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ORIGINAL ARTICLE

CCM molecular screening in a diagnosis context: novel unclassified variants leading to abnormal splicing and importance of large deletions

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Abstract Loss of function mutations in *CCM1*/KRIT1, *CCM2*/MGC4607, and *CCM3*/PDCD10 gene are identified in about 95 % of familial cases of cerebral cavernous malformations and 2/3 of sporadic cases with multiple lesions. In this study, 279 consecutive index patients referred for either genetic counseling or for diagnosis of cerebral hemorrhage of unknown etiology were analyzed for the three cerebral cavernous malformations (CCM) genes by direct sequencing and quantitative studies, to characterize in more detail the mutation spectrum associated with cerebral cavernous malformations and to optimize CCM gene screening. Analysis of the cDNA was performed when possible to detect the consequences of the genomic variations. A pathogenic mutation was identified in 122 patients. *CCM1* was mutated in 80 patients (65 %), *CCM2* in 23 (19 %), and *CCM3* in 19 (16 %). One hundred patients harbored a loss of function point mutation (82 %) and 22 had a large deletion (18 %). Novel unclassified variants were detected in the patients among whom six led to a splicing defect. The causality of three missense variants that did not modify the splicing could not be established. These findings expand the CCM mutation spectrum and highlight the importance of screening the three CCM genes with both direct sequencing and a quantitative method. In addition, six new unclassified variants were shown to be deleterious because they led to a splicing defect. This underlines the necessity of the cDNA analysis when an unknown variant is detected.

Keywords Cerebral cavernous malformations · CCM · KRIT1 · MGC4607 · PDCD10 · Unclassified variants

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Introduction

Cavernous angioma, also known as cerebral cavernous malformations (CCM-OMIM# 116860) are vascular malformations mostly located within the CNS and characterized by abnormal enlarged capillary cavities without

Table 1 CCM point mutations identified by direct sequencing

Gene	Exon/ intron	Nucleotidic change	cDNA analysis	Predicted effect on protein	Number of patients	Classification
CCM1	IVS4	c.103-1G>T	Not performed	Unknown	2	Pathogenic
CCM1	Exon 5	c.151_154del	c.151_154del	p.K51FfsX13	4	Pathogenic
CCM1	Exon 6	c.268C>T	c.268C>T	p.R90X	1	Pathogenic
CCM1	Exon 7	c.457dup	c.457dup	p.T153NfsX10	1	Pathogenic
CCM1	Exon 8	c.535C>T	c.535C>T	p.R179X	7	Pathogenic
CCM1	Exon 8	c.681delC	Not performed	p.C228VfsX17	1	Pathogenic
CCM1	Exon 8	c.702dupT	Not performed	p.G235WfsX12	1	Pathogenic
CCM1	IVS8	c.730-2A>G	c.730_845del (exon 9 deletion)	p.V244GfsX7	1	Pathogenic
CCM1	Exon 9	c.814C>T	Not performed	p.Q272X	1	Pathogenic
CCM1	IVS9	c.845+1_845+4del	c.730_845del (exon 9 deletion)	p.V244GfsX7	2	Pathogenic
CCM1	Exon 10	c.858G>A	c.858G>A	p.W286X	1	Pathogenic
CCM1	Exon 10	c.880C>T	c.880C>T	p.R294X	1	Pathogenic
CCM1	Exon 10	c.902C>G	Not performed	p.S301X	1	Pathogenic
CCM1	Exon 10	c.922C>T	c.922C>T	p.L308F	1	Unknown
CCM1	IVS10	c.989+8G>C	Not performed	Unknown	1	Probably polymorphism
CCM1	Exon 11	990G>A	990G>A	p.W330X	1	Pathogenic
CCM1	Exon 11	c.999_1000delinsT	Not performed	p.L333NfsX9	1	Pathogenic
CCM1	Exon 11	c.1019_1020insTT	Not performed	p.L340FsX3	1	Pathogenic
CCM1	IVS11	c.1146+2T>A	c.990-120_990-49ins990_1146del (exon 11 deletion + ins72 nt)	p.W330X	1	Pathogenic
CCM1	Exon 12	c.1162C>T	c.1162C>T	p.Q388X	1	Pathogenic
CCM1	Exon 12	c.1175dupC	c.1175dupC	p.L393IfsX5	1	Pathogenic
CCM1	Exon 12	c.1201_1204del	Not performed	p.Q401TfsX10	1	Pathogenic
CCM1	Exon 13	c.1255-1_1256delGTA	c.1255_1264del	p.Y419FfsX15	1	Pathogenic
CCM1	Exon 13	c.1267C>T	c.1267C>T	p.R423X	3	Pathogenic
CCM1	Exon 13	c.1306_1310del	c.1306_1310del	p.L436AfsX4	1	Pathogenic
CCM1	Exon 13	c.1362_1363delTC	c.1362_1363delTC	p.Q455RfsX24	3	Pathogenic
CCM1	Exon 13	c.1391G>A	c.1391G>A	p.W464X	1	Pathogenic
CCM1	Exon 14	c.1414_1444del	c.1414_1444del	p.L472NfsX13	1	Pathogenic
CCM1	Exon 14	c.1460G>A	c.1460G>A	p.W487X	1	Pathogenic
CCM1	Exon 14	c.1498C>T	Not performed	p.Q500X	1	Pathogenic
CCM1	Exon 14	c.1513C>T	Not performed	p.Q505X	1	Pathogenic
CCM1	IVS14	c.1564-14C>G	c.1564_1730del (exon 15 deletion)	p.I522X	1	Pathogenic
CCM1	Exon 15	c.1595_1596del	c.1595_1596del	p.P532X	1	Pathogenic
CCM1	Exon 15	c.1608_1633del26	c.1608_1633del26	p.R536SfsX4	2	Pathogenic
CCM1	Exon 15	c.1667delG	c.1667delG	p.S556IfsX6	1	Pathogenic
CCM1	Exon 15	c.1684C>T	Not performed	p.Q622X	1	Pathogenic
CCM1	Exon 15	c.1688_1689delAT	c.1688_1689delAT	p.Y563WfsX4	1	Pathogenic
CCM1	IVS15	c.1730+3A>T	c.1564_1730del (exon 15 deletion)	p.I522X	1	Pathogenic
CCM1	IVS15	c.1730+4_1730+7del	c.1564_1730del (exon 15 deletion)	p.I522X	4	Pathogenic
CCM1	IVS15	c.1730+5G>A	c.1564_1730del (exon 15 deletion)	p.I522X	2	Pathogenic
CCM1	IVS15	c.1731-2A>C	c.1731_1732ins23 (insertion of 23 nt between exons 15 and 16)	p.N77KsX13	1	Pathogenic
CCM1	Exon 16	c.1782A>G	c.1782A>G	no change	1	Polymorphism
CCM1	Exon 17	c.1825dupA	c.1825dupA	p.S609KfsX4	1	Pathogenic
CCM1	Exon 17	c.1977T>A	Not performed	p.Y657X	1	Pathogenic
CCM1	Exon 17	c.2025G>C	c.1819_2025del (exon 17 deletion)	p.N607_K675del	1	Pathogenic
CCM1	IVS17	c.2026-12A>G	c.2026_2142del (exon 18 deletion)	p.L677_A715del	1	Pathogenic
CCM1	Exon 18	c.2054_2058del	Not performed	p.C685YfsX8	1	Pathogenic

Table 1 (continued)

Gene	Exon/ intron	Nucleotidic change	cDNA analysis	Predicted effect on protein	Number of patients	Classification
CCM1	Exon 18	c.2058dupT	Not performed	p.M687YfsX8	1	Pathogenic
CCM1	IVS18	c.2142+1G>C	c.2026_2142del (exon 18 deletion)	p.L677_A715del	2	Pathogenic
CCM1	IVS18	c.2142+2T>G	c.2026_2142del (exon 8 deletion)	p.L677_A715del	1	Pathogenic
CCM2	Exon 1	c.30G>A	Only one full length transcript	Absence	1	pathogenic
CCM2	IVS1	c.30+5_30+6delinsTT	Only one full length transcript	Absence	1	Pathogenic
CCM2	IVS1	c.30+5G>A	Only one full length transcript	Absence	2	Pathogenic
CCM2	Exon 2	c.55C>T	c.55C>T	p.R19X	3	Pathogenic
CCM2	Exon 3	c.205-2A>C	c.205_211del	p.Y69VfsX3	1	Pathogenic
CCM2	Exon 3	c.273delC	c.273delC	p.F91LfsX2	1	Pathogenic
CCM2	Exon 4	c.338T>C	c.338T>C	p.L113P	1	Unknown
CCM2	Exon 5	c.540-541insGG	Not performed	p.S181GfsX34	1	Pathogenic
CCM2	Exon 5	c.546delGinsTA	Not performed	p.S183IfsX53	1	Pathogenic
CCM2	Exon 5	c.593T>G	c.593T>G	p.L198R	1	Pathogenic
CCM2	Exon 6	c.642_655del	c.642_655del	p.Q215CfsX16	1	Pathogenic
CCM2	Exon 7	c.775delG	Not performed	p.E259RfsX33	1	Pathogenic
CCM2	Exon 7	c.780delC	Not performed	p.Y261TfsX31	1	Pathogenic
CCM2	Exon 8	c.804-5C>T	Normal	No change	1	Polymorphism
CCM2	Exon 10	c.1250_1251delAG	Not performed	p.E417GfsX3	1	Pathogenic
CCM2	Exon 10	c.1316T>C	c.1316T>C	p.M439T	1	Unknown
CCM3	Exon 3	c.58A>G	c.58A>G	p.M20V	1	Unknown
CCM3	Exon 3	c.81delT	c.81delT	p.P28LfsX6	1	Pathogenic
CCM3	IVS3	c.97-5T>C[a]	Normal	No change	1	Polymorphism
CCM3	IVS3	c.97-2A>G	c.97_150del (exon 4 deletion)	p.L33_K50del	1	Pathogenic
CCM3	Exon 4	c.103C>T	c.103C>T	p.R35X	2	Pathogenic
CCM3	IVS4	c.151-1G>C	c.151_268del (exon 5 deletion)	p.A51SfsX3	1	Pathogenic
CCM3	Exon 5	c.129_138del	Not performed	p.R45SfsX17	1	Pathogenic
CCM3	Exon 5	c.175dupA	c.175dupA	p.T59NfsX17	1	Pathogenic
CCM3	Exon 5	c.213C>T	c.213C>T	No change	1	Polymorphism
CCM3	Exon 5	c.372_373delAG	Not performed	p.R124SfsX12	1	Pathogenic
CCM3	IVS5	c.269-1G>A	c.151_395del (exon 6 deletion)	p.E90GfsX3	1	Pathogenic
CCM3	Exon 6	c.394_395delinsGATT	mRNA degradation	Absence	1	Pathogenic
CCM3	IVS6	c.396-31_396-13delinsA	c.396_474del (exon 7 deletion)	p.N133HfsX10	1	Pathogenic
CCM3	Exon 7	c.418G>T	c.396_474del (exon 7 deletion)	p.N133HfsX10	1	Pathogenic
CCM3	IVS7	c.474+1G>C	Not performed	Unknown	1	Pathogenic
CCM3	Exon 8	c.496G>T	c.496G>T	p.E166X	1	Pathogenic
CCM3	Exon 9	c.586C>T	c.586C>T	p.R196X	2	Pathogenic

Nucleotide numbering is designed according to the cDNA in which the "A" of the start codon is nucleotide 1. Reference sequences are NM_004912.3 for *CCM1*, NM_031443.3 for *CCM2*, and NM_007217.3 for *CCM3*

[a] Variant identified in a patient harbouring also a deletion of exon 5 of *CCM2*

intervening brain parenchyma [1]. CCM can occur sporadically or in an autosomal dominant fashion, with variable expression and incomplete penetrance. The incidence in the general population has been evaluated to roughly 0.5 % [2]. Clinical symptoms typically appear between 20 and 30 years of age. They include recurrent headaches, focal neurological deficits, hemorrhagic stroke, and seizures [3, 4], but CCM can also be asymptomatic. Familial forms of CCM have been attributed to mutations in three genes, *CCM1*/KRIT1 (krev interaction trapped 1) [MIM# 604214] [5, 6], *CCM2*/MGC4607 (encoding a protein named malcavernin) [MIM# 607929] [7, 8], and *CCM3*/PDCD10 (programmed cell death 10) [MIM# 609118] [9]. Over 90 % of familial CCM patients harbor a mutation in one of the three known CCM genes [10, 11]. Large rearrangement screening methods allow now to find mutations that are not detected

by sequencing [12–16]. Our objective was to establish in more detail the mutation spectrum associated with cerebral cavernous malformations in a large cohort of consecutive patients referred for molecular diagnosis and screened for both point mutations and large rearrangements.

Material and methods

Patients

Two hundred seventy-nine unrelated patients were referred to our laboratory by French hospitals between January 2006 and December 2010 for CCM genes screening. Molecular diagnosis was requested either for genetic counseling in patients showing typical CCM lesions on cerebral MRI, or for patients in whom the diagnosis of CCM was discussed, based on clinical history of cerebral hemorrhages of unknown etiology. Written informed consent was provided by the patients themselves when aged above 18 and by both parents for patients younger than 18 years. One hundred forty-four healthy controls were included for the testing of unknown variants.

Sequencing

Sequencing of the three CCM genes was performed using specific primer pairs amplifying the 16 coding exons of *CCM1* (transcript reference NM_004912.3), the 10 exons of *CCM2* (transcript reference NM_031443.3) and the 7 coding exons of *CCM3* (transcript reference NM_007217.3). Primers are provided in Electronic supplementary material (ESM) Table 1. Sequence products were run on an automated sequencer (ABI 3130, Applied Biosystems, Foster City, CA, USA) and data were analyzed with Seqscape V.2.6 software (Applied Biosystems).

QMPSF conditions

The quantitative multiplex PCR of short fluorescent fragments (QMPSF) method is described in detail elsewhere [17]. Oligonucleotide primer pairs for amplification of short fluorescent fragments corresponding to the 19 exons of *CCM1*, the 10 exons of *CCM2* and the 10 exons of *CCM3* were designed using the Primer Premier Software (Primer Biosoft International, Palo Alto, CA, USA). Primers are provided in ESM Table 1. Four multiplex PCR were set up to check the copy number of all exons of CCM genes. Forward primers were 5′-labelled with the 6-FAM fluorochrome. Each multiplex PCR set contained a control primer set that amplified a short sequence of hydroxymethylbilane synthase gene (*HMBS*), not involved in CCM. Amplicon sizes ranged between 120 and 250 bp. Reactions were done as described previously [18].

PCR products were separated by capillary electrophoresis on an ABI 3130 Genetic Analyzer (Applied Biosystems). Quantification of the area of peaks corresponding to the tested exons and to the internal *HMBS* control was determined using GeneMapper analysis software version 4.0 (Applied Biosystems). The copy number of each tested exon was expressed as the ratio: (area of the peak corresponding to a tested exon for the patient/area of the peak corresponding to *HMBS* for the patient)/(area of the peak corresponding to a tested exon for the control DNA/area of the peak corresponding to *HMBS* for the control DNA). Calculation of ratios was done by the software. A ratio close to 1 is obtained when two copies of the exon are present and a ratio close to 0.5 when only one copy of the tested exon is present (hemizygous DNA).

Long-range PCR and sequencing

Long-range PCR amplifications were performed with the TripleMaster® PCR System (Eppendorf AG, Hamburg, Germany) according to the manufacturer recommendations. Sequencing was performed using standard protocols, on an ABI 3130 DNA analyzer (Applied Biosystems).

cDNA analysis

Total RNAs were extracted from patients' peripheral blood leukocytes either with TRIzol (Invitrogen) or with the PreAnalytix system (Qiagen) according to the manufacturer's procedures. RT-PCR was done using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Sequencing of RT-PCR amplification products was performed using standard protocols on an ABI 3130 DNA analyzer (Applied Biosystems). Primers are provided in ESM Table 1.

In silico analysis

The prediction of the pathogenicity of the mutations was assessed using Human Splicing Finder and MaxEntScan at http://www.umd.be/HSF [19]. The MaxEntScan framework is based on the maximum entropy principle and uses large datasets of human splice sites and takes into account adjacent and non-adjacent dependencies. Human Splicing Finder includes several matrices to analyze splice sites and splicing silencers and enhancers.

Results

Point mutations detected by direct sequencing

Eighty-three different heterozygous variants were identified (Table 1): 20 nonsense mutations, 31 insertions/deletions leading to a frameshift, 12 AG/GT consensus splice site mutations,

Table 2 CCM rearrangements identified by QMPSF

Gene	Exons	Nucleotidic change	cDNA analysis	Other test performed	Predicted effect on protein	Number of patients
CCM1	1–19	c.-892-?_2211+?del	Consequence undetectable		Absence of protein	2
CCM1	1–17	c.-892-?_2025+ ?del	Consequence undetectable		Absence of protein	1
CCM1	1–5	c.-892-?_262 + ?del	Consequence undetectable		Absence of protein	1
CCM1	1–3	c.-892-?_-3+?del	Consequence undetectable		Absence of protein	2
CCM1	11	c.990-?_1146 + ?del	c.990_1146del (exon 11)		p.W330CfsX3	1
CCM1	11–14	c.990-?_1563 + ?del	c.990_1563del (exons 11–14)		p.W330X	1
CCM1	11–17	c.990-?_2025 + ?del	c. 990_2025del (exons 11–17)		p.Y331LfsX31	1
CCM1	15	c.1564-?_1730+?del	c.1564_1730del (exon 15)		p.I522X	1
CCM1	15–17	c.1564-?_2025+?del	c.1564_2025del (exons 15–17)		p.I522_K675del	1
CCM1	18–19	c.2026-?_2211+?del	Consequence undetectable		Unknown	1
CCM2	2	c.31-?_204+?del	c.31_204del (exon 2)		p.P11_K68del	2
CCM2	3–4	c.205-?_472+?del	c.250_472del (exons 3–4)		p.Y69PfsX56	1
CCM2	5	c.472-3096_609+1031del	Not performed	Long-range PCR	p.A158GfsX32	1
CCM2	5	c.472-1993_609+287del	Not performed	Long-range PCR	p.A158GfsX32	1
CCM2	6	c.610-?_745+?del	c.610_745del (exon 6)		p.V204MfsX43	1
CCM2	6–9	c.610-843_1054+1045del	c.610_1054del (exons 6–9)	Long-range PCR	p.A205X	1
CCM3	1–9	c.-398-?_639+?del	Consequence undetectable		Absence of protein	1
CCM3	1B[a]-3	c.-116-?_96+?del	c.-116_96del (exons 1B[a]-3)		Absence of protein	1
CCM3	7–9	c.396-?_639+?del	Consequence undetectable		Unknown	1

[a] PDCD10 exon 1B corresponds to the first exon of the alternative transcript NM_145860

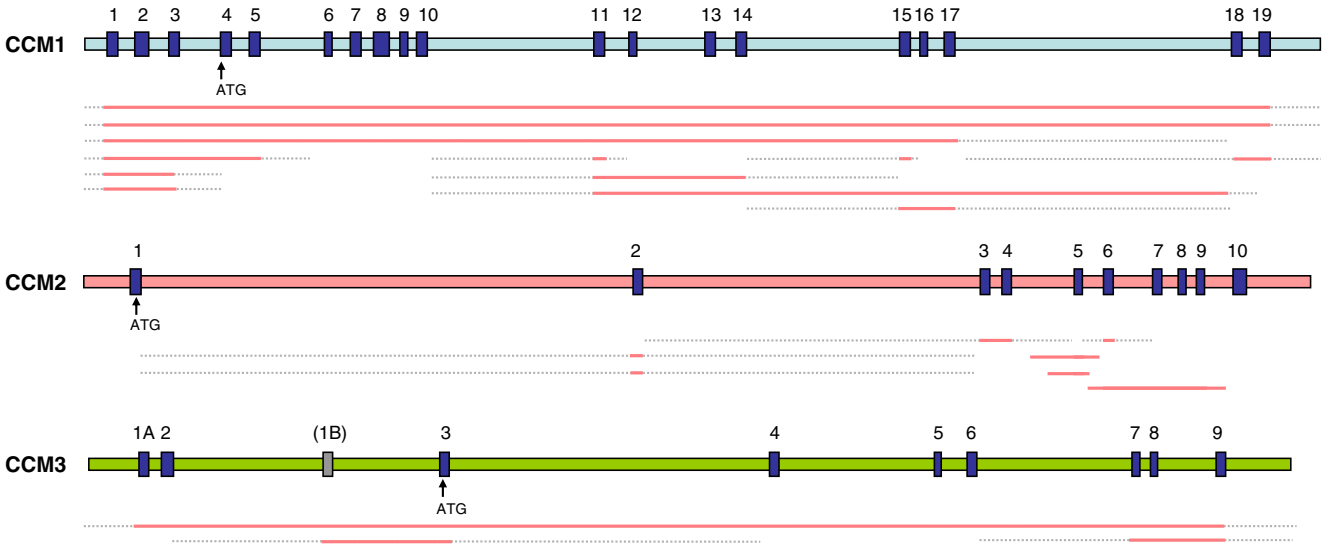

Fig. 1 Schematic representation of the rearrangements detected in the three CCM genes in this study. The *full red lines* indicate the minimum size of the deletions. The *dotted gray lines* indicate the maximum size of the deletions when the break points are not identified

5 missense variants, 4 synonymous variants, and 11 intronic variants located outside the invariant AG/GT splice sites. Loss of function mutations (nonsense mutations, frameshift mutations, and AG/GT consensus splice site mutations) are considered as typical pathogenic variants. The synonymous mutation *CCM2*/c.30G>A, the missense mutation *CCM2*/L198R and the three intronic variants *CCM1*/c.1730+4_1730+7del, *CCM1*/c.2026-12A>G, and *CCM2*/c.30+5_30+6delinsTT had previously been reported in CCM patients [7, 8, 20, 21]. The other variants were novel. The missense mutation *CCM2*/p.L113P was present in three relatives harboring multiple CCM lesions. The three other missense mutations (*CCM1*/p.L308F, *CCM2*/M439T, and *CCM3*/p.M20V) were identified in sporadic CCM patients with multiple lesions for whom parents were not available. The missense variants were absent in 288 control chromosomes.

Large rearrangements detected with QMPSF

QMPSF analysis detected a large deletion in 22 patients, 12 in *CCM1*, 7 in *CCM2*, and 3 in *CCM3*. Deletions varied in size, ranging from a single exon to the whole gene (Table 2 and Fig. 1). Whole gene deletion was found in three patients. *CCM2*/exon 2 deletions had already been reported [13]. It leads to a shorter in frame alternative normal transcript, but the absence of the full length transcript has been shown to be deleterious by preventing the formation of a *CCM1*/*CCM2*/*CCM3* protein complex [11]. Two patients had a deletion of *CCM2*/exon 5; long-range PCR products sequencing showed that the breakpoints were different in these two cases. Two deletions encompassed the ATG start codon (*CCM1*/deletion of exons 1–5 and *CCM3*/deletion of exons 1B-3) and two patients harbored a deletion of the three noncoding exons of *CCM1*. This deletion was not present in 240 control chromosomes and no other mutation was found in the three CCM genes for these two patients. Both of them had typical CCM lesions on cerebral MRI. Clinical data on relatives were not contributory. These deletions are likely responsible of the CCM phenotype in these patients.

cDNA analysis

The consequences on cDNA have been investigated for 61 of the 83 distinct genomic variants and for 11 intragenic large deletions (Tables 1 and 2). Surprisingly, the cDNA of the patient showing an apparently nonsense *CCM3* mutation c.418G>T/p.E140X located in exon 7 was deleted of whole exon 7. The *CCM3*/c.394_395delinsGATT cDNA allele was nearly undetectable by sequencing. All the other nonsense and ins/del exonic mutations identified in genomic DNA were detectable in the cDNA sequence and did not lead to abnormal splicing.

The five missense variants (*CCM1*/p.L308F, *CCM2*/p.L198R, p.L113P, p.M439T, and *CCM3*/p.M20V) were found in the corresponding cDNA. Among the four synonymous variants, the *CCM1*/c.2025G>C variant led to the abnormal splicing of exon 17. The *CCM2*/c.30G>A variant was suspected to lead to an aberrant splicing since sequencing of the cDNA obtained with primers located in exon 1 and 10 did not show the mutation, as well as the other polymorphisms that were detectable on genomic DNA sequences. Nevertheless, the RT-PCR product 5–10 (obtained with a forward primer within exon 5 and a reverse primer within exon 10) showed heterozygosity in exon 8 for one of the patients, in favor of the presence of an alternative transcript. The two other

Table 3 "Unclassified variants" identified in CCM genes

Gene	Location	Nucleotidic change	cDNA	Predicted effect on protein	Number of patients	Classification	Previously reported
CCM1	Exon 10	c.922C>T	c.922C>T	p.L308F	1	Unknown	No
CCM1	IVS10	c.989+8G>C	Not done	Unknown	1	Unknown	No
CCM1	IVS14	c.1564-14C>G	c.1564_1730del (deletion of exon 15)	p.I522X	1	Pathogenic	No
CCM1	IVS15	c.1730+3A>T	c.1564_1730del (deletion of exon 15)	p.I522X	1	Pathogenic	No
CCM1	IVS15	c.1730+4_1730+7del	c.1564_1730del (deletion of exon 15)	p.I522X	4	Pathogenic	Yes [20]
CCM1	IVS15	c.1730+5G>A	c.1564_1730del (deletion of exon 15)	p.I522X	2	Pathogenic	No
CCM1	Exon 16	c.1782A>G	c.1782A>G	No change	1	Polymorphism	No
CCM1	Exon 17	c.2025G>C	c.1819_2025del (deletion of exon 17)	p.N607_K675del	1	Pathogenic	No
CCM1	IVS17	c.2026-12A>G	c.2026_2142del (deletion of exon 18)	p.L677_A715del	1	Pathogenic	Yes [20]
CCM2	Exon 1	c.30G>A	Only one full length transcript	Unknown	1	Pathogenic	Yes [13]
CCM2	IVS1	c.30+5_30+6delinsTT	Only one full length transcript	Unknown	1	Pathogenic	Yes [21]
CCM2	IVS1	c.30+5G>A	Only one full length transcript	Unknown	2	Pathogenic	No
CCM2	Exon 4	c.338T>C	c.338T>C	p.L113P	1	Unknown	No
CCM2	Exon 5	c.593T>G	c.593T>G	p.L198R	1	Pathogenic	Yes [8]
CCM2	Exon 8	c.804-5C>T	Normal	No change	1	Polymorphism	No
CCM2	Exon 10	c.1316T>C	c.1316T>C	p.M439T	1	Unknown	No
CCM3	Exon 3	c.58A>G	c.58A>G	p.M20V	1	Unknown	No
CCM3	IVS3	c.97-5T>C	Normal	No change	1	Polymorphism	No
CCM3	Exon 5	c.213C>T	c.213C>T	No change	1	Polymorphism	No
CCM3	IVS6	c.396-31_396-13delinsA	c.396_474del (deletion of exon 7)	p.D133HfsX10	1	Pathogenic	No

variants (*CCM1*/c.1782A>G and *CCM3*/c.213C>T) are likely polymorphisms since no aberrant transcript was detected. ESM Table 2 gives a meta analysis data on consequences on splicing of the exonic apparently missense or silent mutations reported in CCM patients.

The cDNA analysis of nine patients carrying an intronic variant located outside the invariant AG/GT showed the presence of a shorter transcript caused by an abnormal splicing (Table 3); these mutations were considered as pathogenic. Two *CCM2* intronic variants localized close to exon 1 donor splice site (c.30+5_30+6delinsTT and c.30+5G>A found twice) were suspected to prevent classical splicing because the RT-PCR product showed lost of heterozygosity for different polymorphisms detected in the genomic DNA. These two variants were not present in 280 control chromosomes. At last, the variant *CCM2*/c.804-5C>T and the variant *CCM3*/c.97-5T>C (found in a patient harboring also a deletion of *CCM2* exon 5) were considered as probable polymorphisms since no aberrant transcript was detected. Data are summarized in Table 3. cDNA sequencing was also performed for eight additional patients harboring AG/GT splice site mutations. Among the whole splicing defects (eight in AG/GT and six outside AG/GT), 11 lead to the deletion of the proximal exon and 3 lead to the use of a cryptic splice site. Result of the in silico analyses are given in ESM Table 3.

Discussion

This study reports a large series of patients referred in a diagnostic context for CCM molecular analysis. A mutation considered as pathogenic was found in 122 of 279 of the patients. This mutation rate is lower than the one reported in research CCM series. Indeed, many patients were referred because of cerebral hemorrhages of unknown etiology and did not have typical CCM lesions on cerebral MRI. They are therefore expected to be affected by other conditions.

Among the 122 patients harboring a mutation considered as deleterious, *CCM1* was involved in 80 cases (65.6 %), *CCM2* in 23 cases (18.8 %), and *CCM3* in 19 cases (15.6 %). One hundred patients had a point mutation (82 %) and 22 had a large deletion (18 %), limited to one or a few exons in 19 cases and complete in three cases. Twelve deletions concerned *CCM1* (54 %), seven *CCM2* (32 %), and three in *CCM3* (14 %; Fig. 2). The distribution of the deletions is different from the one reported in the American and in the Italian series [13, 14] and no recurrent deletion has been detected in our cohort.

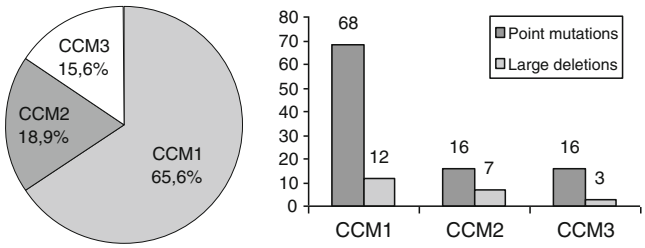

Fig. 2 Distribution of the three CCM genes mutations in the CCM-mutated patients group

The majority of mutations introduce premature termination codon into the mRNA, most likely leading to mRNA decay. Seven *CCM1* mutations were in frame deletions that result from a splicing defect or a large deletion. It may point out important functional domains of the protein; however, in-frame deletions are also known to lead to protein instability and degradation. The in-frame deletion of *CCM2* exon 2 found in two patients in our series and already reported in two others CCM patients [13] emphasizes the importance of the full length transcript for vascular integrity.

Fourteen new unclassified variants (intronic variants outside the consensus AG/GT splice site or exonic missense or silent variants) were identified in patients for whom cDNA analysis was performed. Six of them led to an abnormal splicing and were considered as pathogenic; four variants did not modify the splicing, did not change any amino acid and were considered as probable polymorphisms. Four unreported variants led to an amino acid change but splicing was unchanged. They all involve a conserved amino acid and were absent in 280 control chromosomes. The missense mutation *CCM2* L198R previously reported in a family in which it co-segregated with the presence of CCM was shown to abolish the interaction between *CCM1* and *CCM2* [22] and is up to now the only proven pathogenic missense mutation that does not induce aberrant splicing. The clinical significance of the missense variants found in our series remains to be established.

Although in silico analysis is not sufficient to definitely conclude on the effect of an unknown variant on RNA splicing, results obtained in our cohort using the combination of MaxEntScan and Splice Site finder show that they most often provide good splicing predictions. Houdayer et al. proposed an analysis pipeline to check the unknown variants with a cutoff ratio of 15 % for MaxEntScan and 5 % for Splice Site finder like [23]. Using those cutoff, predictions were exact in 8/10 variants leading to an abnormal splicing, and to 8/8 variants not leading to an abnormal splicing. Experimental analysis of the effect on splicing is however needed in a diagnosis context.

The systematic use of a semi quantitative technique as QMPSF or MLPA, in addition to DNA sequencing, has substantially increased the detection rate of CCM mutations. Nevertheless, cDNA sequencing is required to test the consequences on splicing of unknown variants and is also recommended to confirm large intragenic deletions, especially when they involve only one exon.

Significant progress has been made in the understanding of the mechanisms leading to CCM lesions in transgenic mouse models and preclinical trials are already ongoing. It is therefore important to identify precisely the molecular anomaly in the CCM patients for the constitution of homogeneous cohorts for future clinical trials and this study shows that both genomic DNA and cDNA analyses are needed to reach a good sensitivity. In addition, molecular diagnosis remains helpful in atypical cases showing cerebral hemorrhage but no typical CCM lesions on MRI.

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