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Case Report

Novel *RASA1* mutations in Japanese pedigrees with capillary malformation-arteriovenous malformation

Yosuke Moteki [a,b,c], Hiroyuki Akagawa [d,*], Yasunari Niimi [b], Yoshikazu Okada [c], Takakazu Kawamata [a]

[a] *Department of Neurosurgery, Tokyo Women's Medical University, Tokyo, Japan*
[b] *Department of Neuroendovascular Therapy, St. Luke's International Hospital, Tokyo, Japan*
[c] *Department of Neurosurgery, St. Luke's International Hospital, Tokyo, Japan*
[d] *Tokyo Women's Medical University Institute for Integrated Medical Science (TIIMS), Tokyo, Japan*

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Abstract

Capillary malformation-arteriovenous malformation (CM-AVM, MIM#608354) is a rare autosomal dominant disorder characterized by multiple cutaneous capillary malformations co-occurring with fast-flow vascular anomalies, such as arteriovenous malformation or fistula. Despite the identification of *RASA1* as the first causative gene in Western patients with CM-AVM, there have been no literature reports of Japanese patients with this gene mutation. We herein report two Japanese pedigrees harboring multiple affected members with CM-AVM. Whole-exome sequencing in the two probands identified novel heterozygous mutations in *RASA1*, which were co-segregated with the disease in each family and were not reported in large-scale sequencing databases. One was a frameshift mutation and the other a splice-site mutation causing aberrant splicing, confirmed by a minigene assay. There were no other genes commonly disrupted among these probands. *RASA1* was a major causative gene even in Japanese patients with CM-AVM, although obvious locus heterogeneity was known for this disease.

Keywords: Capillary malformation-arteriovenous malformation; Whole-exome sequencing; *RASA1*; Minigene assay

1. Introduction

Capillary malformation (CM, also referred to as "port-wine stain") is a cutaneous vascular anomaly appearing as a pink to red macule with an incidence of 0.3–0.5% in newborns [1,2]. It is usually a solitary lesion, however, multifocal CMs are associated with hereditary vascular syndrome and complicating fast-flow vascular anomalies, such as arteriovenous malformation (AVM) or arteriovenous fistula (AVF). Eerola et al. first described this autosomal-dominant inherited condition as capillary malformation-arteriovenous malformation (CM-AVM, MIM#608354), and successfully pinpointed *RASA1* (5q14.3, MIM*139150) as the first disease-causative gene from European and North American pedigrees with CM-AVM [3]. However, about half of patients with CM-AVM were reported to have no pathogenic mutation in *RASA1*, suggesting genetic heterogeneity [4,5]. Furthermore, previous genetic studies of CM-AVM were mostly from Caucasian patients, with few East Asian patients [4–6]. In the present study,

* Corresponding author at: Tokyo Women's Medical University Institute for Integrated Medical Sciences (TIIMS), 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan.
E-mail address: akagawa.hiroyuki@twmu.ac.jp (H. Akagawa).

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we performed a comprehensive genetic analysis in two Japanese pedigrees with CM-AVM.

2. Case presentation

The proband of the first pedigree was a ten-month-old boy, presenting with multifocal CMs on the left knee and hand. A pial AVF in the left occipital area had been diagnosed earlier due to respiratory and heart failure at birth, and he was scheduled for endovascular treatment (EVT) after he had grown enough to tolerate EVT. However, because he developed intracranial hemorrhage due to the AVF, EVT was performed and resulted in a good radiological and clinical outcome (Fig. 1).

The proband of the second pedigree was diagnosed with a pial AVF in the right parietal area due to developmental delay at four months of age. He had also multifocal CMs on the head and hip. The vein of Galen was remarkably dilated due to the AVF, which caused acute hydrocephalus at six months of age. EVT successfully improved his hydrocephalus by reducing the abnormal blood flow to the dilated vein of Galen (Fig. 1).

3. Genetic analysis

The genetic analyses in this study were approved by the ethics committees of Tokyo Women's Medical University and St. Luke's International Hospital. After obtaining written informed consent, genomic DNA (gDNA) samples were obtained from the probands and their relatives in the two pedigrees (Fig. 1). The gDNA samples from the two probands were subjected to exome enrichment using a SureSelect Human All Exon V6 kit (Agilent Technologies Inc., Santa Clara, CA, USA), and whole-exome sequencing (WES) was performed using an Illumina HiSeq 2500 sequencer

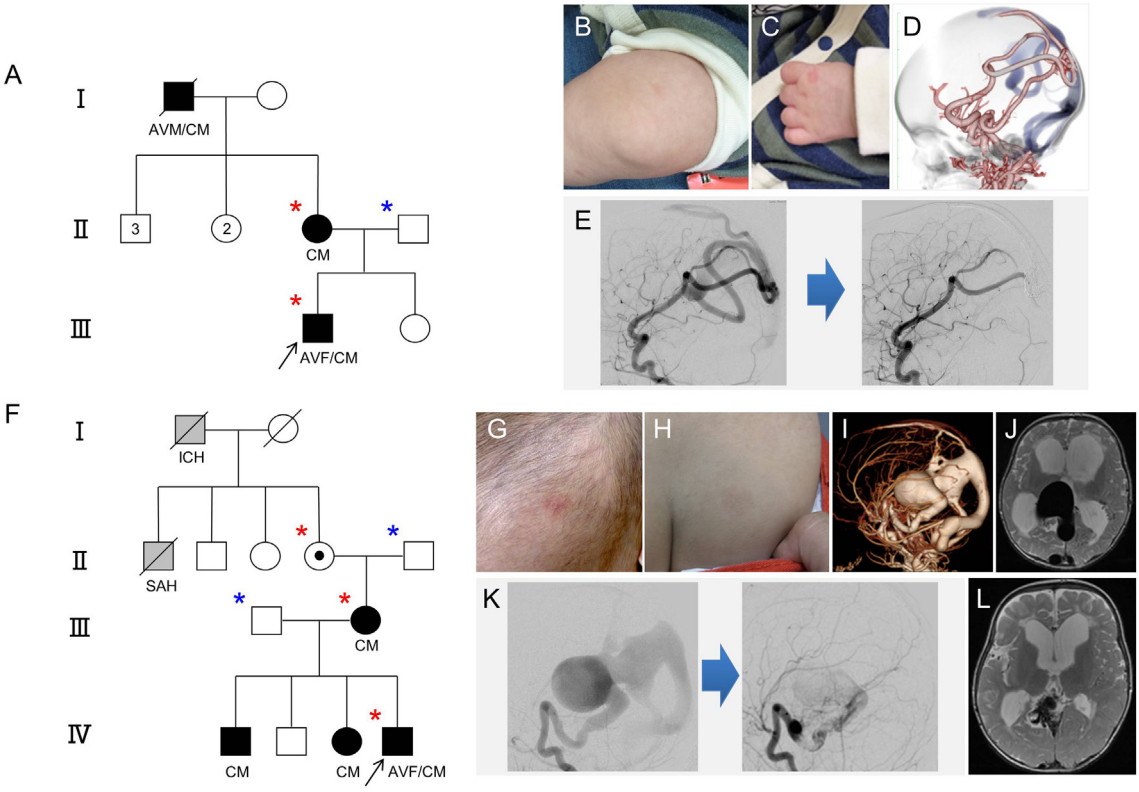

Fig. 1. Clinical manifestation in the patients. (A) The family tree of the first pedigree shows that the proband's mother (II-3) and her father (I-1) had capillary malformations (CMs). The I-1 was also diagnosed with a brain arteriovenous malformation (AVM). DNA samples were obtained from individuals denoted with an asterisk. Red asterisks represent mutation carriers, and blue asterisks represent wild-type at the mutation locus. (B and C) The proband had multifocal CMs on the left knee and hand. (D) Three-dimensional computed tomography angiography (3D-CTA) of the head demonstrates a pial arteriovenous fistula (AVF) in the left occipital area. (E) Conventional angiography shows that the abnormal blood flow from the AVF disappeared after endovascular treatment (EVT). (F) The family tree of the second pedigree shows the proband's brother (IV-1), sister (IV-3), and mother (III-2) had CMs. The proband's maternal great-grandfather (I-1) and great-uncle (II-1) also had intracerebral hemorrhage (ICH) and subarachnoid hemorrhage (SAH), respectively, which seemed to be due to vascular malformation in the brain. (G and H) The proband had multifocal CMs on the head and hip. (I) 3D-CTA of the head demonstrates that the vein of Galen was remarkably dilated due to a pial AVF in the right parietal area. (J) T2-weighted magnetic resonance imaging (MRI) of the head shows hydrocephalus due to the dilated vein of Galen. (K) Conventional angiography shows a marked reduction of the abnormal blood flow to the dilated vein of Galen after EVT. (L) MRI of the head shows an improvement in hydrocephalus with decreasing size of the dilated vein of Galen after EVT (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

(Illumina, San Diego, CA, USA). Sequence variants identified in the WES analysis and subsequent filtering procedures were summarized in Fig. 2. As a result, *RASA1* was the only mutated gene in common between the two probands after the detected variants were filtered against the large public sequencing databases and our control WES datasets from 18 individuals without CM-AVM. Both of these *RASA1* mutations in the two probands were highly deleterious, which were confirmed to be co-segregated with the disease status within the two pedigrees by direct Sanger sequencing (Figs. 1 and 2). The heterozygous c.2925 + 1G > T mutation in the first pedigree alters the consensus splice-donor sequence in the intron 23, and the heterozygous c.724delGinsCT mutation in the second pedigree is a frameshift mutation resulting in a premature termination (p.Gly242LeufsTer23, CCDS34200.1). To the best of our knowledge, these *RASA1* mutations have not been reported earlier in literature according to database screening of the NCBI ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/) and PubMed (http://www.ncbi.nlm.nih.gov/pubmed).

To test the functional impact of the c.2925 + 1G > T mutation on splicing, a minigene assay was performed. The wild-type and mutant 810-bp genomic fragments including the exon 23 and its adjacent intronic sequences were subcloned into the Exontrap pET01 vector (MoBiTec GmbH, Germany), and transiently expressed in Hela or HEK293T cells (Fig. 3). Reverse transcription-PCR covering the 5′ and 3′ exons in the pET01 vector revealed three types of abnormal splicing: the lower band corresponded to complete skipping of the exon23, the middle band to 29-bp skipping at the 3′end of the exon 23, and the upper band to 19-bp insertion of the 5′end of the intron 23 (Fig. 3). The complete skipping of exon 23 resulted in an in-frame deletion of evolutionarily conserved 26 amino acid residues (p.Glu950_Gly975del), whereas the latter two splicing

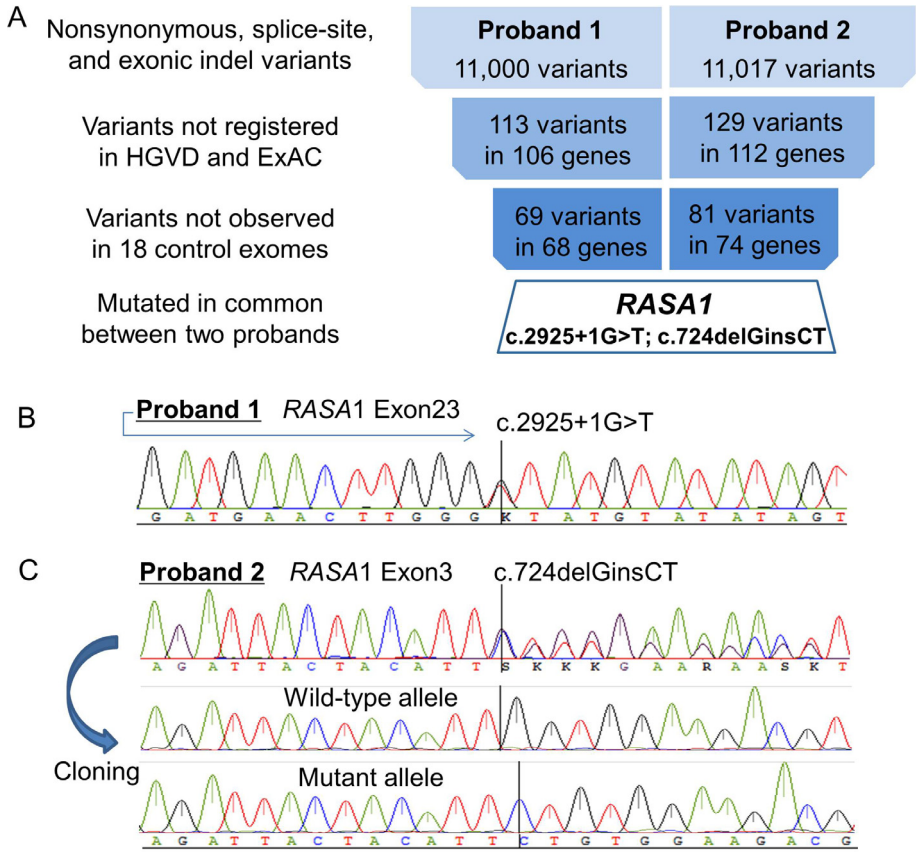

Fig. 2. Results of sequencing analysis. (A) Variant filtering was performed based on the following annotations: exonic or splice regions in the RefSeqGene (http://www.ncbi.nlm.nih.gov/refseq/rsg/), not registered in the Human Genetic Variation Database version 1.4 (HGVD, http://www.genome.med.kyoto-u.ac.jp/SnpDB/index.html) in the Japanese general population and the Exome Aggregation Consortium (ExAC, http://exac.broadinstitute.org/) version 0.3 dataset and not observed in our 18 control exomes without CM-AVM that were captured and sequenced using the SureSelect Human All Exon V6 kit (Agilent Technologies Inc., Santa Clara, CA, USA) and Illumina sequencers (Illumina, San Diego, CA, USA). (B and C) Sequencing chromatograms of heterozygous *RASA1* mutations detected from the two probands. The insertion–deletion (indel) mutation in the second probands was confirmed by TA-cloning into the pMD20-T vector (Takara Bio Inc., Kusatsu, Japan). These chromatograms were visualized using ATGC version 7 (GENETYX CORPORATION, Tokyo, Japan).

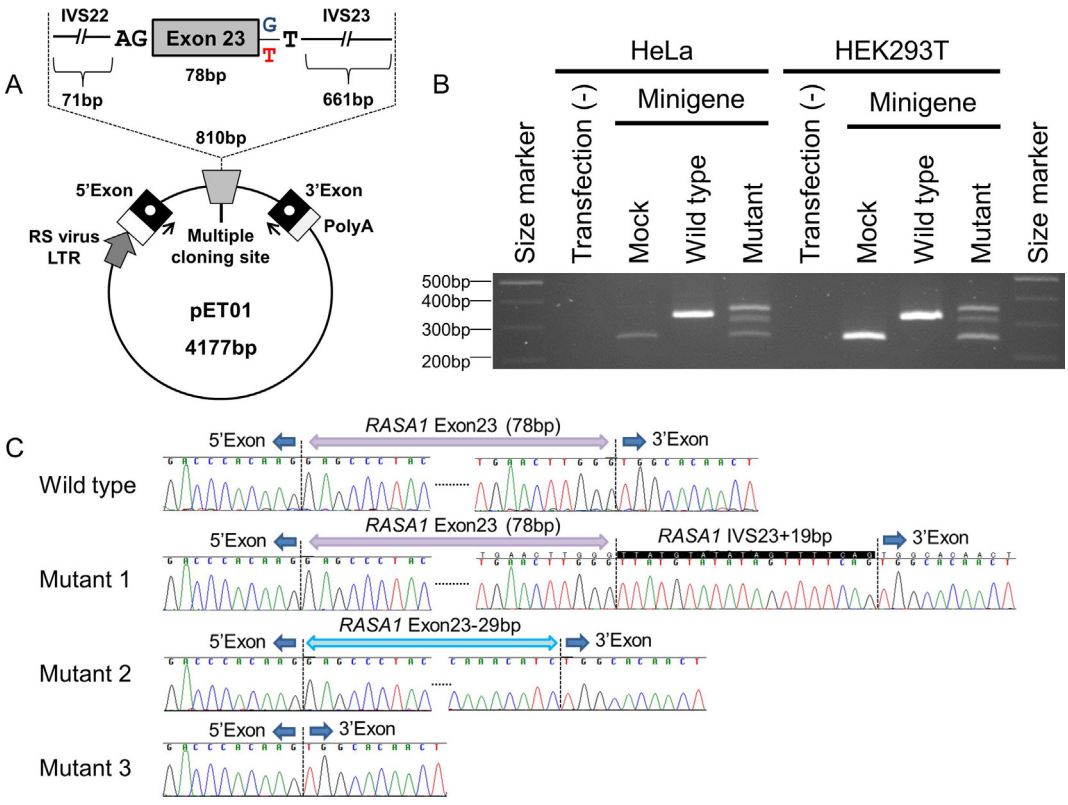

Fig. 3. Minigene assay for the splice-site *RASA1* mutation in the second pedigree. (A) The pET01 construct used in this study. The sequences containing the c.2925 + 1G > T mutation in *RASA1* intron 23 (T allele) or those that did not (G allele) were subcloned into the multiple cloning site of pET01. The arrows under the 3′ and 5′ exons indicate the primer pair used in the reverse transcription-PCR (RT-PCR) after transfection. (B) RT-PCR analysis using HeLa and HEK293 cells transfected with an empty (mock), wild-type or mutant pET01 vector. The RT-PCR products were separated in a 3% agarose gel electrophoresis and were stained with ethidium bromide. (C) Sequencing chromatograms of the RT-PCR products. The mutant RT-PCR products containing three patterns of aberrant splicing were separated by TA-cloning into the pMD20-T vector (Takara Bio Inc.). These chromatograms were visualized using Sequencher version 5.0.1 (Gene Codes Corporation, Ann Arbor, MI, USA).

abnormalities caused immediate premature termination sequences p.Arg966GlnfsTer4 and p.Asn976LeufsTer4, respectively.

4. Discussion

We herein reported the first comprehensive genetic analysis for Japanese pedigrees with CM-AVM. WES confirmed *RASA1* as the disease-causative gene even in Japanese patients with CM-AVM, and identified novel loss-of-function mutations co-segregating with the disease in each family, which were previously unreported mutations in Caucasian and Chinese patients with CM-AVM [4–6].

RASA1 encodes p120-RasGTPase-activating protein (p120-RasGAP), which acts as one of the major negative regulators of the Ras-MAPK-ERK signaling pathway by converting the active GTP-bound state of Ras to the inactive GDP-bound state [3]. This conversion is catalyzed by the conserved RasGAP domain (698–1044 amino-acid residues) in the C-terminal region of p120-RasGAP (InterPro, http://www.ebi.ac.uk/interpro/). Therefore, the splicing abnormalities observed in the first pedigree, as well as the premature termination of translation in the second pedigree, resulted in loss-of-function of *RASA1*, which constitutively activated the Ras-MAPK-ERK signaling pathway, leading to abnormal differentiation of endothelial cells and disorganized vascular development [5,7]. *EPHB4* (7q22.1, MIM*600011), the recently identified second causative gene for CM-AVM (CM-AVM2, MIM#618196), encodes a transmembrane receptor expressed in endothelial cells, which directly interacts with p120-RasGAP to regulate its downstream Ras-MAPK-ERK signaling pathway [5].

It is reported that there was no genotype-phenotype correlation among patients with *RASA1* mutations. Clinical manifestations can range from only a few isolated CMs to more severe symptoms, such as Parkes Weber syndrome, presenting soft tissue and skeletal overgrowth of a limb associated with multiple micro-AVFs, and life-threatening AVFs or AVM in the brain [3–5]. Intrafamilial phenotypic variability was therefore observed, as there were affected members with only

CM in the present pedigrees (Fig. 1). The CM-AVM diagnosis to the present pedigrees was largely based on their family histories, because the CMs found in the two probands were small and inconspicuous (Fig. 1), which highlighted the importance to take detailed family histories from suspected patients and their relatives with CM-AVM.

Such variable expressivity and multifocality observed in patients with CM-AVM are explained by a somatic second hit resulting in a biallelic loss of *RASA1* function in the lesion [4,8,9]. Furthermore, a recent study revealed that the majority of sporadic brain AVMs also harbored somatic activating *KRAS* mutations driving the downstream MAPK-ERK signaling [10]. These lines of evidence suggest that the RAS-MAPK-ERK signaling pathway is the most promising therapeutic target for CMs and AVMs and demonstrates the increasing importance of genetic diagnosis for both germ-line and somatic mutations for future molecular target therapies.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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