

DATA REPORT

**KRIT1** mutations in three Japanese pedigrees with hereditary cavernous malformation

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Cerebral cavernous malformation (CCM) is a vascular malformation in the central nervous system characterized by clusters of enlarged capillary cavities without intervening brain parenchyma. Clinical manifestations can vary from asymptomatic or mild symptoms, such as headache, to more severe symptoms, such as seizures, focal neurological deficits and hemorrhage. The prevalence of CCM in the general population has been reported to range from 0.39 to 0.53% according to large series of autopsies. The prevalence of CCM1 and CCM2/MGC4607 mutations in the general population has been reported to be 0.39% and 0.53%, respectively. The prevalence of CCM3/PDCD10 mutations in the general population has been reported to be 0.53%.

**Cerebral cavernous malformation** is a neurovascular abnormality that can cause seizures, focal neurological deficits and intracerebral hemorrhage. Familial forms of this condition are characterized by de novo formation of multiple lesions and are autosomal-dominantly inherited via **CCM1/KRIT1**, **CCM2/MGC4607** and **CCM3/PDCD10** mutations. We identified three truncating mutations in **KRIT1** from three Japanese families with CCMs: a novel frameshift mutation, a known frameshift mutation and a known splice-site mutation that had not been previously analyzed for aberrant splicing.

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The Ethical Committee of Tokyo Women's Medical University approved the study protocols. After obtaining written informed consent, genomic DNA samples were obtained from 12 participants: the three affected members (II:1, III:1 and III:2) in pedigree A (Figure 1a); the three affected members (II:3, II:5 and III:5), three presumed intrafamilial controls (I:2, II:1 and II:6) and two relatives of unknown disease status (II:7 and III:7) in pedigree B (Figure 1b), and the proband in pedigree C (Table 1). PCR-based direct Sanger sequencing was performed in all coding exons and their exon/intron junctions for the \textit{KRIT1} (NM_194456.1), \textit{MGC4607} (NM_031443.3) and \textit{PDCD10} (NM_007217.3) genes. From this analysis, three pathogenic mutations in \textit{KRIT1} were detected from each of the three families, reflecting high allelic heterogeneity in patients with familial CCMs except for Hispanic-American patients harboring the founder p.Gln248Ter mutation (c.742C$^4$T, rs267607203) in this gene.\textsuperscript{1,7} There were no pathogenic mutations in \textit{MGC4607} or \textit{PDCD10}.

The \textit{KRIT1} mutation in pedigree A was a heterozygous transition at a splice donor site of intron 10 (c.845+1G$^4$A; Figure 2a). This mutation was shared among the three affected participants and was absent from the 1000 Genomes Project database (http://www.1000genomes.org/);\textsuperscript{8} NHLBI GO Exome Sequencing Project.

Figure 1. Clinical manifestations in the individuals in this study. (a, b) Genealogical trees of pedigrees A and B. Black-filled symbols: clearly affected individuals; symbol enclosing a vertical bar: asymptomatic carrier; crossed-out symbols: deceased individuals; arrows: probands. DNA sequencing was carried out in individuals denoted with an asterisk. (c–k) Magnetic resonance imaging of patients. T2-weighted axial brain images of II:1 in pedigree A at diagnosis (e) and those after 10 years (d). T2*-weighted axial brain images of III:5 in pedigree B at diagnosis (e) and those after 7 years (f). T2-weighted axial brain images of his brother (III:6) at onset (g) and those at 4 years after surgery (h). White arrows: \textit{de novo} cerebral cavernous malformations (CCMs); open arrow: a hypothalamic CCM before surgery. The T2-weighted sagittal spinal image of II:3 in pedigree B (i). The T2-weighted sagittal spinal image (j) and T2*-weighted axial brain images (k) of the proband of pedigree C before surgery.
To test the impact of this mutation on splicing, total RNA samples were obtained from peripheral blood leukocytes of the proband (II:1) and an unrelated healthy volunteer without CCMs confirmed by cerebral MRI (PAXgene Blood RNA Kit; Qiagen, Valencia, CA, USA). Complementary DNA (cDNA) was synthesized

### Table 1. Clinical summary of the patients examined for sequencing analysis

| Pedigree | ID | Age at diagnosis | Sex | Type of disease | Neurological symptom | MRI | De novo formation | Follow-up period (years) |
|----------|----|------------------|-----|-----------------|----------------------|-----|-------------------|-------------------------|
| A        | I:1| 53               | M   | Multiple CCM    | Asymptomatic         | Figure 1c and d      | Yes | 10                |
|          | II:1| 22              | M   | Multiple CCM    | Asymptomatic         | N/A | No                | 9                       |
|          | II:2| 17              | M   | Multiple CCM    | Asymptomatic         | N/A | Yes               | 9                       |
| B        | II:3| 63              | M   | SCM             | Numbness in the bilateral lower extremities | Figure 1i | N/A | 1 |
|          | II:5| 50              | M   | Multiple CCM    | Asymptomatic         | N/A | N/A               | Drop-out               |
|          | III:5| 25             | M   | Multiple CCM    | Transient left hemiparesis | Figure 1e and f | Yes | 7 |
|          | III:6<sup>a</sup>| 18 (22)<sup>b</sup> | M   | Multiple CCM    | Consciousness disturbance | Figure 1g and h | Yes | 4 |
| C        | Ped. A| 73           | M   | SCM, multiple CCM | Tetraparesis         | Figure 1j and k      | N/A | 3 |

 Abbreviations: CCM, cerebral cavernous malformation; MRI, magnetic resonance imaging; N/A, not available; SCM, spinal cavernous malformation. *Individuals listed, except for III:6 of pedigree B, were analyzed by direct sequencing of the CCM genes. <sup>a</sup>Age at death.

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**Figure 2.** Results of genetic analysis. (a) DNA sequence chromatogram of the pedigree A proband (II:1), showing the heterozygous c.845+1G>A mutation of KRIT1. (b) Agarose gel electrophoresis of reverse transcriptase PCR (RT-PCR) products covering exons 8–13 of KRIT1, showing altered splicing patterns of the c.845+1G>A allele. (c) Sequencing of clones obtained from the RT-PCR amplicons revealed three types of splicing alterations. (d, e) DNA sequence chromatograms of frameshift KRIT1 mutations detected in pedigrees B and C.
using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Reverse transcription PCR covering a 799-bp cDNA sequence from exon 8 to exon 13 was performed using the following primers: 5′-GTAGTGAATCCAGTACT CATTITGC-3′ and 5′-CGACTTCTCCAGTGTGT-3′, which showed altered splicing patterns for the mutant allele (Figure 2b). Sequencing of the subcloned PCR products (Mighty TA-cloning Kit; TaKaRa, Shiga, Japan) revealed three types of splicing variants. Although the second smallest fragment in the gel electrophoresis was a known splicing isofrom lacking exon 10 (NM_001013406.1), the smallest fragment contained two aberrantly spliced variants having similar nucleotide sizes. One skipped exons 10 and 11, and the other skipped 131 bp in the end of exons 9 and 10, generating the immediate premature termination sequences p.Asp245TrpfsTer4 and p.Gln301AsnfsTer5, respectively (Figure 2c).

In pedigrees B and C, we identified two heterozygous frameshift mutations, i.e., c.1362_1363delTC (Figure 2d) and c.1153delA (Figure 2e), respectively. These mutations also resulted in the generation of premature terminations (p.Gln455ArgfsTer24 and p.Trh385GlnfsTer9) and were not listed in the above-mentioned four public databases. In pedigree B, the c.1362_1363delTC mutation was found in the three affected patients, but was not identified in the three interfamilial controls. II:7 was an asymptomatic carrier who had not undergone cerebral MRI (Figure 1b).

These mutations were screened against the CCM Mutation Database (http://www.angiomaalliance.org/pages.aspx?content = 345&sid = 289), NCBI ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/) and PubMed (Med (http://www.ncbi.nlm.nih.gov/pubmed). The c.845+1G→A mutation in pedigree A and the c.1362_1363delTC mutation in pedigree B have been reported previously; however, until the current study, no functional studies had been performed to validate the impaired splicing of the c.845+1G→A mutation. The c.1153delA mutation in pedigree C had not been previously reported.

Patients with CCM often remain asymptomatic, whereas SCM tends to be more clinically progressive, consistent with the clinical manifestations observed in our patients (Table 1). Early SCM resection has been reported to be associated with good outcomes, although our patient with lumbar SCM early SCM resection has been reported to be associated with good outcomes, although our patient with lumbar SCM (II:3, pedigree B) showed no neurological aggravation during conservative therapy. Therefore, both cerebral and spinal MRI should be considered for patients with multiple CCMs and their potentially affected relatives in order to achieve early diagnosis of subclinical SCM. Conversely, cerebral MRI should be also considered for patients with SCM and their relatives because CCMs occur in 17–42% of patients with SCM, and 12–57% of patients with SCM have a family history of cavernous malformation.

KRIT1, encoded by the KRIT1 gene, interacts with the other two CCM proteins encoded by MGC4607 and PDCD10 to form a heterotrimeric CCM complex. This complex regulates various signaling pathways represented by Rho/ROCK, whose inhibitor MGC4607 may prevent CCM formation in neonatal mice with endothelial-specific deficiency of KRIT1. For future therapies targeting such promising biological pathways, genetic testing and improved knowledge of pathogenic mutations in CCM genes will become increasingly important.

HGV DATABASE
The relevant data from this Data Report are hosted at the Human Genome Variation Database at http://dx.doi.org/10.6084/m9.figshare.hgv.849; http://dx.doi.org/10.6084/m9.figshare.hgv.852; http://dx.doi.org/10.6084/m9.figshare.hgv.855.

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COMPETING INTERESTS
The authors declare no conflict of interest.

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