for the JAM Trial

Clinical requirements
Age: between 16 and 65 years at the time of the initial bleeding episode
Independent in daily life (modified Rankin disability scale score of 0–2)
Intracerebral hemorrhage, intraventricular hemorrhage, or subarachnoid hemorrhage occurring within the preceding 12 months
At least 1 month since the last stroke episode, either ischemic or hemorrhagic
At least 1 month since the completion of acute phase treatment for hemorrhage and for related secondary pathophysiology (eg, hydrocephalus)
Radiological requirements
CT/MRI
Lack of large infarction spread widely over the territory of a main arterial trunk
Lack of contrast enhancement in the infarcted area
Angiography
Angiographic findings should satisfy the diagnostic criteria of the spontaneous occlusion of the circle of Willis (moyamoya disease) published by the Ministry of Health, Labor and Welfare of Japan:
Occlusive lesions should exist in the terminal portion of the intracranial internal carotid artery or in the proximal portion of the anterior or middle cerebral arteries
An abnormal vascular network in the region of basal ganglia and thalamus (moyamoya vessels) is demonstrated in the arterial phase of angiography
These findings should be demonstrated on both sides
Exclusion criteria
Not independent in daily life (modified Rankin disability scale score of 3–5)
Atherosclerotic carotid disease or cardiac arrhythmia that may cause thromboembolic complications
Malignant tumors or organ failure of the heart, liver, kidney, or lung
Unstable angina or myocardial infarction within the past 6 months
Hematologic abnormality showing bleeding diathesis
Uncontrolled diabetes mellitus showing a serum fasting blood glucose level >300 mg/dL, or requires insulin
Hypertension with a diastolic blood pressure of >110 mm Hg
Treated with extracranial–intracranial bypass surgery before enrollment
Pregnancy

CT indicates computed tomography; and JAM, Japan Adult Moyamoya.

Sample Size

At the beginning of the study, the optimal sample size was calculated on the assumption that the incidence of adverse neurological events would be 8%/y in the nonsurgical group and 4%/y in the surgical group. The follow-up period was 5 years, and the sample size of 160 (80 patients per group) was expected to have 80% of the statistical validity required to detect a difference between the 2 groups with a significance level of 0.05. However, this number was reduced to 80 in January 2006 because the number of patients eligible for the study was revealed to be far smaller than expected. The number of new registration was 13.2/y at that time point, which meant that the completion of the study would be later than 2018. Then, the JAM Trial Executive and Steering Committee determined that the original sample size would be impossible to attain within the adequate study period. The calculation of 80% statistical power told us that the number of 80 patients would be able to detect the statistical significance if the event rate of the surgical group was <2.8%/y when that of the nonsurgical group was set to be 8%/y.

Randomization

According to computed tomography and digital subtraction angiography performed at the onset of intracranial hemorrhage, the study office estimated the bleeding point in each case as type A (bleeding from collateral vessels in the anterior circulation) or type B (bleeding from those in the posterior circulation). After informed consent was obtained, a computer-generated randomization scheme was applied, and the patient was assigned to receive either conservative medical care alone (nonsurgical group) or medical care plus extracranial–intracranial bypass (surgical group). To ensure a balance between these groups with respect to the bleeding point, a randomization scheme was performed separately within type A and type B (stratified randomization). This was required because the outcome could differ between hemorrhages of the basal ganglia (type A) and those of the thalamus (type B), although such a difference had not been proven. To avoid inclusion bias and ensure the quality of the trial, all ineligible patients and patients who were eligible but did not participate in this study for whatever reason were reported and checked by the study office. Bypass surgery for these nonparticipants was prohibited unless a legitimate reason compelled it, such as frequent TIAs or progressive ischemic stroke.

Surgical Treatment

Extracranial–intracranial bypass, if assigned to the surgical group, was performed on both sides (each side at some interval within 3 months after enrollment) by a registered neurosurgeon at each institute. As the operative maneuver, a direct anastomotic procedure such as superficial temporal artery–middle cerebral artery anastomosis was required. Indirect bypass procedures can be added to direct bypass; however, indirect bypass alone was not permitted, nor was high-flow bypass such as venous graft or radial artery graft.

Patient Follow-Up

Table 2 shows the follow-up protocol. Each patient was observed for 5 years after enrollment by a pair of neurosurgeons and a neurologist in each participating institute. Postprocedure inpatient events were also handled by this pair. Blood pressure medication was given to patients with hypertension to control it. The use of anticoagulants or antiplatelet drugs was not allowed unless the patient was having significant cerebral ischemic attacks. The patients' medical, neurological, radiological, and functional status was checked and reported every year. Both bleeding time and coagulation time were also monitored.

End Points

The following items constitute primary end points: (1) recurrent bleeding; (2) completed stroke causing significant morbidity; (3) significant morbidity or mortality from other medical cause; or (4) requirement for extracranial–intracranial bypass for a nonsurgical

Table 2. Follow-Up Protocol of the JAM Trial

	Registration	6 mo	1 y	Each Anniversary	Recurrent Bleeding or Other End Point
Neurological examination	●	●	●	●	●
CT/MRI	●	●	●	●	●
MR angiography			●	●	
SPECT	●	●			●
Angiography	●	● (surgical group only)			●
Bleeding time, PT, and APTT	●	●	●	●	●

●indicates examination required; APTT, activated partial thrombin time; CT, computed tomography; JAM, Japan Adult Moyamoya; MR, magnetic resonance; PT, prothrombin time; and SPECT, single-photon emission computed tomography.

patient because of progressive ischemic stroke or crescendo TIAs, as determined by a registered neurologist. Significant morbidity was defined as having a modified Rankin disability scale[6] score of ≥3. The following items constitute a secondary end point: (1) recurrent bleeding occurring later than 3 months after enrollment or (2) related death or significant morbidity. This was because surgical operations were to be performed on each side at some interval within 3 months after enrollment. Asymptomatic bleeding incidentally detected by MRI in the routine follow-up examination was not regarded as an end point. All the adverse events were reported to the central office of the trial, and end points were finally adjudicated in the executive and steering committee consisted of neurologists and neurosurgeons who were not blinded to the allocation.

Statistical Analysis

All statistical analyses were executed by 2 statisticians in the statistical center of the trial (listed in the Appendix). Statistical analysis with a Kaplan–Meier survival analysis and a Cox proportional hazard model was used to compare the length of time without an adverse event for each group. The unpaired 2-group t test, χ^2 for independence test, and Fisher exact probability test were used to compare baseline characteristics of the 2 groups. All analyses were performed with IBM SPSS software, version 20 (IBM Software Group, Chicago, IL).

Ethical Considerations

All institutes participating in the JAM Trial received the approval of the committee of bioethics at each center. The JAM Trial was registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR, ID: C000000166, 2005), which was approved by the International Committee of Medical Journal Editors.

Results

Randomization, Treatment, and Follow-Up

Figure 1 demonstrates the flow diagram of the JAM Trial. During the period of January 2001 to June 2008, 213 patients were assessed for eligibility. After 133 patients were excluded for the reasons listed in Figure 1, 80 patients were enrolled in the JAM Trial and randomized: 42 to the surgical group and 38 to the nonsurgical group. Table 3 summarizes the baseline characteristics of the patients.

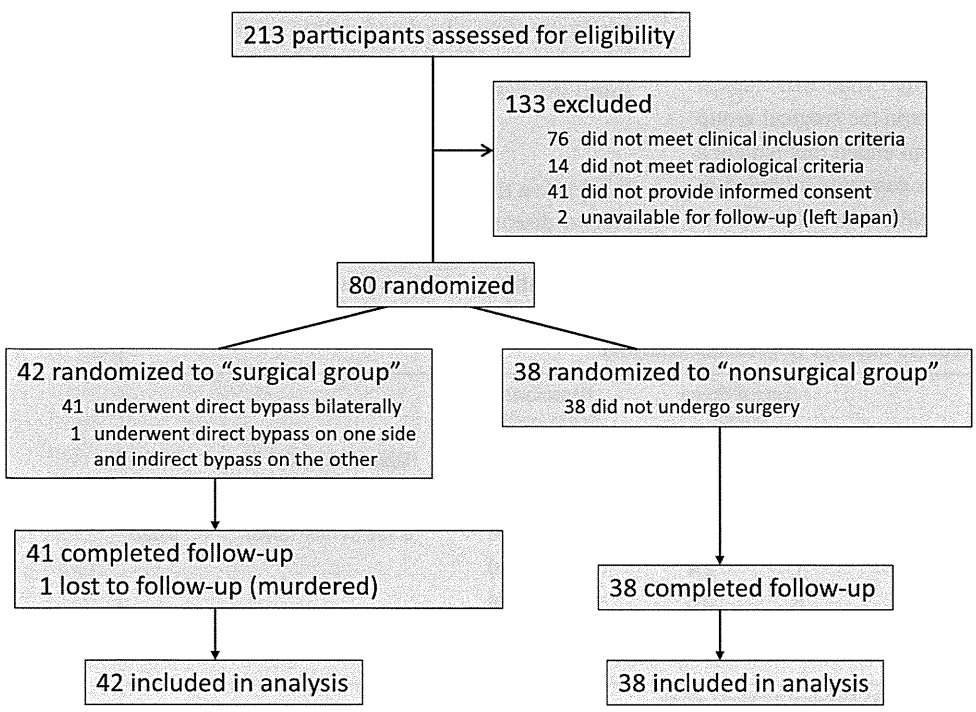

Figure 1. Flow diagram of the Japan Adult Moyamoya (JAM) Trial.