Familial Brain Arteriovenous Malformation Maps to 5p13-q14, 15q11-q13 or 18p11: Linkage Analysis with Clipped Fingernail DNA on High-density SNP Array

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ABSTRACT

Familial arteriovenous malformations (AVM) in the brain is a very rare disease.   It is defined as its occurrence in two or more relatives (up to third-degree relatives) in a family without any associated disorders, such as hereditary hemorrhagic telangiectasia. We encountered a Japanese family with brain AVM in which four affected members in four successive generations were observed.   One DNA sample extracted from leukocytes of the proband and ten DNA samples from clipped finger nails of other members were available. A genome-wide linkage analysis was performed on this pedigree using Affymetrix GeneCip 10K 2.0 Xba Array and MERLIN software.   We obtained sufficient performance of SNP genotyping in the fingernail samples with the mean SNP call rate of 92.49%, and identified 18 regions with positive LOD scores. Haplotype and linkage analyses with microsatellite markers at these regions confirmed three possible disease-responsible regions, i.e., 5p13.2-q14.1, 15q11.2-q13.1 and 18p11.32-p11.22.   Sequence analysis was conducted for ten selected candidate genes at 5p13.2-q14.1, such as *MAP3K1, DAB2, OCLN, FGF10, ESM1, ITGA1, ITGA2, EGFLAM, ERBB2IP*, and *PIK3R1*, but no causative genetic alteration was detected. This is the first experience of adoption of fingernail DNA to genome-wide, high-density SNP microarray analysis, showing candidate brain AVM susceptible regions.

Keywords: arteriovenous malformation, genomewide linkage analysis, fingernail DNA, mutation search, GeneChip^{TM}

1. Introduction

Arteriovenous malformation (AVM) in the brain is a disease defined by the presence of arteriovenous shunt(s) through a nidus of coiled and tortuous vascular connections between feeding arteries and draining veins within the brain parenchyma[1].   This vascular malformation is thought to be congenital, and develops before or after birth[2] from a residual of the primitive artery-vein connection. Its most common symptom is intracranial hemorrhage with an estimated risk of 1.3-3.9% yearly after the diagnosis of AVM[3]. Other signs may include intractable seizures, headache and ischemic steal syndrome. The prevalence of AVM is estimated to be approximately 0.01% and the detection rate ranges between 1.12-1.34 per 100 000 person years[1,2].   Although most cases of AVM are sporadic, a total of 53 patients from 25 families have been reported[4]. Familial brain AVM is defined when it occurs in two or more relatives (up to third-degree relative) in a family without associated disorders such as hereditary hemorrhagic telangiectasia (HHT), is autosomal dominant multisystemic vascular dysplasia[4,5].   It is plausible that familial cases are more frequent and could be overlooked because of asymptomatic conditions in other relatives.

Although several causative genes have been elucidated in some heritable syndromic AVM[6-16], molecular genetic studies of familial or sporadic AVM remain scant. HHT type 1 (HHT1) and HHT type 2 (HHT2) are known to be caused by mutations in *ENG* at 9q34.11 and *ACVRL1* (or *ALK1*) at 12q13.13, respectively[6,7]. Mutations in *RASA1* at 5q14.3 cause capillary malformation-arteriovenous malformation (CM-AVM)[8-12] characterized by small, round-to-oval, pink-red and multiple CM: one-third of CV-AVM patients also has fast-flow lesions such as AVM. Mutations in *PTEN* have been implicated in PTEN hamartoma tumor syndromes including Bannayan-Riley-Ruvalcaba syndrome, in which AVM occasionally presents[13]. Three genes, *KIRIT1* (*CCM1*)[14] at 7q21.2, *MGC4607* (*CCM2*)[15] at 7p13 and *PDCD10* (*CCM3*)[16] at 3q26.1, are responsible for cerebral cavernous malformation (hamartomatous vascular malformations). On the other hand, regarding familial AVM, only two linkage analyses using 6 small families have been published by a research group[17,18], showing seven possible disease-responsible regions, i.e., 6q25 with the highest LOD score, 3p27, 4q34, 7p21, 13q32-q33, 16p13-q12 and 20q11-q13, but failed to identify the causative mutation.   In sporadic brain AVM, microarray study showed that the *VEGFA, ITGA5, ENG* and *MMP9* genes that may involve vascular development or maintenance, are highly expressed in AVM compared with normal brain parenchyma[19-21].

Here we report results of a genome-wide linkage analysis on an AVM family with four affected members in two successive generations.

2. Materials and Methods

2.1. Subjects

A Japanese family consisting of 19 members across four generations included two patients with brain AVM, one patient with pulmonary AVM and one patient with both brain and pulmonary AVM (Figure 1).   The proband (III-3) first exhibited intractable epilepsy at 13 years old and was diagnosed by magnetic resonance imaging (MRI) as having a brain AVM of two centimeters in diameter located in the right frontal lobe (Figure 2). Chest X-ray at the first visit detected a nodular shadow in the right lower lung field, and a diagnoses of pulmonary AVM with a 24% of shunt-rate was made following angiogram made (Figure 2). This was resected when the proband was 14 years old. The proband's brain AVM was treated by gamma knife surgery when she was 19 years old, followed by treatment with antiepileptic medication. Her mother (II-3) died of intracranial hemorrhage due to brain AVM, and the maternal grandfather (I-1) died of a cancer.   Another patient (III-5) had asymptomatic brain AVM, which was accidentally diagnosed by MRI.   His father (II-5) had pulmonary AVM instead of brain AVM.   These four members were assigned to "affected", six members (II-6, III-1, III-6, III-7, IV-1, and IV-2) without AVM confirmed by MRI were "unaffected", and the remaining three (I-2, II-1, and IV-3) who were not assessed by MRI but had neither past history of recurrent epistaxis or gastrointestinal tract bleedings were "unknown". None of the members had any AVM-related diseases, such as HHT. Evaluation of cutaneous lesions was conducted by examination of the proband and by detailed interview of the other family members by the proband and her sister (III-1), who is nurse. A total of 13 members participated in this study under informed consent. All experimental procedures for this study were approved by Committee for the Ethical Issues on Human Genome and Gene Analysis in Nagasaki University.

2.2. DNA Extraction

As a blood sample was available only from the proband, clipped fingernail samples were obtained from 10 of the other 12 members instead. Genomic DNA was extracted from the fingernails using a buffer solution containing urea, DDT and proteinase K, as reported previously[22,23].   Briefly, clipped fingernails were once frozen in liquid nitrogen and crushed into fine powder using Multi-beads Shocker™ (Yasui Kikai, Osaka, Japan).   The nail powder was lysed in a urea-lysis solution (2 M urea; 0.5 % SDS; 10 mM Tris-HCl, pH 7.5; 0.1 M EDTA) containing 1 mg/ml proteinase K and 40 mM DDT at 55 °C overnight.   Nail DNA was extracted with phenol/chloroform, and precipitated with ethanol and sodium acetate. Precipitated nail DNA was dissolved again in extraction buffer (0.5 % SDS; 10 mM Tris-HCl, pH 7.5; 0.1 M EDTA)

containing 1 mg/ml proteinase K, and incubated at 55 °C overnight. DNA was purified again as above, and was suspended in 30 μl of 1x TE buffer.

2.3. SNP Genotyping With Affymetrix 10K 2.0 Array

Blood DNA (250 ng) was processed according to the standard protocol provided by the GeneChip Mapping 10K Xba Assay Kit (Affymetrix, Santa Clara, CA).   Fingernail DNA was processed in a similar manner but with the two following modifications to adapt to the oligonucleotide microarray system[24].   Prolongation of digestion time from 120 minutes as the standard protocol to overnight; and increase of the PCR cycle number from 35 to 45 cycles.   Data acquired from the Affymetrix GeneChip Operating System were analyzed using the Affymetrix GeneChip Genotyping Analysis Software (GTYPE) 4.0 to call genotypes.

2.4. Linkage Analysis With SNP-genotype Data and Haplotype Analysis With Microsatellite Markers

Multipoint LOD scores were calculated using MERLIN software[25], under an assumption that AVM in the family is transmitted in an autosomal dominant mode with reduced penetrance (p = 0.9) and with the disease allele frequency of 0.001.   At loci with a positive LOD score by the GeneChip genotyping, possibly disease-associated haplotypes were constructed using SNP calls.

When SNP information was not informative, microsatellite markers were used for genotyping. Microsatellite markers used were referred to the National Center for Biotechnology Information (NCBI) database.   One each of primer pairs for the markers was labeled with FAM, HEX, or NED (Supplementary table 1), and PCR was performed in a 10 μl mixture containing 5 ng genomic DNA; 0.25 U ExTaq DNA polymerase HS-version (TAKARA Bio Inc., Kyoto, Japan); 200 μM dNTP; 0.5 μM primer; 1x ExTaq buffer on the T1 Thermocycler (Biometra, Goettingen, Germany). PCR products were separated on Genetic Analyzer 3130xl (AppliedBiosystems), and genotyping was carried out using GeneMapper software (AppliedBiosystems).   At the regions where the affected individuals have a disease-associated haplotype, two-point LOD score was calculated by MLINK program (included in FASTLINK software version 4.0P)[26].

2.5. Mutation Analysis

Some genes located within candidate regions identified by the linkage analysis were selected for further mutation analysis. A few other genes, albeit outside the regions, were also subjected to mutation analysis.   Primer pairs for such genes were designed

using Primer3-web 0.3.0 (http://frodo.wi.mit.edu/primer3/input.htm), according to their genomic sequences retrieved from the University of California, Santa Cruz (UCSC) Genome Browser Home (http://genome.cse.ucsc.edu/). PCR was carried out in a 15 μl reaction mixture containing 5 ng DNA; 0.25 U ExTaq DNA polymerase HS version; 200 mM dNTP; 0.5 μM each primer; 1x ExTaq buffer on the T1 Thermocycler. PCR products were subjected to direct sequencing, using BigDye Terminator v3.1 Cycle sequencing Kit (AppliedBiosystems) and Genetic Analyzer 3130xl. Electropherograms of sequences were aligned with ATGC software (GENETYX Corp., Tokyo, Japan) to inspect base alterations.

2.6. Search for Genomic Aberration

To search for copy number change within the candidate loci identified by linkage analysis, we used Affymetrix® Genome-Wide Human SNP Array 5.0 (920,568 probes; Affymetrix). Genomic DNA extracted from white blood cell of proband was processed according to manufacture's protocol. Intensity data from each probes were obtained from Affymetrix® Genotyping Console 3.0 as a CEL files. Unpaired copy number analysis of whole genome was carried out using Partek Genomics Suite (Partek, MO, USA) and regions with copy number change were determined by Hidden Markov Model at default settings.

3. Results
3.1. Linkage and Haplotype Analyses

The mean SNP call rate was 92.49% in 11 fingernail DNA samples, compared to 98.11% in a blood DNA sample from the proband. Incorrect SNP calls may result in seemingly inconsistent parent-child transmissions, but the call rates obtained are actually enough for further studies. We thus advanced to calculate LOD scores using these data.

The linkage analysis using MERLIN software revealed 18 regions with positive LOD scores (>0.00). Of the 18 regions, 14 with the following conditions were excluded: those without any functional full-length RefSeq genes; those in small size (< 200 kb); and those in which some affected members did not have a common haplotype. Consequently, four loci, 5p13.2-q14.1, 15q11.2-q13.1, 18p11.32-p11.22 and 19q13.33-q13.42, remained as possibly linked regions (Figure 1 and Figure 3).

We then genotyped with microsatellite markers and calculated two-point LOD scores, considering the affected, unaffected, and the unknown family members. We confirmed three of the four candidate loci. They were a 48-Mb region between markers rs1366265

and rs1373965 at 5p13.2-q14.1, a 6-Mb region between rs850819 and rs818089 at 15q11.2-q13.1, both giving the maximum two-point LOD score of 1.632 ($\theta = 0$), and a 9-Mb region between rs486633 and rs1942150 at 18p11.32-p11.22 with the maximum LOD score of 0.851 ($\theta = 0$) (Table 1). As a possibly disease-associated haplotype on 19q13.33-q13.42 was transmitted to two definitively unaffected individuals (III-6 and IV-1), chromosome 19 was ruled out from the candidacy (Table 1, Figure 1).

3.2. Mutation Analysis of Candidate Genes

Within the 48-Mb region at 5p13.2-q14.1, there are about 200 RefSeq genes. Ten (*MAP3K1*, *DAB2*, *OCLN*, *FGF10*, *ESM1*, *ITGA1*, *ITGA2*, *EDFLAM*, *ERBB2IP*, and *PIK3R1*) from these genes were focused and selected as candidates for brain AVM, since they concern development or maintenance of vessels, are associated with other heritable vascular disorders such as HHT, or are expressed in the brain with AVM[19-21] .Mutation analyses in these 10 genes revealed no pathologic mutation in the proband, although other affected members were not examined because of insufficient amount of their DNA.   Although the genes endoglin isoform 1 precursor (*ENG*), activin A receptor type II like 1 (*ALK1*) and RAS p21 protein activator 1 (*RASA1*) are not located in the candidate region, we investigated whether any of them are involved in the etiology of AVM in the family as a partial symptom of HHT or AVM-CM. Direct sequencing of these three genes failed to show any causative variants.
  Copy number analysis of proband revealed one increased copy number loci at 12q and decreased at 2p, 3q, 4q, 6p, 7q and 22q (data not shown). But all these alterations were reported previously as copy number polymorphisms (http://projects.tcag.ca/variation/) and out of our candidate loci. In addition, neither deletions nor microdeletions were detected at 9q34.11 of *ENG*, 12q13.13 of *ALK1* and 5q14.3 of *RASA1*.

4. Discussion

We have reported a family consisting of two affected members with brain AVM, one with pulmonary AVM and one with both brain and pulmonary AVM.   The condition in this family met the criteria of familial brain AVM and seems to be inherited in an autosomal dominant mode.   We tried to assign the location of a putative disease-gene by linkage analysis and search for mutations by subsequent candidate gene approach.

The linkage analysis of the family revealed three candidate regions (5p13.2-q14.1, 15q11.2-q13.1, and 18p11.32-p11.22) with relatively high LOD scores of 1.632, 1.632 and 0.851, respectively (Table 1).   However, neither region was conclusive. This insufficient mapping may have arisen from the small pedigree size, and/or from

incomplete ascertainment of affected members, e.g., probable existence of asymptomatic affected persons among the "unknown" members. Indeed, as for a candidate locus at 5p13.2-q14.1, the proband's maternal grandmother (I-2) and son (IV-3) had a haplotype common to the three affected members (Figure 1), but they were fallen into the "unknown" individuals. If DNA from IV-2 was available and if MRI examinations of VI-3 and I-2 were carried out, we would have obtained more definitive results. As we performed linkage analysis using high density SNP genotyping, 14 small regions not containing RefSeq genes or miRNAs showed a positive LOD score. It is possible that an unidentified transcribed RNA in one of these regions could cause familial AVM, but these regions are candidate loci with a lower priority than those containing known genes. Thus, the three regions have remained at present as the equally possible loci for AVM. The three regions do not overlap with a previously reported candidate locus of familial brain AVM, i.e. 6p25[17], and do not contain genes responsible for syndromic AVM (heritable disorders involving AVM) or cerebral cavernous malformations, such as *ENG*[6], *ALK1*[7], *RASA1*[8-12], and *PTEN*[13], *KRIT1*[14], *MGC407*[15], *PDCD10*[16].

We then searched for mutations in 10 genes within 5p13.2-q14.1, among which *MAP3K1, DAB2* and *OCLN* encode proteins playing roles in the TGF-β signaling pathway, and *FGF10, ESM1, ITGA1, ITGA2, EGFLAM, ERBB2IP* and *PIK3R1* were those expressed in brain AVM tissues by previous microarray analysis[19-21]. Nevertheless, no pathologic mutation was found in any of them. Because the presence of both brain AVM and pulmonary AVM in this pedigree is reminiscent of Hereditary Hemorrhagic Telangiectasia, we analyzed *ENG* and *ALK1* for mutations and genomic aberrations, which may cause HHT1 and HHT2 respectively[6,7]. The proband did not have any mutations in the coding exons or intron/exon boundaries of either gene, nor any genomic aberrations at those loci. We also analyzed *RASA1* because this may cause CM-AVM, which is characeraized by multiple CM and AVM[8-12]. No causative mutation or genomic aberration was detected in the proband. Although other genes, such as *KRIT1*, *MGC407* and *PDCD10,* have been shown to cause slow-flow lesions *i.e.,* cerebral cavernous malformation [14-16], they were not investigated in the present study, because the clinical manifestations in our family did not meet the criteria for these diseases.

Participation of family members and compliance with guidelines for human genome researches are critical to conduct a linkage analysis. Whole-blood samples cannot occasionally be available in some family members because of their far domicile. In such the case, fingernail DNA is useful, since clipped fingernails can be mailed in a usual way, and stored long at a room temperature, as indicated previously[22,27]. The

present study is the first experience to adopt fingernail DNA to genome-wide high-density SNP microarray analysis. The performance obtained from fingernail DNA was sufficient, showing all SNP call rates of >86%. According to the manufacture's protocol, samples with a SNP call rate of <85% should further be evaluated before including the data in downstream analysis. Incorrect SNP calls may make serious problems in linkage analysis. For instance, SNPs with parent-child transmission inconsistency may be omitted, leading to a reduced LOD score.

In conclusion, we have assigned the familial AVM locus to three alternative regions, 5p13.2-q13.2, 15q11.2-q13.1 and 18p11.32-p11.22, by a genome-wide, high-density, SNP-based linkage analysis with fingernail DNA in an AVM family. However, mutation analyses of some genes in the regions failed to identify any pathological changes.

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Table 1.   Two-point LOD scores for brain AVM at various loci

Locus	Recombination fraction (θ)					
	0.00	0.01	0.02	0.03	0.04	0.05
AVM5pl17xAC	0.032	0.030	0.029	0.027	0.026	0.025
D5S418	0.551	0.535	0.518	0.501	0.484	0.467
AVMch5p25xAC	1.334	1.301	1.268	1.234	1.201	1.167
AVM5pr18xGT	0.511	0.491	0.472	0.452	0.433	0.414
AVMch5c27xGT	1.630	1.597	1.564	1.531	1.497	1.463
AVM5c18xAC	1.373	1.344	1.314	1.285	1.255	1.225
D5S407	1.632	1.599	1.566	1.532	1.499	1.465
D5S647	1.154	1.121	1.089	1.056	1.023	0.991
AVM5qr19xCA	0.810	0.790	0.769	0.748	0.727	0.706
D15S1021	0.171	0.164	0.157	0.150	0.143	0.137
D15S128	0.876	0.858	0.841	0.823	0.805	0.787
D15S986	0.812	0.791	0.770	0.749	0.728	0.707
D15S975	0.400	0.387	0.374	0.361	0.348	0.335
D15S1002	1.330	1.298	1.266	1.234	1.202	1.170
D15S1019	1.632	1.599	1.566	1.532	1.499	1.465
D18S59	0.199	0.214	0.225	0.234	0.241	0.246
D18S459	0.142	0.136	0.131	0.125	0.120	0.114
D18S1132	0.677	0.663	0.650	0.636	0.623	0.609
D18S452	0.851	0.832	0.813	0.794	0.774	0.755
D18S471	0.240	0.231	0.222	0.214	0.205	0.197
D19S927	-0.302	-0.277	-0.254	-0.234	-0.216	-0.200
D19S418	-2.655	-2.453	-2.257	-2.078	-1.919	-1.778
D19S605	-0.648	-0.574	-0.512	-0.460	-0.414	-0.374

Supplementary table 1.   Primer sequences used in the present study

| Name	Item	Position	Band	Forward primer (5'->3') Reverse primer (5'->3')
AVM5pl17xAC	17xAC	chr5:39266704-39266738	5p13.1	CAGGGTACAGCTGAGAATTGC GGACAATCAGTCATTCCATTCA
AVMch5p25xAC	25xAC	chr5:42961358-42961407	5p12	TTCTGGGACCTCTTTACTCA ACACGGAGACAACAGAAGAC
AVM5pr18xGT	18xGT	chr5:44815955-44815990	5p12	AAAAGCAACCCACTGTCACC TGTCCATTACTTCTGACCCTGA
AVM5c27xGT	27xGT	chr5:53278755-53278808	5q11.2	ATGTGGGATCTCATTGTTGT GGGAATTTTCTGCCTTTAAT
AVM5c18xAC	18xAC	chr5:54022345-54022381	5q11.2	CATCCCACATTATAAAATGAGTCAGA CATCCACCTCAGCCTAGAGAA
AVM5qr19xCA	19xAC	chr5:70838370-70838407	5q13.2	TCATGATTGGTGATTTGGTGA TGACAGAGCAAGAGCAAGACCCTTGT

Figures Legends:
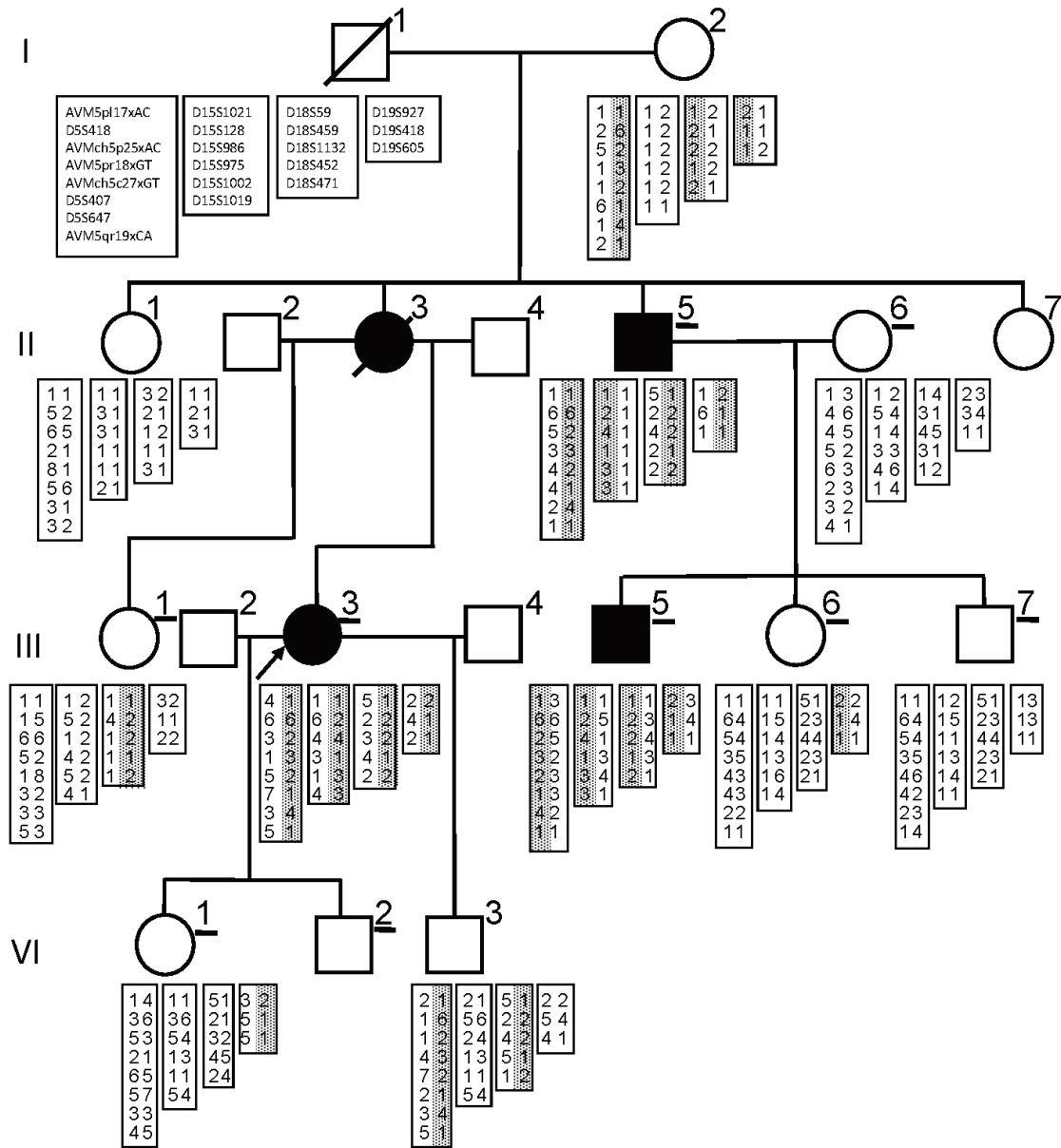
Figure 1. Results of haplotype analysis at polymorphic loci in four regions, 5q13.2-q14.1, 15q11.2-q13.1, 18p11.32-p11.22 and 19q13.3-q13.42. Underlined individuals indicate those examined by MRI, and DNA was unavailable from individuals without haplotypes. Polymorphic alleles are numbered and candidate disease-associated haplotypes are shown by dotted boxes. Primer sequences designed for CA repeat amplification are available in supplementary table.
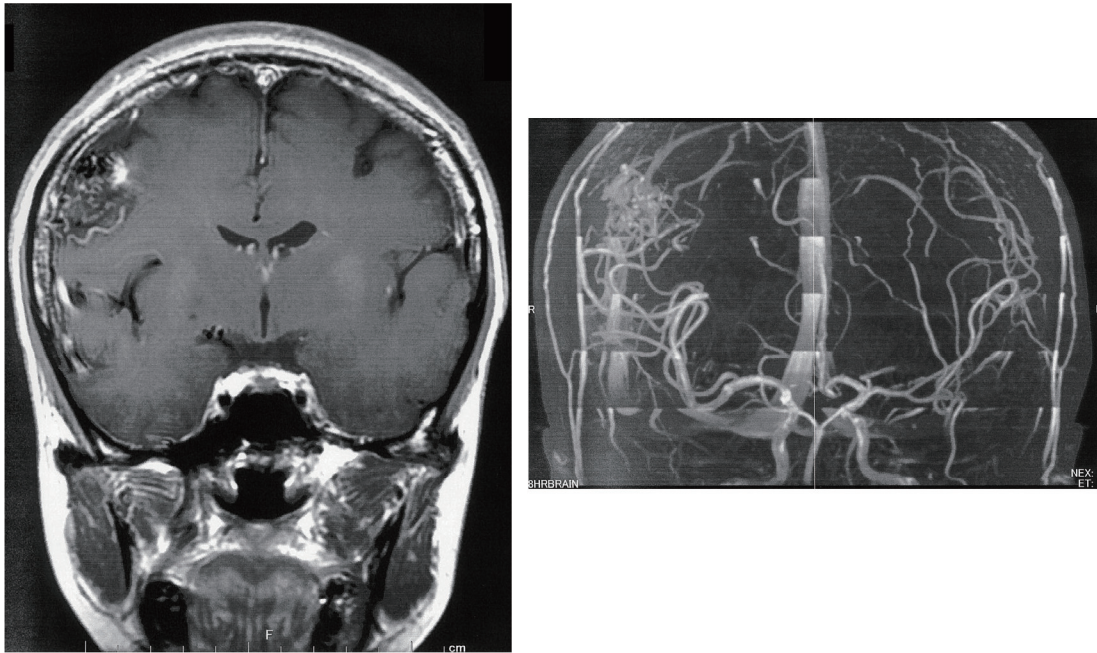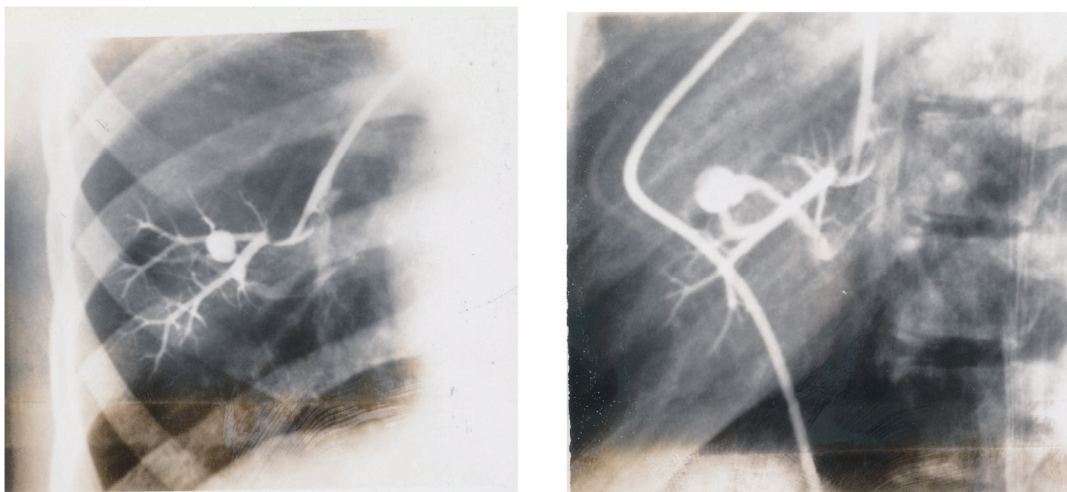
Figure 2. Imaging of the brain and pulmonary AVM in the proband. (A) MRI scan and MR angiogram of the proband. The AVM is located right frontal lobe measured 2.0 x 1.3 cm. (B) Pulmonary angiograms of the proband. The pulmonary AVM is located in the right lower lobe (rtS8b) with 24% of shunt-rate.
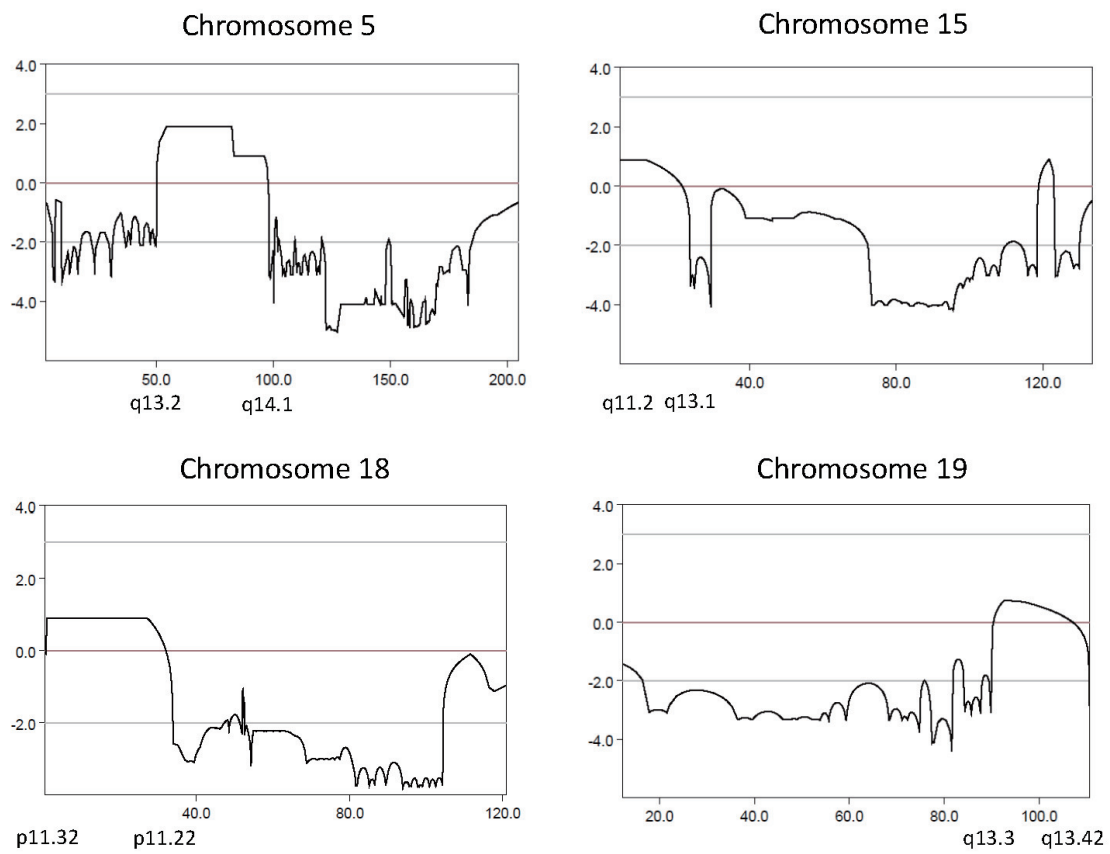
Figure 3. Multipoint LOD scores calculated by MERLIN in four chromosomal regions, 5q13.2-q14.1, 15q11.2-q13.1, 18p11.32-p11.22 and 19q13.3-q13.42.