Neurosurg Rev (2007) 30:155–160
DOI 10.1007/s10143-006-0057-1

CASE REPORT

CCM1 gene deletion identified by MLPA in cerebral cavernous malformation

Sabine Gaetzner · Sonja Stahl · Oguzkan Sürücü · Anne Schaafhausen · Birgit Halliger-Keller · Helmut Bertalanffy · Ulrich Sure · Ute Felbor

Received: 30 August 2006 / Revised: 2 October 2006 / Accepted: 2 October 2006 / Published online: 23 December 2006

Abstract Familial cerebral cavernous malformations (CCMs) occur with a frequency of 1 in 2000 and may cause recurrent headaches, seizures, and hemorrhagic stroke. Exon-scanning-based methods have identified intragenic mutations in three genes, *CCM1*, *CCM2*, and *CCM3*, in about 70% of familial CCM. To date, only two large *CCM2* and a single large *CCM3* deletion have been published. In addition to direct sequencing of all three *CCM* genes, we applied a newly developed multiplex ligation-dependent probe amplification gene dosage assay (MLPA) designed to detect genomic *CCM1–3* deletions/duplications. Direct sequencing did not reveal a mutation in the index case who presented with multiple CCMs that had caused a generalized tonic-clonic seizure with Todd's paralysis and headaches at the age of 5. In contrast, MLPA analyses detected a large deletion involving the entire *CCM1* coding region in the proband and further affected members of this German CCM family. The MLPA results were corroborated by analyses of single nucleotide polymorphisms (SNPs) within the *CCM1* gene. Thus, we here present the first report on a *CCM1* gene deletion. Our results confirm a loss-of-function mutation mechanism for *CCM1* and demonstrate that the use of MLPA enables a higher *CCM* mutation detection rate which is crucial for predictive testing of at-risk relatives.

S. Gaetzner · S. Stahl · A. Schaafhausen · B. Halliger-Keller · U. Felbor (✉)
Department of Human Genetics, University of Würzburg, Biozentrum, Am Hubland,
97074 Würzburg, Germany
e-mail: felbor@biozentrum.uni-wuerzburg.de

O. Sürücü · H. Bertalanffy · U. Sure
Department of Neurosurgery, University of Marburg, Marburg, Germany

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

Introduction

Familial cerebral cavernous malformations (CCMs) (MIM 116860, 603284, 603285) are autosomal dominantly inherited vascular abnormalities with genetic heterogeneity and likely interaction among gene products [15]. Exon-by-exon screening approaches found *CCM1* mutations in 43–54% of familial CCMs [2, 12]. Up to 22% were shown to carry a *CCM2* mutation [4, 8] and less than 10% a *CCM3* mutation [1, 5, 9, 12]. Two large *CCM2* deletions [4] and one deletion involving the entire *CCM3* gene [1] initially contributed to identification of the *CCM2* and *CCM3* genes via loss-of-heterozygosity mapping. Since large genomic deletions escape detection by conventional, nonquantitative polymerase chain reaction (PCR)-based mutation analysis, we adopted the multiplex ligation-dependent probe amplification (MLPA) gene dosage assay to screen for large deletions/duplications in the *CCM1–3* genes. MLPA allows the relative quantitation of up to 50 different target DNA sequences in a single reaction and has been proven to be a reliable and sensitive method [7, 10]. We here present identification of a large, heterozygous deletion that encompasses the entire *CCM1* coding region.

Patients and methods

The index case is an 8-year-old boy (III-1, Fig. 1a) who experienced a generalized tonic-clonic seizure with Todd's paralysis and headaches at the age of 5. Magnetic resonance

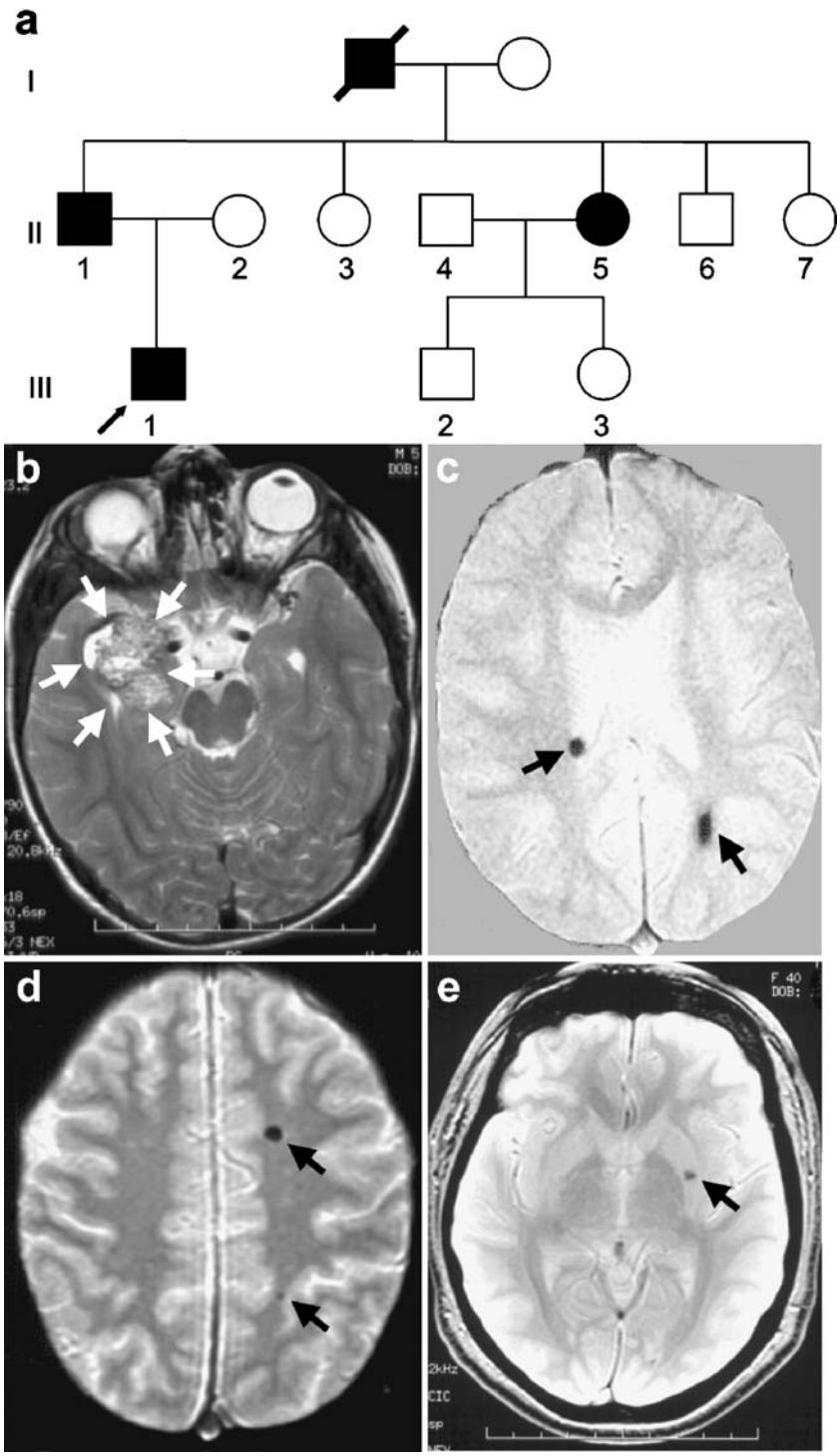

Fig. 1 **a** Pedigree of the German cerebral cavernous malformation (CCM) family (*black circle*=affected female, *black square*=affected male). **b** Axial T2-weighted magnetic resonance image (MRI) shows a large, right temporomesial cavernous malformation (*white arrows*) in the index case (*arrow* in **a**) before microsurgical excision. **c** Additional asymptomatic cavernous malformations (*black arrows*) were diagnosed in both cerebral hemispheres by gradient echo MRI. **d** Axial gradient echo MRI of the boy's father shows two small asymptomatic cavernous malformations, left frontal and parietal (*arrows*). **e** One aunt (II-5 in **a**) also revealed an asymptomatic cavernous malformation in the left basal ganglia (*arrow*)

imaging (MRI) of the brain showed multiple cavernous malformations, including a right temporomesial lesion (Fig. 1b,c). This symptomatic lesion with a diameter of 3.5 cm was completely excised microsurgically via a pterional approach. The postoperative course of the patient was uneventful. After 6 months, he did not require further antiepileptic medication.

Family history revealed a paternal grandfather with multiple intracranial lesions and fatal hemorrhage at age 47. The patient's father is clinically unaffected, but MRI revealed multiple small cavernous malformations which have not required surgical intervention so far (Fig. 1d). Neuroimaging of three further asymptomatic aunts (II-3, II-5, and II-7; Fig. 1a) revealed a small

cavernous malformation in the basal ganglia of aunt II-5 (Fig. 1a,e).

Genetic testing was approved by the local ethics committees (University of Würzburg, Study 21/05; Philipps-University Marburg, Study 149/05). With informed consent, genomic DNA was extracted from peripheral blood lymphocytes. All coding *CCM1–3* exons were directly sequenced on a Beckmann CEQ 8800 capillary electrophoresis system according to published protocols, with slight modifications [1, 2, 4]. Screening for large *CCM* alterations requires two MLPA kits (SALSA MLPA Kits P130 & P131 CCM; MRC Holland, Amsterdam, The Netherlands). The protocol provided by MRC Holland was followed without further optimization. *CCM1–3* MLPA analyses of four control individuals in each test and all ten available family members were carried out using an ABI Prism 310 genetic analyzer. Haplotype analyses were performed for the index case and his parents using 19 intragenic single nucleotide polymorphisms (SNPs) (rs975707, 1064819, 1064820, 1064821, 11984192, 17164451, 2027950, 1034575, 10223994, 10282603, 10274699, 6953959, 12113704, 11542682, 1052043,

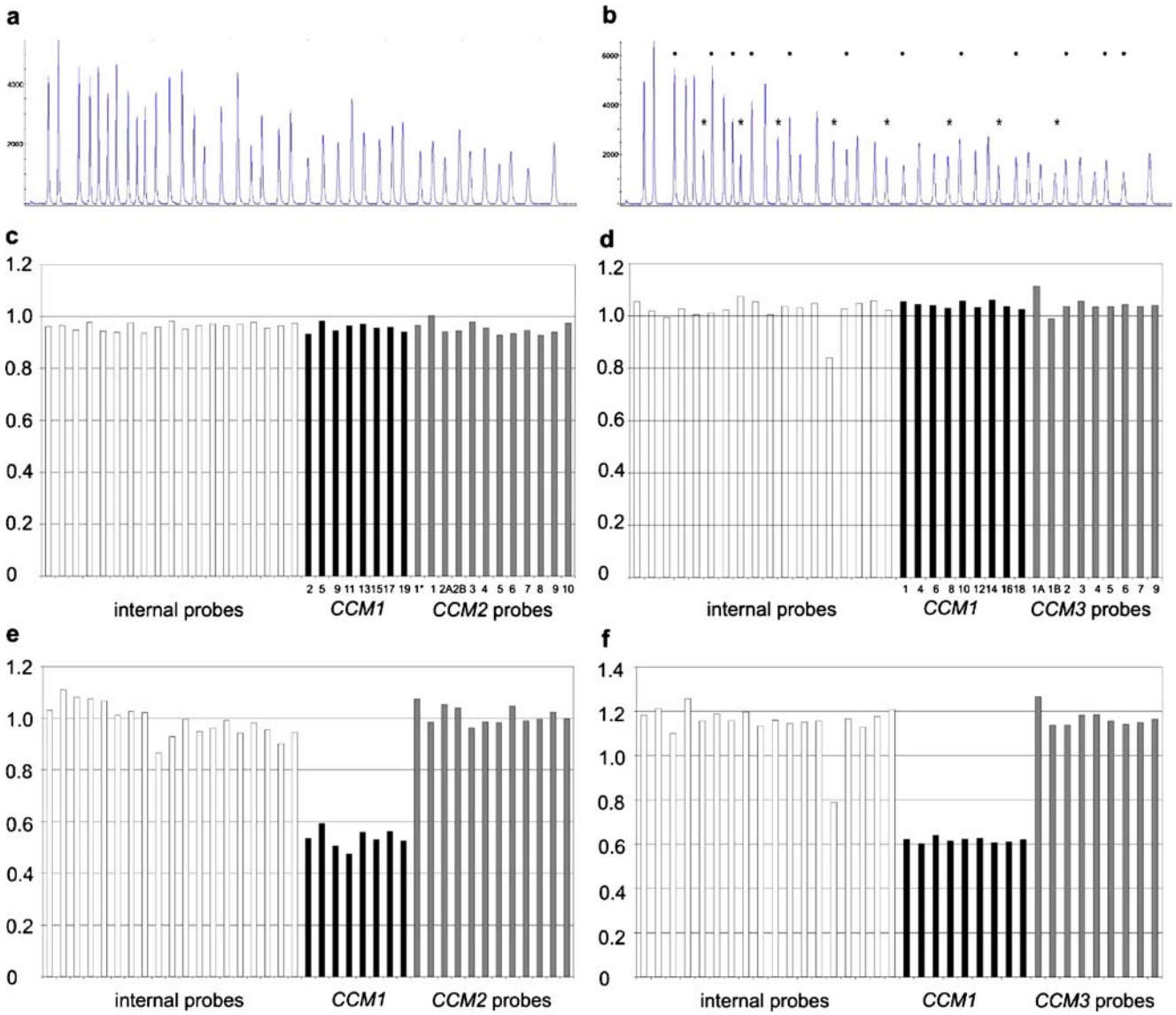

Fig. 2 Multiplex ligation-dependent probe amplification gene dosage assay (MLPA) data demonstrating a heterozygous deletion of the entire *CCM1* gene in the index case. Example of: **a** normal, and **b** pathological MLPA raw data (SALSA MLPA Kit P130). The electropherograms show reduced peaks for all *CCM1* exons in the proband (*asterisks*), whereas *CCM2* peaks (*dots*) are comparable between control and patient. Peaks from internal controls are not highlighted. **e, f** Quantitative analyses demonstrate that the relative peak areas are decreased to approximately 50% in the patient's two noncoding and 15 coding *CCM1* exons analyzed (*black*) when compared with internal (*white*) and **c, d** external controls and to **e** *CCM2* (*grey*) and **f** *CCM3* (*grey*) probes. Numbers below the columns in **c, d** indicate the *CCM1–3* exons analyzed according to the manufacturer's protocol

1063658, 1063659, 11542681, 1063660) and five polymorphic markers flanking the *CCM1* locus (D7S2410, D7S1813, D7S2189, D7S646, and noninformative D7S689).

Results

Direct sequencing of all coding *CCM1*, *CCM2*, and *CCM3* exons and adjacent splice sites did not reveal any pathological intragenic alterations in the index patient. In contrast, only the index case but none of the controls displayed a large deletion encompassing all *CCM1* exons when tested by MLPA (Fig. 2b,e,f). *CCM2* and *CCM3* peaks and ratios did not differ between proband and controls (Fig. 2a,c,d). A second independent MLPA analysis included all ten family members of the second and third generation (Fig. 1a). The heterozygous *CCM1* deletion was confirmed in the three affected family members only (data not shown). Thus, the *CCM1* deletion was reproducible, segregates with the disease, and was not transmitted to children III-2 and III-3 and uncle II-6 (Fig. 1a), rendering neuroimaging unnecessary for these individuals and their offspring.

To further confirm the deletion detected by MLPA, haplotype analyses were performed with 19 intragenic *CCM1* SNPs, none of which was found to be heterozygous in the patient and his affected father. Only three SNPs turned out to be informative. While the patient's mother (II-2) is homozygous for G at rs975707, the father (II-1) is homo- or hemizygous for C (c.1-3078G>C). Since their son did not inherit a paternal C allele (Fig. 3), he is hemizygous for the maternal G allele. Similarly, the mother carries a homozygous C at rs2027950 and a homozygous T at rs6953959 (c.989+4389C>T), whereas the father's sequence revealed a G (c.989+63C>G) and a C, respectively. The proband only shows the maternal C and T alleles. On the basis of the order of microsatellite markers linked to the disease locus and intragenic SNPs as D7S2410-D7S1813-D7S2189-rs975707-rs2027950-rs6953959-D7S646, the proband and his mother share the haplotype 1-2-2-G-C-T-1. The son inherited the disease haplotype 2-3-3-del-del-del-2 from his father, and this haplotype clearly lacks a second allele for three intragenic *CCM1* SNPs (Fig. 3).

Discussion

The *CCM1* deletion was found in a total of five CCM families in which four novel intragenic *CCM* mutations had been identified by direct sequencing ([11] and unpublished data). Based on microsatellite genotyping and cDNA analyses, previous publications reported that two out of ten *CCM2* mutations [4] and one out of eight *CCM3* mutations [1] were large deletions. An additional *CCM3* mutation was described as possibly being due to a deletion of the genomic region that encompasses exon 5 [1]. Furthermore, a genomic deletion involving the 3' end of *CCM1* exon 18 and part of intron 18 was detected [6]. We anticipate that a significant proportion of CCM patients will display large deletions or duplications that remain undetected by direct sequencing of genomic DNA.

MLPA is a suitable and efficient method for identifying such *CCM* gene alterations. MLPA has been reported to be more precise, accurate, and time effective than real-time PCR [3]. Furthermore, deletions larger than the entire coding region, such as the *CCM1* deletion presented in this report, would escape detection by RNA-based reverse transcriptase (RT)-PCR analysis using primers from the coding region, as has been described for deletions of the *NF1* gene causing neurofibromatosis type 1 [14]. Fluorescence in situ hybridization (FISH) of chromosomes is much more laborious than is MLPA and would not be sensitive enough to detect single- or multiexon deletions/duplications. However, if sequencing of genomic DNA and MLPA fail to detect a mutation, RNA-based analysis is a complementary method that allows detection of splice mutations caused by, e.g., point mutations that activate a cryptic splice motif [13] or alterations located deep within a large intron. Thus, the application of MLPA and, in some

	II-1		III-1		II-2	
D7S2410	4	2	2	1	1	3
D7S1813	1	3	3	2	2	4
D7S2189	1	3	3	2	2	4
rs975707	C	del	del	G	G	G
rs2027950	G	del	del	C	C	C
rs6953959	C	del	del	T	T	T
D7S646	4	2	2	1	1	3

Fig. 3 Haplotype analysis of the proband (III-1), his father (II-1), and his mother (II-2). While heterozygosity (i.e., the existence of two different alleles, a maternal and a paternal) for microsatellite markers flanking the *CCM1* locus (D7S2410, D7S1813, D7S2189, D7S646) could be demonstrated for the index patient and his father, the proband carried only the maternal intragenic single nucleotide polymorphism (SNP) haplotype G-C-T, indicating hemizygosity (presence of only one copy of the respective DNA sequence) at the *CCM1* locus for both father and son. The haplotype associated with the cerebral cavernous malformation (CCM) phenotype was determined as 2-3-3-del-del-del-2 (*boxed*) and lacks a second allele for three informative SNPs within the *CCM1* gene (rs975707, rs2027950, rs6953959) (*del*= deletion), thus confirming the Multiplex ligation-dependent probe amplification gene dosage assay (MLPA) results

instances RT-PCR, in addition to sequencing of genomic DNA is important for improving the mutation detection rate in CCM patients which is the basis for predictive testing of at-risk relatives.

Acknowledgements The authors thank the patients and their family for their cooperation and MRC Holland for developing the MLPA kits. Ute Felbor receives an Emmy Noether-grant from the Deutsche Forschungsgemeinschaft (Fe 432/6-5) and Sonja Stahl a stipend from the Graduiertenkolleg 1048.

References

1. Bergametti F, Denier C, Labauge P, Arnoult M, Boetto S, Clanet M, Coubes P, Echenne B, Ibrahim R, Irthum B, Jacquet G, Lonjon M, Moreau JJ, Neau JP, Parker F, Tremoulet M, Tournier-Lasserve E (2005) Mutations within the programmed cell death 10 gene cause cerebral cavernous malformations. Am J Hum Genet 76:42–51
2. Cavé-Riant F, Denier C, Labauge P, Cecillon M, Maciazek J, Joutel A, Laberge-Le Couteulx S, Tournier-Lasserve E (2002) Spectrum and expression analysis of *KRIT1* mutations in 121 consecutive and unrelated patients with Cerebral Cavernous Malformations. Eur J Hum Genet 10: 733–740
3. Damgaard D, Nissen PH, Jensen LG, Nielsen GG, Stenderup A, Larsen ML, Faergeman O (2005) Detection of large deletions in the LDL receptor gene with quantitative PCR methods. BMC Med Genet 6:15
4. Denier C, Goutagny S, Labauge P, Krivosic V, Arnoult M, Cousin A, Benabid AL, Comoy J, Frerebeau P, Gilbert B, Houtteville JP, Jan M, Lapierre F, Loiseau H, Menei P, Mercier P, Moreau JJ, Nivelon-Chevallier A, Parker F, Redondo AM, Scarabin JM, Tremoulet M, Zerah M, Maciazek J, Tournier-Lasserve E (2004) Mutations within the *MGC4607* gene cause cerebral cavernous malformations. Am J Hum Genet 74:326–337
5. Guclu B, Ozturk AK, Pricola KL, Bilguvar K, Shin D, O'Roak BJ, Gunel M (2005) Mutations in apoptosis-related gene, PDCD10, cause cerebral cavernous malformation 3. Neurosurgery 57:1008–1013
6. Laberge-le Couteulx S, Jung HH, Labauge P, Houtteville JP, Lescoat C, Cecillon M, Marechal E, Joutel A, Bach JF, Tournier-Lasserve E (1999) Truncating mutations in *CCM1*, encoding KRIT1, cause hereditary cavernous angiomas. Nat Genet 23:189–193
7. Lalic T, Vossen RH, Coffa J, Schouten JP, Guc-Scekic M, Radivojevic D, Djurisic M, Breuning MH, White SJ, den Dunnen JT (2005) Deletion and duplication screening in the DMD gene using MLPA. Eur J Hum Genet 13:1231–1234
8. Liquori CL, Berg MJ, Siegel AM, Huang E, Zawistowski JS, Stoffer T, Verlaan D, Balogun F, Hughes L, Leedom TP, Plummer NW, Cannella M, Maglione V, Squitieri F, Johnson EW, Rouleau GA, Ptacek L, Marchuk DA (2003) Mutations in a gene encoding a novel protein containing a phosphotyrosine-binding domain cause type 2 cerebral cavernous malformations. Am J Hum Genet 73:1459–1464
9. Liquori CL, Berg MJ, Squitieri F, Ottenbacher M, Sorlie M, Leedom TP, Cannella M, Maglione V, Ptacek L, Johnson EW, Marchuk DA (2006) Low frequency of *PDCD10* mutations in a panel of CCM3 probands: potential for a fourth *CCM* locus. Hum Mutat 27:118
10. Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G (2002) Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res 30:e57
11. Sürücü O, Sure U, Gaetzner S, Stahl S, Benes L, Bertalanffy H, Felbor U (2006) Clinical impact of *CCM* mutation detection in familial cavernous angioma. Childs Nerv Syst 22:1461–1464
12. Verlaan DJ, Roussel J, Laurent SB, Elger CE, Siegel AM, Rouleau GA (2005) *CCM3* mutations are uncommon in cerebral cavernous malformations. Neurology 65:1982–1983
13. Verlaan DJ, Siegel AM, Rouleau GA (2002) *Krit1* missense mutations lead to splicing errors in cerebral cavernous malformation. Am J Hum Genet 70:1564–1567
14. Wimmer K, Yao S, Claes K, Kehrer-Sawatzki H, Tinschert S, De Raedt T, Legius E, Callens T, Beiglbock H, Maertens O, Messiaen L (2006) Spectrum of single- and multiexon *NF1* copy number changes in a cohort of 1,100 unselected NF1 patients. Genes Chromosomes Cancer 45:265–276
15. Zawistowski JS, Stalheim L, Uhlik MT, Abell AN, Ancrile BB, Johnson GL, Marchuk DA (2005) CCM1 and CCM2 protein interactions in cell signaling: implications for cerebral cavernous malformations pathogenesis. Hum Mol Genet 14:2521–2531

Comments

Hildegard Kehrer-Sawatzki, Ulm, Germany

The results reported by Gaetzner et al. are important in the context of mutation screening in families or sporadic cases suffering from recurrent headaches, seizures, and hemorrhagic stroke attributable to cerebral cavernous malformations. These lesions are frequently caused by mutations in one of the three *CCM* genes, *CCM1*, *CCM2*, or *CCM3*. Gaetzner et al. successfully applied the MLPA technique and identified a deletion of the *CCM1* gene in a family with several affected members. Since smaller and larger deletions are often difficult or even impossible to identify by the analysis of polymorphic markers, the MLPA technique applied in this study proved to be an efficient method to identify such alterations unambiguously. Thus, the MLPA technique is an important addition to the current mutation detection protocols if sequencing of exons failed to reveal mutations. The manuscript is written very well and the results are presented in a clear and illustrative manner.

Hidetoshi Kasuya, Tokyo, Japan

The exact mechanism of pathogenesis for familial cavernous malformation is not known. Mutations at three loci (*CCM1*, *CCM2*, and *CCM3*) have been shown in familial cavernous malformation. Three *CCM* genes likely act through the same molecular pathway because familial cavernous malformations caused by different gene mutations are pathologically and phenotypically indistinguishable. There is growing evidence that CCM1 may play a role in regulating ß1 integrin-mediated angiogenesis through this product, which is involved with a bidirectional signaling pathway between the extracellular matrix and the cytoskeleton that uses an integrin-mediated cascade [1]. Many mutations have been reported in *CCM1*: frameshifts, nonsense mutations, changes in the invariant splice junctions, missense mutations, and 84-base pair deletion [2]. MLPA allows the detection of midsize alterations by simultaneously screening for the loss or duplication of up to 50 target sequences. By using this newly developed technique, Gaetzner et al. successfully present the first report on a *CCM1* gene deletion larger than the entire coding region, which would escape detection by the techniques reported before.

References

1. Dashti SR, Hoffer A, Hu YC, Selman WR (2006) Molecular genetics of familial cerebral cavernous malformations. Neurosurg Focus 21(1):E2

2. Verlaan DJ, Davenport WJ, Stefan H, Sure U, Siegel AM, Rouleau GA (2002) Cerebral cavernous malformations: Mutations in *Krit1*. Neurology 58: 853-8574

Dietmar Krex, Dresden, Germany

The pathogenesis of familial cerebral cavernous malformation is based on genetic variants within three genes, *CCM1*, *-2*, and *-3*, which is in contrast to most other cerebral vascular malformations where disease-causing genes still have to be determined. Therefore, predictive testing of at-risk relatives is possible by the analysis of blood samples; a goal that has to be achieved, for instance, for arterio-venous malformations or intracranial aneurysms.

However, valid diagnostics are hampered, as genetic variants within *CCM* genes not only comprise various small mutations but also large genomic deletions, which might lead to false negative results by standard sequencing techniques. Gaetzner et al. show that, by using the multiplex ligation-dependent probe amplification gene dosage assay (MLPA), this particular shortcoming of missing genomic deletions can be overcome, making the analysis more accurate. As MLPA is an established technique based on commercially available kits, it can be widely and easily used for improving the predictive value of genetic testing. We are looking forward to results from larger cohorts tested in that comprehensive manner.