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ORIGINAL COMMUNICATION

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Study of cerebral cavernous malformation in Spain and Portugal

High prevalence of a 14 bp deletion in exon 5 of *MGC4607* (*CCM2* gene)

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Abstract *Objective* We aimed to study clinical, radiological and molecular genetic features of patients with cerebral cavernous malformations (CCMs) from the Iberian Peninsula. *Methods* We screened *Krit1(CCM1)*, *MGC4607(CCM2)*, and *PDCD10(CCM3)* by systematic SSCP and direct sequencing of coding exons in 48 nuclear families and 30 sporadic cases of CCM from Spain and Portugal. *Results* Screening of CCM patients detected nine different mutations in 19 families. We found four new mutations in *Krit1*. Three of them were caused by either a small insertion or deletion, which lead to frameshift and premature termination codons. We also found a missense L308H mutation located in a highly conserved sequence within the ankyrin domain of *Krit1*. In *CCM2*, we found a redundant 14 bp deletion in exon 5 of *MGC4607* which predicts a truncated protein at residue 230. We did not find mutations in CCM3. *Conclusions* Finding that the 14 bp deletion was present in eleven families from the Iberian Peninsula indicates a high prevalence of this mutation. This redundant CCM2 mutation is worth considering in molecular diagnosis and genetic counselling of cerebral cavernous malformations.

Key words Cerebral cavernous malformations · CCM · MGC4607 · KRIT1 · PDCD10

Introduction

Cerebral Cavernous Malformations (CCMs) are enlarged vascular cavities without intervening brain parenchyma with an estimated prevalence in the general population close to 0.1– 0.5 percent. Single or multiple malformations may develop, which can lead to cerebral haemorrhage, seizures, headache and other neurological symptoms.

Genes responsible for CCM were mapped [4] and located on 7q21.2 *(CCM1, Krit1)* [7, 12], 7p13 (*CCM2, MGC4607*) [8] and 3q26.1 (*CCM3, PDCD10*) [1]. Truncating mutations in *CCM1*, the gene encoding *Krit1* protein, cause hereditary cavernous malformations [7, 12] and more than 100 different mutations have been described. An important feature is the stereotyped nature of the mutations on the putative *Krit1* protein since almost all of them lead to premature stop codons [2, 3].

MGC4607, a gene encoding malcavernin, was reported as the mutated *CCM2* gene responsible for familial CCM [8]. Malcavernin contains a phosphotyrosine binding domain (PTB) similar to the *Krit1* binding partner ICAP1α. *Krit1* interacts with ICAP1α and with malcavernin via their respective PTB [14]. Mutations within *PDCD10* (programmed cell death 10) has been identified at *CCM3* as cause of CCM [1].

Nearly 100 different mutations have been described in CCM patients with a very low degree of redundancy among different nuclear families. An exception is the 742T $\rightarrow$ C transition of the *Krit1* gene, a highly prevalent mutation in the Hispanic-Mexican population [12] which was not detected in patients of Spanish and Portuguese descent [11].

We screened *Krit*, *MGC4607* and *PDCD10* coding exons in 48 CCM nuclear families and 30 sporadic cases from Spanish and Portuguese populations. We found five new mutations, four in the *Krit1* gene, and the other was a highly redundant deletion within the PTB domain in exon 5 of *MGC4607* gene.

Methods

Patients

We collected 78 consecutive families (Spanish (57) and Portuguese (21)), which comprise 143 patients with clinical symptoms and gradient-echo MRI of CCMs. Clinical assessment of patients included information on cerebral haemorrhage, seizures, headache and other neurological symptoms, in addition to MRI. The patients were classified as having hereditary or sporadic CCM on the basis of MRI and familial characteristics. Patients with at least one affected relative and/or multiple cavernous malformations on MRI were classified as having hereditary CCM. MRI showed multiple cavernous malformations in 113 patients from 48 families (38 Spanish and 10 Portuguese). A total of 30 patients (19 Spanish and 11 Portuguese) were considered sporadic forms, since they had single CCMs and no affected relatives. Written consent was obtained from the patients and their relatives included in this study.

Molecular genetic analysis

Genomic DNA was extracted from peripheral blood using standard procedures after salting out proteins. We sought linkage to *CCM1* in a group of families with multiple CCM by the analysis of haplotypes segregation with markers previously described [10]. The initial search of mutations was done by SSCP of coding and partial intronic sequences with primers previously described for *Krit1* [7, 12] and *MGC4607* [5]. We screened *PDCD10* exons with a set of 7 pairs of primers (sequences available upon request). Aliquots of the amplified DNA were electrophoresed in 10% acrylamide under variable denaturing and buffer conditions. Finding of polymorphic bands in the SSCP, suggestive of heterozygous point mutations, was followed by sequencing the DNA fragment. When SSCP shifts were found and the mutation was characterized we did not sequence further exons. When no SSCP shifts were detected, we sequenced the entire CCM1, CCM2 and CCM3 coding exons. Partially automated sequencing of both the sense and antisense strands, extending to 30–40 bases of introns, were done with either 5'-labeled IRD800 primers or with IRD800-labeled ddNTP according to the protocols of the SequiTherm kit (Epicentre Technologies, Madison, Wisconsin). Electrophoretic separation in acrylamide gels and analysis were performed in a LI-COR DNA4000 sequencer. We screened the 742C $\rightarrow$ T transition of exon VI (Q455X), described as a founder mutation in Hispanic-American families, in the 78 index patients and in 90 healthy controls. Exon VI of *Krit1* was PCR amplified and digested with MseI as previously described [10].

The 14 bp deletion in exon 5 of MGC4607 was analyzed by SSCP and restriction polymorphism (RFLP). DNA aliquots from patients and controls were amplified by PCR with primers [5] forward (5'CCTTCCACT GTGCTAAACT) and reverse (5'GGAGC-CAGTTCTGAGTA AG) at 55°C annealing T^a. The PCR products were denatured at 95° for 5 min in a loading buffer mixture containing 50% phormamide. Samples were run under non-denaturing conditions in 10% acrylamide gels at 120 V for 90 min. Gels were sylber green stained (SYBR, Molecular Probes, Oregon). For RFLP study, PCR products were digested overnight at 30°C with ApaI, which recognizes the sequence GGGCCC, and the fragments were separated in 10% acrylamide gels. Additional details are given in the legend to figure 1.

Results

KRIT1 (CCM1)

The segregation of haplotypes in families with multiple CCM provided informative linkage to *CCM1* in 21% (10 out of 48) families. The screening of coding exons by SSCP and sequencing identified four new mutations and three others previously described. Six of them were located within exons 10, 11, 13 and 17. One of these was a single nucleotide substitution (923 T $\rightarrow$ A) causing a missense mutation (L308H). The other five were nonsense small exonic insertions/deletions leading to frameshift and truncated proteins

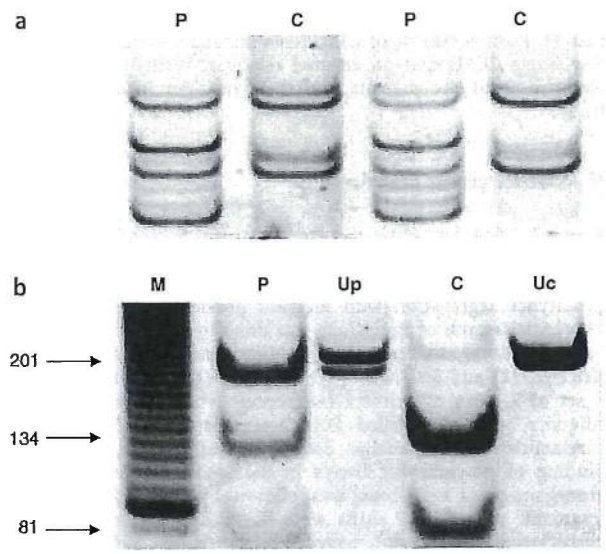

Fig. 1 **A.** *SSCP polymorphism of exon 5 of MGC4607.* DNA aliquots from a group of patients and controls were amplified by PCR with primers as described in Methods. P refers to unrelated CCM patients and C refers to healthy controls. **B.** *Restriction fragment polymorphism of the 14 bp deletion.* Exon 5 of MGC 4607 was amplified and digested under conditions described in Methods. M was a 10 bp ladder molecular weight marker. P and C refer to patient and healthy control respectively. Up and Uc refer to the undigested aliquots of patient and control. The wild type chromosome splits in two fragments of 81 and 134 bp. The mutated chromosome loses the restriction site of ApaI and results in a single fragment of 201 bp. The presence of three bands corresponding to both the wild type and mutated chromosome, 215 and 201 bp respectively, can also be appreciated in the mutated undigested chromosome (Up lane)

at residues 334, 381, 478, 493 and 636. Further, one proband carried a deletion at the splicing donor site in intron 15. MRI of the patients harbouring the new mutations in *Krit1* (CVE59, CVE66, CVE86 and CVE87) showed multiple malformations. None of these mutations were recurrent in our cohort of CCM patients (table 1). Furthermore, Q455X, which was described as a common mutation in Hispano-Mexican families, was not detected in the Iberian Peninsula population studied.

MGC4607 (CCM2)

Non-*Krit1* index patients were studied for SSCP and posterior sequencing of *MGC4607*. We detected two nonsense mutations; one of these, located in exon 2 and consisting of a 4bp-deletion (169–172delAGAC), was found in a proband of Italian descents. The deletion causes a frameshift and a stop codon (57fs58X, see table 2).

We found a very demonstrative SSCP within exon 5 of *MGC460* (see figure 1A) in eleven non-*Krit1* probands. Sequencing of the exon showed a 14bp deletion (554-567delGTGCAGTTGGGCCC) not previously described. This deletion causes frameshift and a stop codon (185fs230X, see table 2). The eleven index patients belong to apparently unrelated families from the Iberian Peninsula. Five of them are from different regions of Spain and the other six are from Portugal. MRI showed single malformations in four out of eleven probands, although in two of them gradient-echo MRI was not possible (see table 2). The other seven patients had multiple malformations. Because of the high recurrence of this mutation, we checked 50 chromosomes of unrelated healthy individuals by both SSCP and restriction fragment polymorphism and, as expected, the 14-bp deletion was absent in chromosomes of the healthy controls.

PDCD10 (CCM3)

We have studied CCM3 by SSCP analysis of all CCM1- and CCM2-negative patients. In addition we screened exon by exon the recently discovered *PDCD10* gene in CCM1- and CCM2-negative index patients. Both SSCP and direct sequencing failed to detect mutations in these patients.

Discussion

Screening of CCM patients by SSCP and sequencing, or direct sequencing of the entire coding exons,

Table 1 Distribution of mutations within Krit1 gene

Pedigree	No. of affected individuals	Ages at MRI	No. of lesions	Exon/Intron	Nucleotide change	Mutation consequence	Predicted amino acid change
CVE59	2	40,39	Multiple	10	923T → A*	Missense	L308H
CVE87	7	61,45,26, 21,20,11,9	Multiple	10	966-969InsCACC*	Frameshift	323fs334X
CVE66	1	68	Multiple	11	1114-1115delCA*	Frameshift	372fs381X
CVE79	1	28	Multiple	13	1360-1363delTCTC	Frameshift	453fs493X
CVE36	1	18	Two	13	1362-1363delTC	Frameshift	454fs478X
CVE10	5	82,46,15, 11,4	Multiple	17	1904InsA	Frameshift	Y635Xfs636
CVE86	3	41,31,17	Multiple	IVS15	IVS15+4delAGTA*	Altered splice site	NA

NA, not analyzed. Asterisks refer to mutation non-reported previously

Table 2 Distribution of mutations within MGC4607 gene

Pedigree	No. of affected individuals	Ages at MRI	No. of lesions	Exon/ Intron	Nucleotide change	Mutation consequence	Predicted amino acid change
CVE8	2	54,22	Single	5	14bpdel554-567	Frameshift	185fs230X
CVE25	3	78,44,35	Multiple	5	14bpdel554-567	Frameshift	185fs230X
CVE35	1	37	Multiple	5	14bpdel554-567	Frameshift	185fs230X
CVE37	2	57,55	Multiple	5	14bpdel554-567	Frameshift	185fs230X
CVP55*	1	43	Single	5	14bpdel554-567	Frameshift	185fs230X
CVP57	1	67	Multiple	5	14bpdel554-567	Frameshift	185fs230X
CVP58	1	61	Multiple	5	14bpdel554-567	Frameshift	185fs230X
CVP75	1	64	Multiple	5	14bpdel554-567	Frameshift	185fs230X
CVP76	1	52	Single	5	14bpdel554-567	Frameshift	185fs230X
CVP77*	1	50	Single	5	14bpdel554-567	Frameshift	185fs230X
CVE84	1	54	Multiple	5	14bpdel554-567	Frameshift	185fs230X
CVE100	1	41	Multiple	2	169-172delAGAC	Frameshift	57fs58X

CVE and CVP refer to pedigrees of Spanish and Portuguese descent respectively. The 169-172delAGAC has been described in a family of Italian descent [8]. Asterisk in CVP55 and CVP77 denotes that gradient-echo MRI was not possible

detected nine different mutations in 19 families which were below the expected number of families linked to CCM1, CCM2 and CCM3 genes. Five of these mutations have not been previously reported. We found four new *Krit1* mutations, one missense and three nonsense. One of these is the 923T → A missense mutation located within the ankyrin domain which changes the leucine in position 308 for a histidine residue. This is a highly conserved aminoacid in paralog and ortholog genes both in vertebrate and invertebrate species. This mutation was not detected in 168 unrelated chromosomes indicating that it is not a common polymorphism in our population. The nature of *Krit1* mutations is highly stereotyped since all of them lead to premature termination codon, including missense mutations that in fact, lead to splicing errors [2, 3]. We failed to find aberrantly spliced Krit1 transcripts in lymphocytes of the CV59 index patient, a carrier of 923T → A missense mutation. The other three mutations predict either truncated proteins or alternative splicing, in coincidence with most previously reported mutations. Q455X, a common mutation described in Hispano-Mexican families [12], was not detected in the Iberian Peninsula population. This could be expected since Spanish patients do not share the Hispano-Mexican haplotype [6].

The analysis of *MGC4607* in our cohort revealed two different mutations. The exon 2 deletion, 169–172delAGAC, was found in a patient of Italian descent and has been previously reported in another population [8]. The other mutation is a 14 bp deletion located in exon 5 within the PTB domain which, to our knowledge, has not been detected in other cohorts. Regarding this new mutation, three points are interesting: i) the high frequency of this mutation is comparable to the recurrent Q455X described in Hispano-Mexican families [12]; ii) single cavernous malformation were found in 36 % of probands (four out of eleven), although gradient-echo MRI was not possible in two of them; iii) Only 27 % of probands (three out of eleven) harbouring the 14 bp deletion had affected relatives. It is worth pointing out that some relatives were not reachable for clinical or radiological study and that gradient-echo MRI was not possible in all patients. Taking into account these considerations and the low clinical and radiological penetrance of CCM we can not discard the existence of other affected relatives.

CCM2 has been studied in different populations with a relative prevalence ranging from 13–20% [5, 8]. In other studies, there are many patients in which CCM-mutations have not been identified after screening the three *CCM* genes [1, 9, 13]. Several explanations have been proposed for this apparent defective detection: i) the existence of mosaicism or genomic deletions non-detected by the exon-by-exon sequencing procedure [1]; ii) the existence of an additional fourth CCM locus close to PDCD10 [9].

Finding that the 14 bp deletion was present in eleven families from the Iberian Peninsula indicates a high prevalence of this mutation. This redundant CCM2 mutation is worth considering in molecular diagnosis and genetic counselling of cerebral cavernous malformations. These results suggest that it would be worthwhile to analyse the CCM mutations reported here, particularly the redundant 14 bp deletion in *MGC4607*, in other cohorts of CCM patients.

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