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Update on Novel CCM Gene Mutations in Patients with Cerebral Cavernous Malformations

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Abstract Cerebral cavernous malformations (CCMs) are lesions affecting brain microvessels. The pathogenesis is not clearly understood. Conventional classification criterion is based on genetics, and thus, familial and sporadic forms can be distinguished; however, classification of sporadic cases with multiple lesions still remains uncertain. To date, three CCM causative genes have been identified: CCM1/KRIT1, CCM2/MGC4607 and CCM3/PDCD10. In our previous mutation screening, performed in a cohort of 95 Italian patients, with both sporadic and familial cases, we identified several mutations in CCM genes. This study represents further molecular screening in a cohort of 19 Italian patients enrolled by us in the few last years and classified into familial, sporadic and sporadic with multiple lesions cases. Direct sequencing and multiplex ligation-dependent probe amplification (MLPA) analysis were performed to detect point mutations and large genomic rearrangements, respectively. Effects of detected mutations and single-nucleotide polymorphisms (SNPs) were evaluated by an in silico approach and by western blot analysis. A novel nonsense mutation in CCM1 and a novel missense mutation in CCM2 were detected; moreover, several CCM2 gene polymorphisms in sporadic CCM patients were reported. We believe that these data enrich the mutation spectrum of CCM genes, which is useful for genetic counselling to identify both familial and sporadic CCM cases, as early as possible.

Keywords Brain vascular pathology · CCM gene variants · Early diagnosis · Genetic test update · Predictive medicine

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Introduction

Cerebral cavernous malformations (CCMs, OMIM no. 116860) are vascular lesions that involve the central nervous system (CNS) capillaries, without impairment of normal cerebral parenchyma. Histologically, CCMs are characterised by enlarged vessels deep in a dense collagenous matrix. An absence of pericytes and deficiency at adherens and tight junctions are characteristics of these malformations, resulting in an impairment of blood–brain barrier (BBB) integrity (Lee & Spetzler 1989). The incidence of CCM is estimated to be about 0.1–0.5% worldwide and represents from 10 to 20% of all cerebrovascular malformations; however, these data would appear to be underestimated due to the high rate of asymptomatic patients (approximately 40% of the total) (Labauge et al. 2007). Clinical manifestations include intracerebral haemorrhages, as a result of impaired endothelial cell junctions and seizures, recurrent headaches and focused neuronal deficits, closely related to lesion localisation (Rigamonti et al. 1988).

Lesions can arise sporadically or be present in a familial syndromic form. Sporadic forms, usually characterised by single lesions, develop between the third and fifth life decade. In familial forms (familial cerebral cavernous malformations (FCCM)), instead, multiple lesions are already present at

paediatric age (Lee & Spetzler 1989). FCCM are inherited with an autosomal dominant pattern and are mostly linked to germline mutations within the three loci: *CCM1/KRIT1* on 7q21-22 (Notelet et al. 1997), *CCM2/MGC4607* on 7p15-13 and *CCM3/PDCD10* on 3q26.2-27 (Craig et al. 1998). Clinical penetrance is estimated to be about 88% for *CCM1*, 100% for *CCM2* and 63% for *CCM3* (Haasdijk et al. 2012).

CCM genes encode for three interacting proteins involved in different cellular pathways contributing to correct angiogenesis (Wüstehube et al. 2010). Regulation of intercellular junctions (Draheim et al. 2014) and apoptosis (Harel et al. 2009; Schleider et al. 2011), response to stress (Guazzi et al. 2012; Zawistowski et al. 2005; Fidalgo et al. 2012) and modulation of vascular endothelial growth factor (VEGF) pathways (DiStefano et al. 2014; Wüstehube et al. 2010; You et al. 2013), representing the main pathways of BBB development and integrity, are maintained by CCM protein activity.

With regard to mutation rate, in familial cases, 56, 16 and 17% are linked to *CCM1*, *CCM2* and *CCM3*, respectively, while in sporadic cases, these percentages are 33, 10 and 14%, respectively. No germline mutations were reported in 11 and in 43% of familial and sporadic cases, respectively (Spiegler et al. 2014).

To date, about 200 germline mutations at the three known CCM genes have been described in literature as causative mutations of CCMs (Kumar et al. 2014; D'Angelo et al. 2013; D'Angelo et al. 2011). In the last few years, the number of patients who have come to our attention has increased greatly. Here, we report results obtained from CCM gene molecular screening, performed on the more recent cohort of 19 patients.

Methods

Sample Collection

Nineteen patients coming from the neurological and neurosurgical clinics of "Gaetano Martino" Hospital (Messina) were considered for this study: 4 familial forms, 12 sporadic cases and 3 sporadic cases with multiple lesions, in 2015. DNA blood samples were obtained with written informed consent from all patients and normal controls. For underage patients, consent was obtained from the parents. Study protocol followed the guidelines of the local ethics committee, and the investigation was conducted with the ethical requirements defined in the Helsinki Declaration.

Familial Cases

FC1 The proband of the family 1 (Fig. 1a, left panel, II:2) is a 21-year-old woman manifesting recurrent headache events since she was 2 years of age. MRI, carried out at the age of 18, showed a single CCM lesion in the right parietal region. No alterations were identified at neurological examination. Pedigree of the family is shown in Fig. 1a: the father (Fig. 1a, left panel, I:1), a 41-year-old man, was neurologically asymptomatic until at the age of 39, when he suffered from a cerebral ischemia. An MRI showed a lesion in the right anterior paraventricular area; more retention cysts were detected in the right maxillary sinus.

No MRI alteration, instead, was observed in the proband's mother (Fig. 1a, left panel, I:2) although she reported recurrent episodes of headaches. No MRI data are available for the two sisters, despite the elder (Fig. 1a, left panel, II:1) having reported frequent migraine events since paediatric age.

FC2 The proband (Fig. 1b, left panel, II:4) was a 51-year-old woman. At the age of 24, she was affected by hemispheric cerebral haemorrhage and consequent local epileptic seizures. Brain MRI performed at the age of 37 revealed multiple CCM lesions in the supratentorial and subtentorial regions. Family members were investigated for CCM gene mutations: her husband (Fig. 1b, left panel, II:5), a healthy 55-year-old subject with no available MRI; her 18-year-old son, with negative MRI (Fig. 1b, left panel, III:1); her daughter, an asymptomatic 9 years old (Fig. 1b, left panel, III:3) showing brain MRI positive for multiple cavernous angiomas in the right posterior temporal cortex. Another daughter (Fig. 1b, left panel, III:2) died at 18 months of age, of encephalorrhagia. No cutaneous, retinic and spinal cavernomas were reported for the family members. None of the family members, except the proband, reported referable clinical symptoms of CCM.

FC3 The proband is a man of 48 years (Fig. 1c, left panel, III:1). He complained of balance disorder, left eye nystagmus and vertigo at the age of 20. A cerebellar CCM lesion was removed. However, 2 years ago, in 2014, he underwent two operations to remove four new lesions, located in the left temporal (two lesions), in the right temporal and in the right fronto-parietal regions. No other family member appears to show symptoms, except the proband's paternal grandmother (Fig. 1c, left panel, I:2) who died of an intracerebral haemorrhage. However, CCM gene screening was performed on his parents (Fig. 1c, left panel, II), his brother (Fig. 1c, left panel, III:2) and his daughter (Fig. 1c, left panel, IV:1).

FC4 The proband is a 55-year-old female (Fig. 1d, left panel, III:1) reporting a 1-month history of headaches, general malaise with nausea and vomiting, tinnitus and muscle weakness on the right side. At admission, the patient presented dysphagia for both liquids and solids. Neurological examination showed nystagmus, defects in perception of sounds and right brachio-crural hemiparesis. Although difficult to interpret, MRI showed an unusually 2-cm large hypointense lesion at the medulla oblongata with acute and subacute intralesional

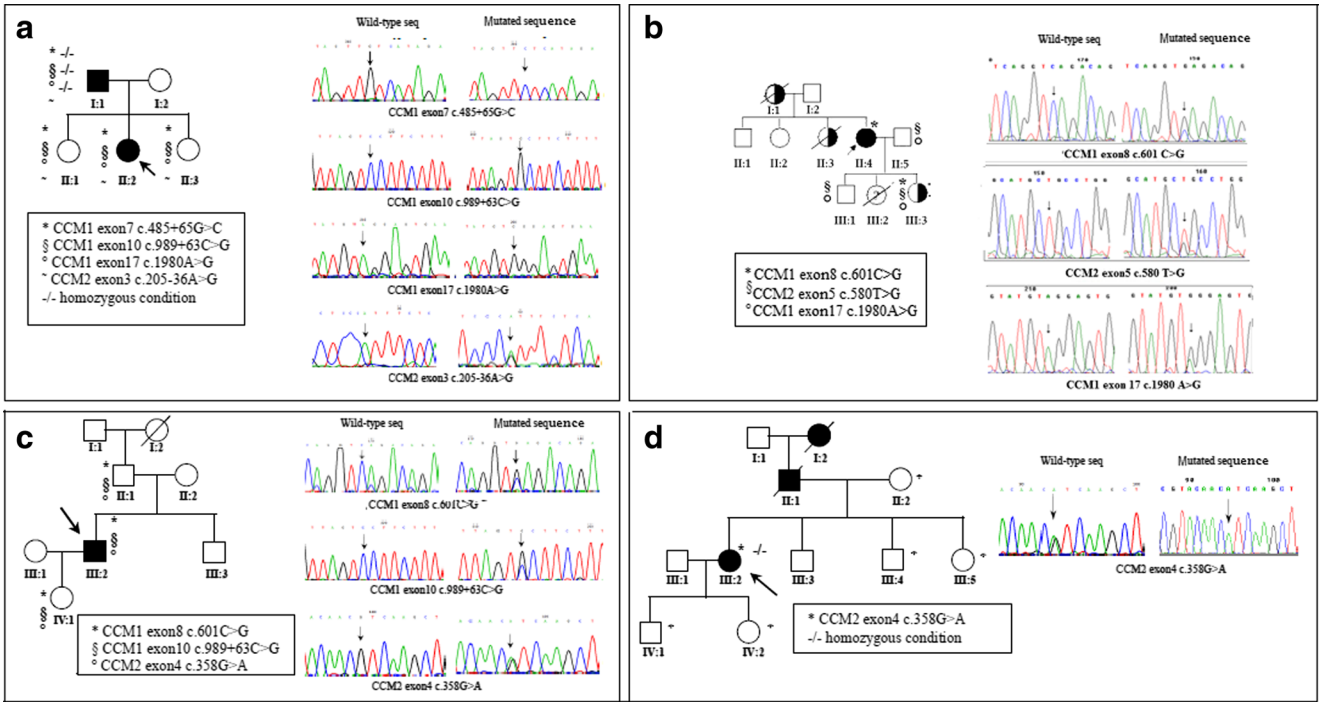

Fig. 1 Families' pedigrees showing variant segregation. In each *panel*: on the *left*, pedigree of family: *blackened circles* and *squares* indicate affected members and *diagonal line* deceased individuals. *Arrows* indicate the proband of each family; on the *right*: electropherograms showing detected variants. Wild-type and mutated sequences are reported; the *arrows* indicate the nucleotide substitutions. **a** FC1. **b** FC2: *question mark* indicates uncertain diagnosis and *half-blackened circle* affected asymptomatic individual. **c** FC3. **d** FC4

bleeding. Family history reported that the patient's father (Fig. 1d, left panel, II:1) died of a cerebral haemorrhage, as well as the maternal grandmother. Consequently, genetic testing on the patient and her relatives was required. The latter include the following: mother of 84 years (Fig. 1d, left panel, II:1); three brothers of 53, 52 and 48 years (Fig. 1d, left panel, III:2, III:3, III:4); and two sons of 31 (Fig. 1d, left panel, IV:1) and 25 years (Fig. 1d, left panel, IV:2). No lesions were found at MRI investigation.

Sporadic Cases

Twelve patients (named from sporadic case (SP) 1 to SP12) affected by sporadic forms were included in this study. Data of anamneses are collected in Table 1.

Sporadic Cases with Multiple Lesions

Three patients (multiple lesion (ML) 1, ML2, ML3) presenting with multiple lesions but without family history of multiple cavernomas, epilepsy, cerebral haemorrhages or focal neurological deficits were classified as sporadic cases with multiple lesions. In particular, the first (ML1) is an 80-year-old man who presented with mental confusion and memory deficits. Computed tomography and MRI images showed more than 70 subtentorial and supratentorial cavernomas (Fig. 2). The second (ML2) is a 33-year-old woman who developed recurrent headaches and vomiting at the age of 15. A single lesion was, at the time, removed. However, following some episodes of bleeding, at 32 years old, ten CCM lesions were detected. The last case (ML3) is a 39-year-old man who manifested seizures, vertigo and a stutter about 3 years ago. Multiple cavernomas were detected in bilateral fronto-parietal and rear temporal regions and at cerebral peduncle.

Molecular Analysis

Direct Sequencing

Molecular analysis was performed by Sanger sequencing. DNA was isolated from peripheral blood; coding exons and intron–exon boundaries of CCM1, CCM2 and CCM3 genes were screened using the pairs of primers designed according to the published nucleotide sequences of GenBank (accession no. NG_012964.1, NG_016295.1 and NG_008158.1, respectively). All PCR products were sequenced on a 3500 Genetic Analyzer (Applied Biosystems), using the BigDye Terminator v3.1 chemistry, following the manufacturer's instructions.

Multiplex Ligation-Dependent Probe Amplification Assay

Multiplex ligation-dependent probe amplification (MLPA) assay was performed to exclude large genomic rearrangements.

Table 1 Clinical features of sporadic CCM patients

	Gender	Age	Symptom age	Symptoms	Number of lesions	Localisation
SP1	M	19	Asymptomatic		1	Left lateral ventricle
SP2	M	41	39	Seizures, dysphasia	1	Left temporal-basal
SP3	F	73	Asymptomatic		1	Right paraventricular
SP4	M	55	52	Seizures	1	Left deep temporal
SP5	F	58	55	Seizures, limbs tremor, headache	2	Right deep temporal, right frontal
SP6	F	44	42	Headache, giddiness	1	Right thalamic
SP7	F	49	47	Seizures	1	Right parasagittal
SP8	F	57	57	Headache	1	Subcortical
SP9	F	46	No data available			
SP10	M	34	20	Seizures	2	Right fronto-parietal
SP11	M	14	Asymptomatic		1	Cerebellum
SP12	M	49	47	Vertigo, seizures	1	Right temporal-mesial

Two different MLPA kits (SALSA MLPA Kits, P130 and P131 CCM, MRC Holland) were used. For visual inspection, peak heights were compared between the sample and controls to find any alteration in relative peak heights within the test sample. For the normalised peak area calculations, each peak area was normalised by dividing the individual peak area by the total peak area of all peaks for that sample; statistical analysis was performed by the Coffalyser software.

PCR-Restriction Fragment Length Polymorphism

To estimate the population frequency of the novel variants detected in our cohort, 300 controls from noncorrelated healthy subjects were screened by PCR-restriction fragment length polymorphism (RFLP). In particular, the new nonsense c.596C>G *CCM1* variant results in the creation of a restriction site for MnlI endonuclease, in the 445-bp wild-type fragment.

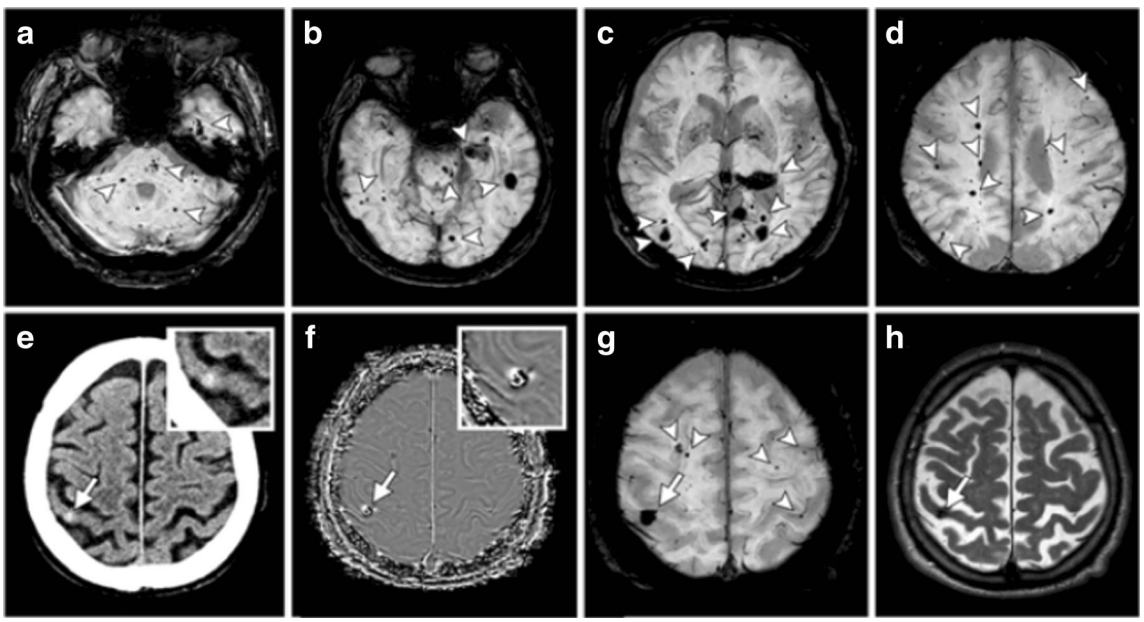

Fig. 2 CT and MRI of multiple cavernomas detected in ML3 patient. **a**–**d** MR images of minimum intensity projections (MinIP) susceptibility-weighted imaging (SWI) show multiple subtentorial and supratentorial cavernomas (*arrowheads*) in different axial levels. **e**–**h** Single axial slices of the same axial level showed a partially calcified cavernoma in the right postcentral cortex (*arrows*), studied, respectively, by noncontrast-enhanced computed tomography (**e**), SWI phase (**f**), combined postprocessed magnitude and phase MinIP SWI (**g**) and TSE T2-weighted images. The *right upper sides* of **e**, **f** are magnified images of right parietal cavernoma. Note how SWI phase (**f**) can easily distinguish between calcified and noncalcified parts of cavernoma (respectively, *white* and *black portions*). Other cavernomas are also shown in **g** (*arrowheads*)

If a variant is present, digest product results in two fragments of 205 and 240 bp.

Conversely, the new missense c.580T>G *CCM2* variant introduces a cutting site for Acil endonuclease. The entire wild-type 217-bp fragment contains a restriction site for the enzyme. After digestion, two fragments (84 and 133 bp) were obtained for wild-type samples, while three fragments (84, 67 and 66 bp) for mutated ones.

Western Blot Analysis

Western blot analysis was performed to test the effect of nonsense c.596C>G *CCM1* mutation. Proteins were extracted from blood, and equivalent amounts (50 μg) of each sample were electrophoresed in a 12% (*w*/*v*) discontinuous polyacrylamide minigel. Proteins were separated electrophoretically and transferred to nitrocellulose membranes. For immunoblotting, the membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) for 1 h and then incubated with primary polyclonal antibody against Krit1 1:100 (Abcam) overnight at 4 °C. The membranes were incubated with mouse anti-rabbit IgG-HRP 1:5000 (Santa Cruz Biotechnology).

In Silico Analysis

We decided on an in silico approach to test the effect of bothnovelc.580T>G variant and all single-nucleotide polymorphisms (SNPs) detected in the CCM genes. We focused on splicing changes and protein folding.

Splicing Regulatory Element Predictions

Analysis was performed using the Human Splicing Finder (HSF) tool (http://www.umd.be/HSF3/index.html) that integrates all matrices to detect splice sites and regulatory splice motifs of wild-type sequences compared to mutated ones.

Protein Predictions

To assess the amino acid substitution compatibility, we used the PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) tool. To predict the secondary structure of the polypeptide, we used the PSI-blast-based secondary structure PREDiction (PSIPRED) tool (http://bioinf.cs.ucl.ac.uk), an accurate secondary structure prediction method, incorporating two feedforward neural networks which perform an analysis on output obtained from Position-Specific Iterated Basic Local Alignment Search Tool (PSI-BLAST). We submitted both the full original and the mutated amino acid sequences. Moreover, we used Raptor X (http://raptorx.uchicago.edu) to predict protein tertiary structures: given a query sequence, RaptorX predicts its secondary and tertiary structures as well as solvent accessibility and disordered regions. RaptorX also predicts the binding sites of a protein sequence, based upon the predicted 3D mode.

Results

Familial Cases

FC1

Molecular analysis performed on the young woman showed a heterozygous condition for four SNPs: rs17164451 (c.485+65G>C), rs2027950 (c.989+63C>G) and rs11542682 (c.1980A>G) in *CCM1* and rs2304689 (c.205-36A>G) in *CCM2*. Her mother showed neither mutations nor SNPs, while her father was homozygous for the three *CCM1* polymorphisms and heterozygous for the *CCM2* polymorphism. Also, both sisters were carriers of the same polymorphisms (Fig. 1a, right panel).

FC2

Screening of CCM genes was performed on the proband, her husband and two children: an 18-year-old son, with negative MRI, and a 9-year-old daughter, with an MRI diagnosis of CCM, but asymptomatic. In the proband, we identified the c.601C>G mutation in exon 8 of the CCM1 gene (p.Q201E; p.Q201RfsX2), originally published as a missense mutation (p.Q201E) (Davenport et al. 2001) and then found to create an alternative splice site, causing aberrant splicing and leading to a frame shift and protein truncation (Verlaan et al. 2002). In the daughter, we detected the same *CCM1* mutation (inherited from the mother) and the c.1980A>G (p.V660V) (rs11542682) in exon 17. Moreover, we found a novel missense variant in exon 5 of the CCM2 gene, c.580T>G (p.C194G), a change that did not obviously disrupt the open reading frame and was not near a splice junction (Fig. 1b, right panel). No mutation was found in *CCM3*. A novel variant in exon 5 of the CCM2 gene was also identified in the son. In the proband's husband, father of the two children, we identified the c.1980A>G polymorphism in *CCM1* and the c.580T>G variant in *CCM2* (Fig. 1b, right panel). This variant is not reported in the follow databases: Angioma Alliance (http://www.angiomaalliance.org/index.aspx), HGMD (http://www.hgmd.cf.ac.uk/ac/index.php), dbSNP (http://www.ncbi.nlm.nih.gov/snp) and Ensembl (www.ensembl.org). RFLP screening confirmed that the c.580T>G variant is not a polymorphism, since it was not found in any of the 300 healthy controls.

Uniprot database (www.uniprot.org) showed that the c.580 T>G variant affects the phosphotyrosine-binding domain (PTB) of malcavernin. With regard to splicing alteration, in

silico analysis performed by the HSF tool shows how the c.580T>G mutation can potentially affect splicing due to an alteration of an exonic splicing enhancer (ESE) in exon 5 of CCM2 gene (http://www.umd.be/HSF3/4DACTION/AW3_input_ChoixGene). However, complementary DNA (cDNA) analysis was not performed due to difficulty in recruiting patient's sample again.

With regard to protein predictions, PolyPhen-2 was employed. This combines information on sequence features, multiple alignment with homologous proteins and structural parameters and contacts to make a prediction of SNP effects on protein. It gives a score of 0.997 (deleterious replacement if score is 1), so the substitution is predicted to be probably damaging (http://genetics.bwh.harvard.edu/ggi/pph2/0eed0d82654135960645568b8b6161352a21d56e/3600356.html). The mutation involves amino acid 194. PSIPRED analysis showed that in wild-type sequence, C194 is part of a sheet motif; p.C194G substitution leads to an exclusion of this amino acid from the sheet (not shown). Alteration of malcavernin tertiary structure is confirmed by RAPTOR X prediction, and Fig. 3a shows the misfolding of PTB domain in a mutated protein.

FC3

CCM causative mutation c.601C>G (p.Q201E; p.Q201RfsX2) in *CCM1* exon 8 was also detected in the proband of the third family reported in this study, together with intronic rs2027950 (c.989+63C>G) in CCM1 gene and rs11552377 (c.358G>A, p.V120I). Regarding family members, *CCM1* mutation was only detected in the proband's father (asymptomatic) and in his 6-year-old daughter who had no lesions at MRI investigation (Fig. 1c, right panel).

FC4

The variant c.358G>A in exon 4 of CCM2 gene was detected in the patient and in five of six family members. Among them, only the patient carried the variant in homozygous state (Fig. 1d, right panel). This variant is reported on Ensemble Genome Browser (http://www.ensembl.org/index.html) as a polymorphism (rs11552377) that leads to substitution p.V120I and resides in the phosphotyrosine-binding domain (PTB) of malcavernin protein. Previously, in a case–control study performed on sporadic CCM patients, this polymorphism was found to be associated with a more benign course of CCM disease (D'Angelo et al. 2012).

From in silico analysis, the HSF tool predicted a possible alteration of an ESE site with consequent splicing aberration.

Moreover, as shown by the PSIPRED analysis, c.358G>A variant could affect a sheet motif.

Results of secondary structure prediction revealed a clear distribution of alpha helix, beta sheet and coil. In the presence of polymorphism V120I, a beta sheet is involved in the 120–160 region.

Upon structural prediction of native and mutant malcavernin protein by using the RaptorX program, the results revealed significant structural changes. When two structures of malcavernin protein with native V120 residue and I120 mutant were superimposed, an alteration of the folding in position 120 caused by a change in the number and the position of intramolecular H bonds of the PTB domain was observed (Fig. 3b).

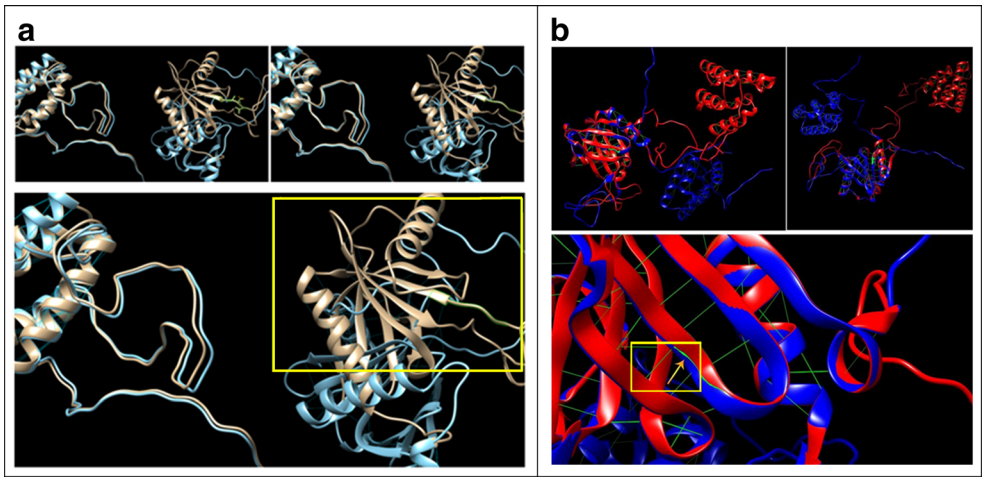

Fig. 3 Three-dimensional structure of malcavernin protein generated by RaptorX 3D prediction program. Each *panel* shows overlapping between wild-type and mutated tertiary structures. **a** p.C194G: the misfolding of PTB domain in mutated protein (in *blue*) is highlighted by the *yellow box*. **b** p.V120I: superimposed structures of malcavernine protein with native V120 residue and I120 residue substitution. The *arrow* indicates the region of the PTB domain where the V120I substitution resides and the *yellow box* the alteration of the intramolecular hydrogen bonds at the level of substitution

Sporadic Cases

No mutations were detected by direct sequencing of CCM genes performed on sporadic cases; however, several polymorphisms, except in sample SP5, were reported. Also, MLPA showed no rearrangements. Global results are collected in Table 2.

In silico analysis to assess potential splicing changes shows that rs11542682 (c.1980A>G p.Val660Val) in CCM1 gene may potentially affect splicing (http://www.umd.be/HSF3/4DACTION/AW3_input_ChoixGene), as well as rs2304689 (c.205-36A>G), rs11552377 (c.358G>A p.V120I) and rs2289367 (c.915G>A p.Thr305Thr) in CCM2 gene (http://www.umd.be/HSF3/4DACTION/AW3_input_ChoixGene). CCM3 SNPs do not seem to affect splicing.

Sporadic Cases with Multiple Lesions

A novel nucleotide substitution c.596C>G (p.Ser199*) was found in exon 8 of *CCM1* of ML1 sample (Fig. 4a). The variant was not observed in 300 healthy controls, nor in the following publically available databases: CCM mutation database Angioma Alliance (http://www.angiomaalliance.org/pages.aspx?content=348#.VImlG9KGAg), HGMD (http://www.hgmd.cf.ac.uk/ac/index.php), dbSNP (http://www.ncbi.nlm.nih.gov/snp/) and Ensembl (http://www.ensembl.org/Homo_sapiens/Gene/Sequence?db=core;g=ENSG00000001631;r=7:92198969-92246166). No variations were detected in *CCM2* and *CCM3*.

The C>G transition at position 596 in exon 8 of CCM1 cDNA changes the "TCA" codon at position 199, encoding for a serine residue, to the "TGA" stop codon, which leads to a truncated protein. The mutation effect was proven by western

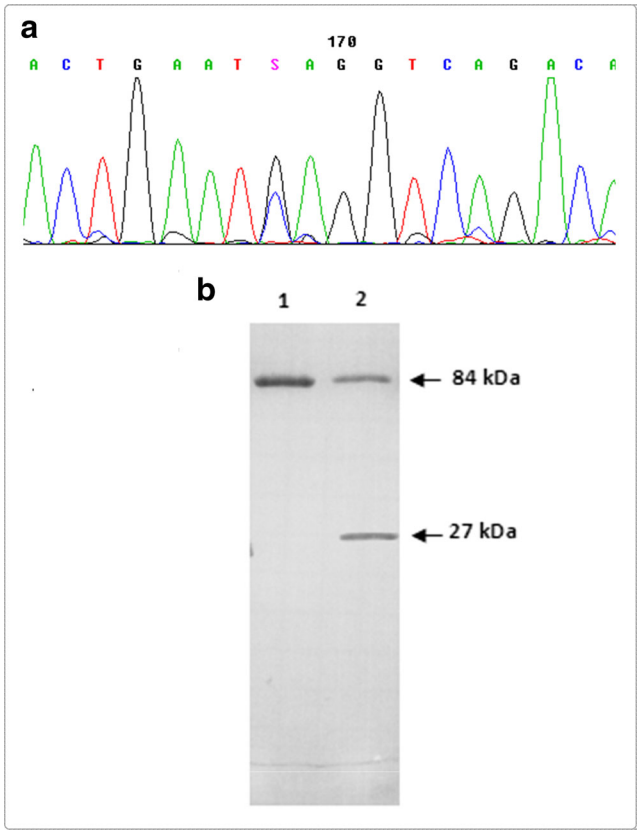

Fig. 4 Case ML1. **a** Electropherogram of *CCM1* exon 8 showing the c.596C>G substitution. Nucleotide numbering reflects cDNA with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence. **b** Western blotting analysis: western blotting of extracts (50 μg protein) from control's blood (*left*) and patient's (*right*) with primary polyclonal antibody. The *upper arrow* indicates the 84-kDa wild-type krit1 protein. The *lower one* indicates the truncated form at ~27 kDa

Table 2 Genotypes of CCM sporadic patients for the most represented CCM gene polymorphisms in our cohort

Gene	SNP	SP1	SP2	SP3	SP4	SP5	SP6	SP7	SP8	SP9	SP10	SP11	SP12
CCM1	rs17164451 c.485+65G>C	G/G	G/C	G/G	G/G	G/G	G/G	G/G	G/G	G/C	G/G	G/G	G/G
	rs2027950 c.989+63C>G	G/G	C/C	C/C	G/G	C/C	C/G	C/G	C/G	G/G	C/G	C/G	C/G
	rs11542682 CM105502 c.1980A>G p.Val660Val	A/A	A/G	A/A	A/A	A/A	A/A	A/A	A/A	A/G	A/A	A/A	A/A
	rs181340604 c.*272G>A	G/G	G/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G
CCM2	rs2304689 c.205-36A>G	A/A	G/G	A/A	A/G	A/A	A/A	A/G	A/A	A/G	A/A	A/A	A/G
	rs11552377 c.358G>A p.V20I	G/A	G/G	G/G	G/G	G/G	A/A	G/G	G/A	G/G	G/A	G/G	G/G
	rs746984207 c.472+9G>C	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/C	G/G	G/G
	rs73107990 c.472+127C>T	C/C	C/T	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/T
	rs145003686 c.804-5C>T	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/T	C/C	C/C
	rs2289367 c.915G>A p.Thr305Thr	G/G	A/A	A/A	G/A	G/G	G/G	G/A	G/G	G/A	G/G	G/A	G/A
	rs2289369 c.915+119C>T	C/C	T/T	T/T	C/T	C/C	C/C	C/T	C/C	C/T	C/C	C/C	C/T
CCM3	rs200180968 c.268+53C>T	C/C	C/C	C/C	C/C	C/C	C/T	C/C	C/C	C/C	C/C	C/C	C/C
	rs184532453 c.269-119C>A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/A	C/A	C/C	C/C	C/C

blot analysis, performed on the patient and on a negative control. The immunoblot showed a band for full-length krit1 protein of 84 kDa in both the control and patient. This latter reveals a ~27 kDa band, corresponding to predicted truncated protein detected (Fig. 4b).

No mutations were detected in ML2 and ML3 patients. However, rs55967204 c.745+98G>C intronic SNP was detected in ML3 in a homozygous condition. Due to the very low frequency of this SNP in our cohorts of patients screened in the last 10 years, we wanted to investigate if it can affect splicing. However, the HSF tool predicted that it has no effect (http://www.umd.be/HSF3/4DACTION/AW3_input_ChoixGene).

Discussion

This study reports results of CCM gene mutation analysis performed on a cohort of 19 patients affected by CCM. We divided our patients by genetics, since different pathogenetic mechanisms have been proposed to explain development of different CCM forms (Bravi et al. 2016). Germline CCM gene mutations are reported in about 90% of patients affected by familial forms, and this percentage has been confirmed by several studies; conversely, data on sporadic forms are highly discrepant (Spiegler et al. 2014; Riant et al. 2013; D'Angelo et al. 2011). This could result from an omitted distinction between sporadic patients with single lesions from those with multiple ones. In relation to our data, we detected no mutations in sporadic patients with single lesions; instead, a novel *CCM1* c.596C>G (p.Ser199*) nonsense mutation was found in one of the three sporadic patients with multiple lesions. The percentage of CCM gene-negative patients would have been lower if we had considered sporadic cases with single lesions together with sporadic cases with multiple CCMs.

The pathogenesis of sporadic cases with multiple lesions is truly controversial; although these may really be familial forms improperly diagnosed because of incomplete penetrance, the mutation rate is lower in sporadic cases with multiple lesions compared to familial ones (Labauge et al. 2007). Another hypothesis may be the onset of de novo mutations. In our cases, only in one patient did we detect the novel germline mutation c.596C>G (p.Ser199*) in exon 8 of *CCM1*. It introduces a stop codon causing a truncated krit1 protein of 198 amino acids vs the 736 amino acids of full-length protein.

Among sporadic patients with single lesions, several SNPs were detected (Table 2); HSF in silico analysis showed a high probability of splicing alteration for rs2304689 (c.205-36A>G), rs11552377 (c.358G>A p.V120I) and rs2289367 (c.915G>A p.Thr305Thr) alleles. Moreover, we previously reported an association study in which statistically significant differences were shown in rs2304689 and rs2289367 frequencies between patients and controls (D'Angelo et al. 2012).

Regarding familial cases, in family 2, another novel missense mutation, c.580T>G (p.C194G) in exon 5 of CCM2 gene, was detected, not in the proband, who harbours a single mutation in the CCM1 gene, c.601C>G (p.Q201E; p.Q201RfsX2), but in the healthy husband, in the healthy son and, together with c.601C>G in CCM1 gene, in the affected daughter (Fig. 1b); however, she is asymptomatic.

The c.580T>G was also detected in the 18-year-old brother and in the father, both healthy at the moment. Moreover, in the proband, CCM lesions were diagnosed at the age of 24, while in her daughter, carrier of two alterations (the same as the proband, p.Q201RfsX2 and the novel p.C194G), multiple CCM lesions were diagnosed at the age of 9. Therefore, we cannot exclude that the coexistence of the two mutations might be linked to early onset of CCM lesions.

Although we suspect that the p.C194G in malcavernin protein may be associated with low penetrance in the healthy status of the proband's husband and son, to date, this cannot be considered a disease-associated mutation. Incomplete penetrance and variable expressivity are peculiar to familiar forms.

In our cases, incomplete penetrance of CCMs is once again proven in the third family that we examined. The same c.601C>G (p.Q201E; p.Q201RfsX2) variant in exon 8 of *CCM1* gene was detected in the proband, in his father (asymptomatic) and in his daughter with no CCM lesions. Interfamilial and intrafamilial variable expressivity among subjects who carry the same mutation makes it hard to predict genotype–phenotype correlations for diagnostic purposes on the progeny of affected patients. Moreover, clinical heterogeneity of CCM disease could also be attributed to modifier gene activity (Choquet et al. 2014).

No mutations were, instead, detected in families 1 and 4; the two *CCM2* gene SNPs rs2304689 (c.205-36A>G) and rs11552377 (c.358G>A, p.V120I) were found in the probands of the two families, respectively. Potential pathogenic roles of these SNPs were previously described. Regarding rs2304689, we hypothesise that, in a heterozygous condition, it is not sufficient to develop a familial syndrome, despite it being a risk factor and can alter splicing. One hypothesis is involvement of other/s still undetected gene/s.

For rs11552377, our attention was focused on the homozygous status of the proband, the only living affected member of the family. This polymorphism, falling in the PTB domain of malcavernin, changes the folding of the protein and interferes with Krit1/malcavernin/pdcd10 complex formation. Therefore, we think that the homozygous condition for *CCM2* gene c.358G>A polymorphism can lead to a *CCM2* loss of function, a deleterious genetic state playing a role in the onset of CCM disease.

In conclusion, we performed molecular analysis for the three CCM genes on a cohort of 19 Italian patients affected by CCM. Based on genetics, the patients were classified into

familial cases (4 families), sporadic cases (12 patients) and sporadic cases with multiple lesions (3 patients). Several polymorphisms were found in sporadic patients, confirming their role as risk factors. Moreover, we detected a novel *CCM1* nonsense mutation and a novel *CCM2* missense mutation; the role of the latter in CCM pathogenesis should be validated. Increasing the already large collection of CCM mutations represents a valid tool for geneticists and for updating genetic tests. Early identification of asymptomatic CCM patients, their follow-up and the risk assessment of lesion development in family members could reduce cerebral damage due to late diagnosis.

Compliance with Ethical Standards DNA blood samples were obtained with written informed consent from all patients and normal controls. For underage patients, consent was obtained from the parents. Study protocol followed the guidelines of the local ethics committee, and the investigation was conducted with the ethical requirements defined in the Helsinki Declaration.

Conflict of Interest The authors declare that they have no conflicts of interest.

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