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SHORT COMMUNICATION

Large germline deletions and duplication in isolated cerebral cavernous malformation patients

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Abstract Cerebral cavernous malformations (CCM) are vascular lesions that predispose to headaches, seizures, and hemorrhagic stroke. Hereditary CCMs are usually associated with the occurrence of multiple CCMs and occur with a frequency of 1:2,000 to 1:10,000. In this study, eight isolated cases with multiple CCMs but no *CCM1-3* point mutation were analyzed using the multiplex ligation-dependent probe amplification assay. Four genomic rearrangements were identified including a previously unreported large duplication within the *CCM1* gene and a novel deletion involving the entire coding region of the *CCM2* gene. Consequently, systematic screening for *CCM* deletions/duplications is recommended.

Keywords Vascular malformations · Cerebral cavernous malformation · MLPA · Deletion · Duplication

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Cerebral cavernous malformations (CCM) consist of abnormally dilated, immature blood vessels with a lobulated appearance. Due to their fragile cytoarchitecture, they tend to rupture and to cause intracranial hemorrhages. Clinical symptoms include recurrent headaches, seizures, and hemorrhagic stroke and may occur in early childhood. However, CCMs generally manifest during the second to fourth decade of life. Neurosurgical resection is usually indicated in the following situations: rapid cavernoma growth, repetitive hemorrhages or a major hemorrhage, occurrence of intractable epilepsy, or neurological deficits [1].

Patients with familial CCM often present with multiple lesions whose number and size increase with age. The prevalence of familial CCM has been estimated to be 1:2,000–1:10,000 [2]. Familial CCM is caused by autosomal dominant, inactivating mutations in three genes, *CCM1*, *CCM2*, and *CCM3*. A mutation detection rate of 94% is achieved for familial CCM if RNA-based screening techniques are used in addition to exon-by-exon sequencing. At least 57% of isolated cases with multiple lesions also harbor detectable mutations in *CCM1*, *CCM2*, or *CCM3* [2]. One explanation for the lack of detectable mutations in the remaining patients is the existence of large genomic rearrangements that escape detection by direct sequencing and reverse transcriptase PCR analyses of the coding regions. Only a few genomic deletions have thus far been identified (Table 1) [3–5]. These have been associated with incomplete penetrance and the same intrafamilial phenotypic variability ranging from complete lack of symptoms to fatal hemorrhage [5], as has been observed for patients with small mutations.

We have applied the multiplex ligation-dependent probe amplification (MLPA) technique, which permits the detection of large genomic rearrangements [6] to a previously analyzed Swiss cohort of 14 isolated patients affected with

Table 1 Genomic *CCM* deletions/duplications

Number	Gene	Exons involved	Type of mutation	Reference
1	*CCM1*	2–20[a]	Deletion	[5]
2	*CCM1*	2–20	Deletion	This paper
3	*CCM1*	7–17	Duplication	This paper
4	*CCM1*	18[b]	Deletion	[12]
5	*CCM2*	1–10+5′-UTR	Deletion	This paper
6	*CCM2*	1+5′-UTR	Deletion	[3]
7	*CCM2*	1+5′-UTR	Deletion	[3]
8	*CCM2*	1+5′-UTR	Deletion	This paper
9	*CCM3*	1–10	Deletion	[4]
10	*CCM3*	5[c]	Deletion	[4]

UTR Untranslated region
[a] Exon numbering according to NM_194456 for *CCM1*, [3] for *CCM2*, and [4] for *CCM3*.
[b,c] In-frame 84 and 174-bp cDNA deletions, respectively

multiple CCMs [7]. In this cohort, only four *CCM1* and two *CCM3* mutations were previously identified ([7] and unpublished data). Using an exon scanning mutation detection strategy based on denaturing high-pressure liquid chromatography as well as direct sequencing, none of these individuals revealed a mutation in the *CCM2* gene [8]. We demonstrate in this paper the identification of two previously overlooked genomic *CCM2* deletions as well as one large *CCM1* deletion and one multi-exon duplication within the *CCM1* gene applying the MLPA method.

Materials and methods

DNA was collected with informed consent and approval of the Committee for the Protection of Human Subjects at Dartmouth College (Hanover, NH, USA). The criteria for inclusion in the study were (1) neuroradiologic diagnosis of multiple CCMs by MRI, (2) histologic verification of at least one neuroradiologically diagnosed CCM, and (3) negative family history for typical clinical manifestations of CCMs. This cohort is referred to as Swiss cohort, as the majority of individuals analyzed were Swiss patients attending an epilepsy outpatient clinic.

MLPA analyses were performed according to the manufacturer's instructions using two MLPA kits (SALSA MLPA Kits, P130 and P131 CCM, MRC Holland). The *CCM1-3* MLPA analyses of three control individuals in each test and the eight patient samples were carried out according to the manufacturer's instructions using an ABI Prism 310 genetic analyzer and GeneMapper 3.7 data collection software. In addition to visual examination of the peaks, MLPA signals were exported to Microsoft Excel. The individual peak area of each PCR product was divided by the mean peak area of all internal controls. Then, the quotient of each patient sample was divided by the mean of five corresponding quotients from the healthy controls.

Primers for long-range PCR were *CCM1*ex17forward (5′-ATCGTACCTGTTACCAAACTG-3′) and *CCM1*ex7reverse (5′-ATAAATAATGATGCTTCTCTGC-3′). The following *CCM2* single nucleotide polymorphisms (SNPs) were analyzed: rs 2107732, 2304689, 11552376, 11552377, 2289366, 2289367, 2289368, 2304691, and 3214691.

Results

Heterozygous changes in exon copy number were observed in four individuals. One patient suffered from partial epilepsy with complex partial seizures due to multiple CCMs since the age of 24 years (Fig. 1). In this patient, an increase in copy number of *CCM1* exons 7–17 was found, suggesting a large, multiexon duplication of *CCM1* exons 7 through 17. Notably, this duplication was observed independently in contiguous MLPA probes in both MLPA kits (Fig. 2e,f) and not in the controls (Fig. 2a,c,d) or the further patients (Fig. 2b,g–l). To confirm this first large duplication within a *CCM* gene, long-range PCR was performed with primers localized in exons 17 and 7 of the *CCM1* gene. A 1,853-bp PCR product could be amplified from the patient's DNA but not from control DNA (Fig. 3). Sequencing of the entire product revealed breakpoints at

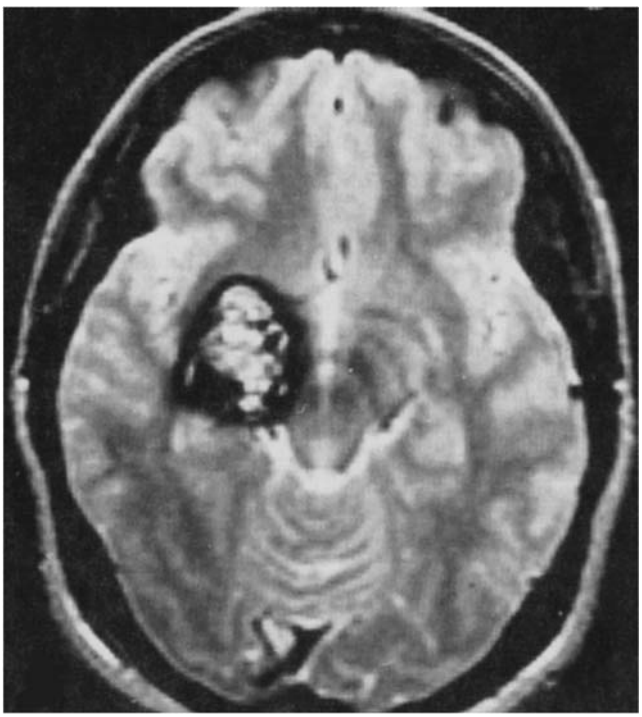

Fig. 1 T1-weighted magnetic resonance imaging shows the typical appearance of a cavernous malformation (hypo/hyperintense) with perilesional hemosiderin deposits (hypointense rim) in the right temporal lobe

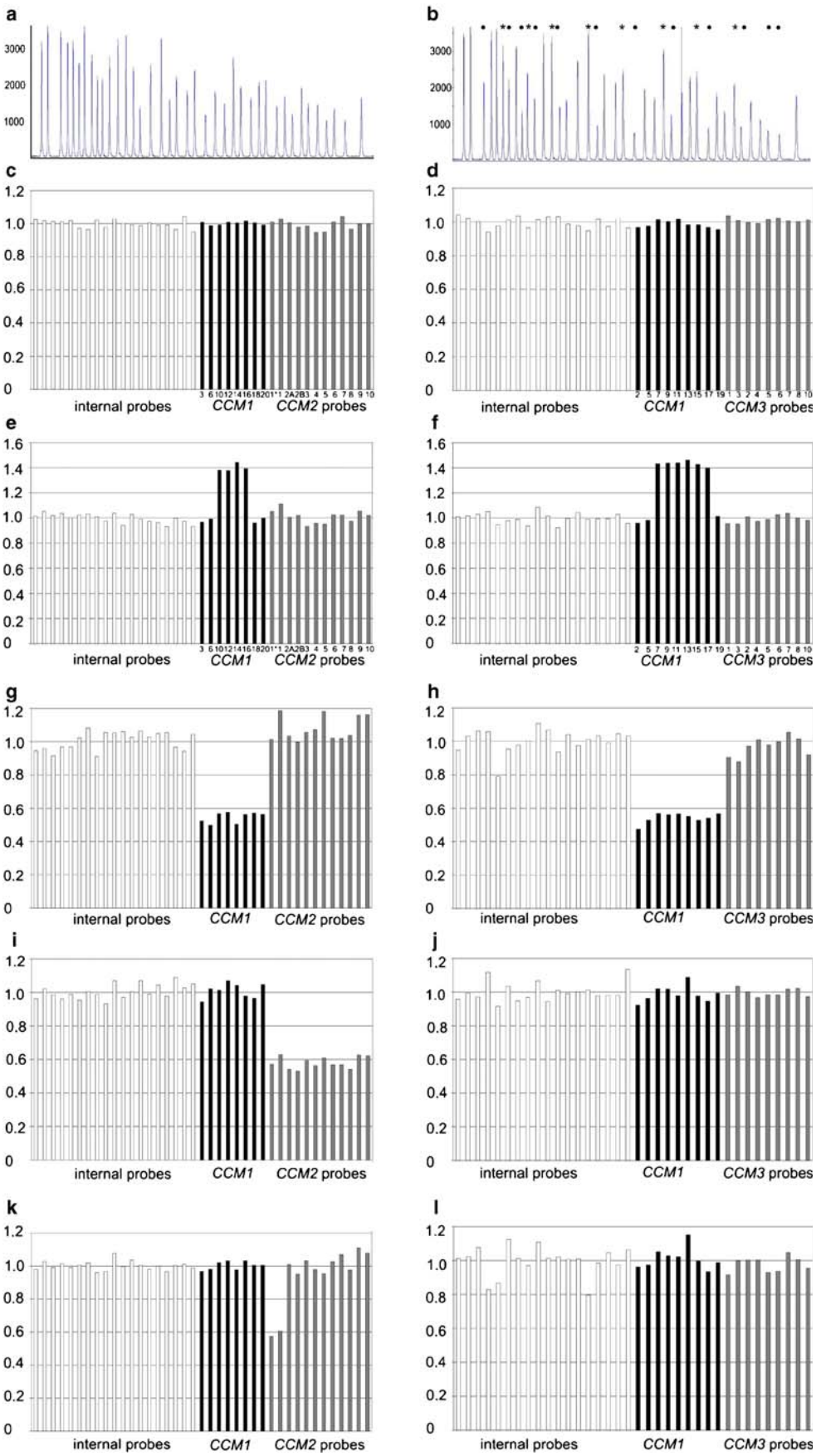

Fig. 2 MLPA data demonstrating heterozygous copy number changes in four sporadic cases. Example of **a** normal and **b** pathological MLPA raw data (SALSA MLPA kit P130). The electropherograms show reduced peaks for all *CCM2* exons in the proband (*dots*), whereas *CCM1* peaks (*asterisks*) are comparable between the control and the patient. Peaks from internal controls are *not highlighted*. **c**–**l** Quantitative analyses demonstrate that the relative peak areas are either increased by 50% (**e**, **f**) or decreased to approximately 50% (**g**–**l**) in affected codons when compared to internal (*white*) and external controls (**c**, **d**). All MLPA analyses were performed in duplicate. Numbers *below the columns* in (**c**–**f**) indicate the *CCM1-3* exons analyzed. **e**, **f** Duplication of *CCM1* exons 7–17, **g**–**l** deletions of the *CCM1* (**g**, **h**), and *CCM2* (**i**, **j**) genes, as well as *CCM2* exon 1 and the 5′ untranslated region (**k**, **l**)

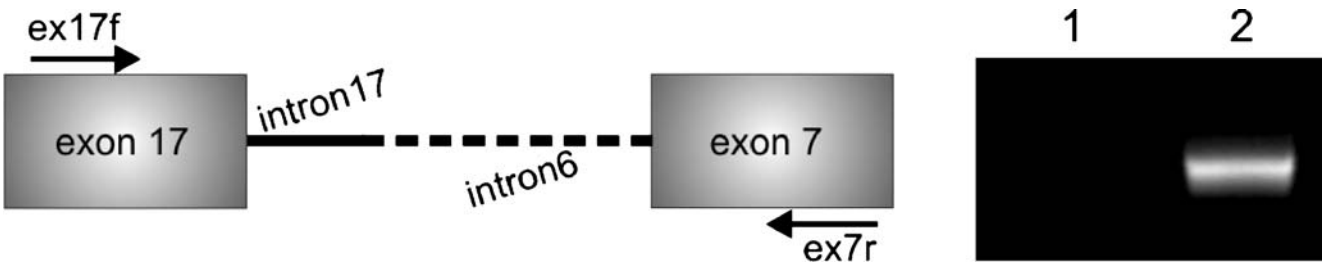

Fig. 3 Confirmation of the *CCM1* duplication. PCR amplification of almost the entire intron 17 fused to the 3′ 1,258 bp of intron 6 using primers *CCM1*ex17forward (ex17f) and *CCM1*ex7reverse (ex7r) yielding a 1,853 bp product in patient (*2*) but not in control DNA (*1*) upon separation on an agarose gel

positions c.1818+455 and c.263-1259 in introns 17 and 6, respectively (data not shown). This novel multi-exon duplication event likely leads to a *CCM1* frameshift mutation (p.N607EfsX6).

In a further patient with multiple CCMs manifesting with partial epilepsy at the age of 16 years, a heterozygous deletion of all *CCM1* exons was found, whereas *CCM2* and *CCM3* peaks and ratios did not differ between the patient and the controls (Fig. 2g,h). A similar deletion of the *CCM1* gene was previously detected in a German CCM family and was confirmed with three informative SNPs [5]. It could be demonstrated that the two *CCM1* gene deletions had occurred twice independently because the Swiss patient did not share the German disease haplotype 2-3-3-2 based on the order of microsatellite markers D7S2410–D7S1813–D7S2189–D7S646 linked to the disease locus (data not shown).

A novel heterozygous deletion of all contiguous *CCM2* exons was reproducibly identified in another case, whereas the *CCM1* and *CCM3* exons showed no copy number changes (Fig. 2i,j). In support of these results, none of the nine intragenic SNPs analyzed revealed heterozygosity. This patient with multiple supratentorial CCMs experienced an ataxia due to an additional infratentorial CCM at age 50. Finally, two adjacent MLPA probes designed for *CCM2* exon 1 and the 5′-upstream region were reduced in the fourth patient (Fig. 2k,l) who presented with partial epilepsy with simple partial seizures due to multiple CCMs at the age of 19 years. Two large deletions including the first exon of the *CCM2* gene have previously been published [3]. It remains to be determined whether a common founder exists for the Swiss patient and one of the two French families.

Discussion

Using MLPA, two novel and two recurrent genomic alterations were found in a total of 14 isolated patients with multiple lesions. In this group, four *CCM1* and two *CCM3* intragenic mutations had been previously identified ([7] and unpublished data). Thus, our mutation detection rates for the three genes (*CCM1*, 6/14=42%; *CCM2*, 2/14=14%; *CCM3*, 2/14=14%) are in agreement with the results obtained from the large French cohort published most recently: *CCM1*, *CCM2*, and *CCM3* mutations were found in 53, 15, and 10% of combined multiple and isolated CCM [2]. Compatible with the French results of 57% for isolated patients with multiple CCMs after direct sequencing, polymorphic marker, and transcript analyses, the combination of sequencing and MLPA analyses enabled us a mutation detection rate of 71%. Our results, therefore, underline that the majority of patients with multiple CCMs harbor germline mutations that can be transmitted to their offspring.

Neuroimaging has demonstrated that 77% (17/22) of isolated patients with multiple CCMs had an asymptomatic parent with small CCMs [9]. The identification of *CCM* mutations permits us to determine if the mutation has been inherited from an asymptomatic parent. Predictive genetic testing can then be offered to further at risk family members before onset of symptoms. However, the clinical course of the disease remains unpredictable. We did not notice phenotypic differences between subjects with small inactivating mutations and large genomic rearrangements. Furthermore, wide intrafamilial clinical variability has been described for small and large *CCM* mutations [5].

Only a few *de novo* mutations have been published more recently [4, 10, 11]. Somatic mosaicism due to postzygotic *de novo* mutations may in part explain the obvious discrepancy of mutation detection rates between familial and isolated CCM cases. The undiagnosed 6% of the familial cases [2] may be attributable to the occurrence of mutations within regulatory regions of *CCM1-3*, the existence of a fourth *CCM* gene or limitations of the screening methods applied.

Five genomic *CCM* deletions had previously been identified by one French laboratory that applied RNA-based mutation analysis complemented with analysis of polymorphic markers [3, 4, 12] (Table 1). With the use of

MLPA in a small German [5] and the Swiss cohort presented here, the total percentage of known genomic *CCM* rearrangements increased from 4% (5 out of 140 published *CCM* mutations [13]) to 7% (10/145; Table 1). It can be anticipated that a systematic screen for genomic alterations in other cohorts will result in a higher proportion of this type of mutations that can be caused by homologous recombination and nonhomologous DNA end joining [14, 15]. Future studies will have to reveal whether it might be more cost effective to perform MLPA before full sequence analyses of *CCM1-3*.

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Disclosure The authors have reported no conflicts of interest.

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