CCM2 gene polymorphisms in Italian sporadic patients with cerebral cavernous malformation: A case-control study

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Abstract. Cerebral cavernous malformations (CCMs) are vascular lesions of the CNS characterized by abnormally enlarged capillary cavities that can occur sporadically or as a familial autosomal dominant condition with incomplete penetrance and variable clinical expression attributable to mutations in three different genes: *CCM1 (Krit1), CCM2 (MGC4607)* and *CCM3 (PDCD10)*. Among our group of CCM Italian patients, we selected a cohort of sporadic cases negative for mutations in CCM genes. In this cohort, five variants in *CCM2* gene were detected, which proved to be the known polymorphisms in intronic regions (IVS2-36A>G and IVS8 +119 C>T) and in coding sequence (c.157 G>A in exon 2, c.358 G>A in exon 4 and c.915 G>A in exon 8). Therefore, we undertook a case-control study to investigate the possible association of these polymorphisms with sporadic CCMs. The five polymorphisms were identified in 91 CCM sporadic patients and in 100 healthy controls by direct sequencing methods using lymphocyte DNA. Polymorphisms IVS2-36A>G and c.915 G>A showed statistically significant differences in frequencies between patients and controls [(χ^2, 6.583; P<0.037); (χ^2, 14.205; P<0.001)]. The prevalence of the wild-type genotype was significantly lower in the CCM group than in the control sample. Patients with the A/G and G/G genotypes (IVS2-36A>G) had a significant increase for CCM risk (OR, 3.08; 95% CI, 1.5-5.9 and OR, 4.3; 95% CI, 1.4-22.6) and the same was observed for the polymorphism c.915 G> A (genotype G/A OR, 6.1; 95% CI, 3.0-12.6 and genotype A/A OR, 2.79). In addition, the polymorphisms c.358 G>A in exon 4 (χ^2, 15.977; P<0.04) and c.915 G>A in exon 8 (χ^2, 18.109; P<0.02) were significantly associated with different types of symptoms. Haplotype analysis, performed only on polymorphisms c.358 G>A (p.Val120Ile), c.915 G>A (p.Thr305 Thr) and IVS2-36A>G, shows that haplotype GAG (+--) significantly increased among CCM sporadic patients compared to the control group. Significant differences between patients and controls were observed only for IVS2-36A>G and c.915 G>A polymorphisms indicating their possible association with sporadic CCMs and an increased risk of CCM. On the other hand, polymorphisms c.358 G>A and c.915 G>A were associated with a more benign course of the disease. These data were confirmed by the haplotype GAG (+--) frequencies.

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Introduction

Cerebral cavernous malformations (CCMs; OMIM 116860) are vascular abnormalities, predominately localized in the brain, characterized by abnormally enlarged sinusoidal channels, with a simple endothelial lining devoid of elastin and smooth muscle (1). CCMs affect up to 0.5% of the human population (2,3) although only 20-30% of affected individuals develop symptomatic disease (4,5). CCMs have been reported in infants, children, and old people, but the majority of patients present with symptoms between the second and fifth decades. The most common manifestations include seizures, recurrent headaches, hemorrhagic stroke and focal neurological deficits (2,3). CCM can arise sporadically or may be inherited as an autosomal dominant condition with incomplete penetrance and variable clinical expression.

Three genes, *CCM1 (KRIT1), CCM2 (MGC4607)* and *CCM3 (PDCD10)*, encoding the proteins krev/rap1 interacting trapped 1 (Krit1), malcavernin/OSM, and programmed cell death 10 (PDCD10)/CCM3 respectively, are involved in CCM development since mutations in these genes cause loss of function of gene proteins and result in CCM (6-10). CCM proteins are expressed in most tissues, including microvascular endothelium, and regulate diverse aspects of endothelial cell morphogenesis and blood vessel stability, such as cell-cell junctions, cell shape and polarity, or cell adhesion to the extracellular matrix. Krit1 interacts with Rap-1A (Krev-1) (11), a member of the Ras superfamily of GTPases, with integrin cytoplasmic domain associated protein (ICAP1) α and

the phosphotyrosine binding (PTB) domain of malcavernin (10,12).

The malcavernin protein may function as a scaffolding protein for MAP kinases (mitogen-activated protein kinase) that are essential in p38 activation responding to osmotic stress including MEKK (mitogen-activated protein kinase kinase kinase 3) and MKK3 (mitogen-activated protein kinase kinase); it also binds to Rac and actin (13,14).

Finally, PDCD10 forms a complex with Krit1 and malcavernin proteins. It interacts directly with malcavernin independent of Krit1-malcavernin interaction (15). Malcavernin then links Krit1 and PDCD10 which alone would have little affinity with each other (13,16) and directly interacts with PDCD10 in the signalling pathways essential for vascular development and for CCM pathogenesis (13). Moreover, mutations in the PTB domain of malcavernin (amino acids 66-224), on conserved residues critical for NPXY/F motif binding, are deleterious and can lead to inactivation of the protein.

It has been demonstrated, that a noted *CCM2* missense mutation, p.L198R, located in the PTB domain, was able to inhibit the malcavernin interaction with Krit1 (17). Similar results have been observed with p.F217A, an engineered mutation (18). In addition, the latest data on a mutant protein lacking, demonstrated that an intact malcavernin domain, located N-terminally of the PTB domain (amino acid residues 11-68), is essential for Krit1 binding to malcavernin (16).

While familial CCMs are characterized by the presence of multiple lesions, an evolutive condition as assessed by the strong correlation between patient age and number of lesions (3,19,20), sporadic cases mostly have a single lesion (Fig. 1) and often occur in association with developmental venous anomalies. They are not inherited and do not carry a CCM gene germline mutation and do not have any known clinically affected relative. Sporadic cases with multiple lesions may be due to unrecognized familiarity (occult germ line mutations), multiple CCMs in association with a single developmental venous anomaly, or multiple CCMs after craniospinal irradiation.

Furthermore, familial cerebral cavernous malformations may show a very variable phenotype due to a highly variable penetrance of mutations. This variability seems related to the localization of mutations and may determine subtle or incomplete clinical manifestations obscuring the pattern of genetic transmission. As a result sometimes either mimicking a recessive trait in a given family or a sporadic condition in a family with an apparently negative history (21). In our recent study on mutation analysis of *CCM1, CCM2* and *CCM3* genes in a cohort of Italian patients with cerebral cavernous malformation, both sporadic and familial, sixteen mutations were identified, and only two in sporadic patients (22).

In addition, we found that only the sporadic patients negative for mutations in the three CCM genes and whose relatives were negative to MRI, had five variations in the *CCM2* gene (data not shown), which proved to be the known polymorphisms; some detected previously by Denier *et al* (17) in families with CCM negative to molecular screening for *CCM1* mutation, and by Pileggi *et al* (23) in the affected members of a large Italian family harbouring a CCM1 mutation and carrying genetic variations in *CCM1, CCM2* and *CCM3* gene. Three of these polymorphisms are located in the coding sequence of the *CCM2* gene (c.157 G>A in exon 2, c.358 G>A in exon 4 and c.915 G>A in exon 8).

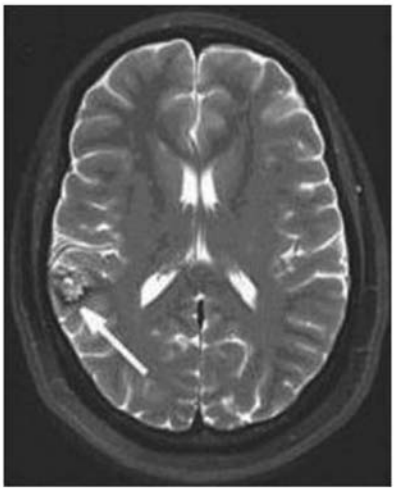

Figure 1. Radiological features of cerebral cavernous malformations (CCMs). T2 weighted MRI shows a right posterior temporoparietal cavernous malformation that exhibits the characteristic 'mulberry' appearance (arrow).

c.157 G>A and c.358 G>A are non-synonymous polymorphisms leading to substitutions p.Val53Ile and p.Val120Ile respectively; p.Val120Ile resides in the PTB domain of the malcavernin protein while p.Val53Ile in the domain located N-terminally to PTB domain that, as mentioned above, is essential for krit1 binding to malcavernin.

c.915 G>A is a synonymous polymorphism leading to a silent alteration at amino acid position 305 (p.Thr305Thr) which, as is well known, while not affecting the protein structure, might be liable to alter gene function. Nothing has been reported, in literature, on the polymorphisms IVS2-36A>G and IVS8 +119 C>T residing in intronic regions and so able to lead to exon skipping or to create new splice sites.

Therefore, in this study, we decided to undertake a case-control study to analyze the possible relationships between these polymorphisms and sporadic cerebral cavernous malformations, induced mainly by the fact that our polymorphism detection is related to a cohort of patients who were sporadic and negative to CCM genes mutation analysis, performed by direct DNA sequencing and subsequent Multiplex Ligation-Dependent Probe Amplification (MLPA) analysis, and that little is known in literature on these polymorphisms.

Materials and methods

Patients. A total of 91 CCM patients (49 male and 42 female) were recruited. Detailed clinical and neuroimaging information on patients and their relatives were collected through direct interview by review of the medical charts, before CCM gene molecular analysis and after providing their written informed consent.

On the basis of pedigree analysis and in the absence of relatives positive to biomolecular investigation and/or MRI (standard spin echo and fast turbo spinecho T1- and T2-weighted axial, coronal, and/or sagittal images) the patients were considered sporadic. In each patient, lesions were identified based on typical charateristics and were classified according to number, localization and size. Lesions were single in 87 patients (95.6%) and multiple in 4 patients (4.4%).

Table I. Demographic and disease data on CCM sporadic patients and controls.

A, CCM sporadic patients (n=91)

		Age at onset of symptoms[a]		
Gender	n	Mean ± SD	Range	Median
Male	47	41.7±22.5	1-79	43
Female	36	41.9±17.9	14-69	44
Overall	83	41.7±20.6	1-79	43
Lesion number	4/91>1 Lesion			
Lesion seat				
72/91Supratentorial				
12/91 Subtentorial				
7/91 Spinal-cerebellar				
Male + female (asymptomatic) 2+6				
Asymptomatic	8			
> One symptom	4			
Symptoms				
Headache	32			
Epilepsy	22			
Cerebral hemorrhage	18			
Focal neurological deficits	7			

B, Healthy controls (n=100)

		Age		
Gender	n	Mean ± SD	Range	Median
Male	54	43.5±17.9	10-78	42
Female	46	43.1±12.9	16-60	44
Overall	100	43.36±15.7	10-78	42

[a]The age at onset of symptoms in all patients (with the exception of asymptomatic patients) coincides with the age at diagnosis. P>0.05 indicates no significant differences. Statistical analysis was tested by the Student's t-test. CCM, cerebral cavernous malformation; SD, standard deviation.

The latter had two lesions. In 72/91 (79.1%) patients, lesions were located supratentorially (frontal, temporal, parietal, occipital). In 12/91 (13.2%) lesions were subtentorial (mid-brain) and in 7/91 (7.7%), were spinal-cerebellar (Table I). The data available did not reveal the existence of patients with skin, spinal cord, retinal, hepatic or vertebral lesions. Additional clinical data included: age of symptoms onset and neurological events classified in: headache, epilepsy, cerebral hemorrhages and focal neurological deficits.

Headache was reported in 32 patients, epilepsy in 22, cerebral hemorrhages in 18 and focal neurological deficits in 7; 4 patients have more than one symptom, while 8 patients were asymptomatic (Table I). With the exception of the asymptomatic patients, the age at onset of symptoms, in all patients examined, coincides with the age at diagnosis and the patient's age at the time of recruitment. The mean age at onset of symptoms was 41.7±20.6 years (median, 43.0 years; range, 1-79 years) (Table I).

The control group consisted of 100 unrelated, randomly selected, ethnically matched, healthy individuals. Demographic data on controls are summarized in Table I. Age at onset of symptoms of male vs. female was matched in the CCM patients, as well as age of male versus female in the controls. Overall patients age at onset of symptoms versus overall healthy control age were matched. Table I indicates no significant differences (P<0.05). This study was approved by the Scientific Ethics Committee of the Azienda Ospedaliera Universitaria-Policlinico 'G. Martino' Messina. Informed consent was obtained from all patients and controls.

Polymorphisms of the CCM2 gene. Among the five polymorphisms of CCM2 gene identified, two are located in non-coding regions (introns): IVS2-36A>G (rs2304689) and IVS8 +119 C>T (rs2289369); three are in the coding region: two non-synonymous, c.157 G>A in exon 2 (rs2107732) leading to the substitution of a valine by isoleucine at amino acid posi-

Table II. Oligonucleotide primer sequences used for genotyping.

Gene	Site	Polymorphism	Primers (5'-3')	Ta (˚C)	Expected amplicon size (bp)
CCM2	Exon 2	c.157 G>A	(F) CTA CTT CTG TTT GTT AAC CAT A (R) AGA GTG TCT GGT GGA TAC AAG C	51.7	329
CCM2	Exon 3	IVS2-36A>G	(F) GAA GCA CTT GGT TTG TGC TC (R) AGC CAA GTG TAC CCA TAA TGT GA	52.4	299
CCM2	Exon 4	c.358 G>A	(F) TTT GTC ACA TGT GTG ACA TC (R) ACC CAA CAC GAA GCT GCA A	53.0	386
CCM2	Exon 8	c.915 G>A IVS8+119 C>T	(F) GAA GCC ACC CGC TCA CAT (R) AGC AAA ATT GAC CAA GAG T	56.3	291

Ta, amplification T.

Table III. Association between c.358 G>A and c.915 G>A polymorphisms and symptoms in CCM patients.

				Genotypes n (%)[a]			
Exon	Polymorphism	Amino acid substitution	Symptom	GG	GA	AA	Total
4	c.358 G>A	p.Val120Ile	Headache	20 (31.2)	12 (60.0)	0	32
			Epilepsy	19 (29.7)	3 (15.0)	0	22
			Cerebral hemorrhages	15 (23.4)	3 (15.0)	0	18
			Other symptoms	2 (3.12)	2 (10.0)	3 (100)	7
			Asymptomatic	8 (12.5)	0	0	8
				64	20	3	87
8	c.915 G>A	p.Thr305Thr	Headache	2 (6.4)	30 (56.7)	0	32
			Epilepsy	10 (32.2)	8 (15.4)	4 (100)	22
			Cerebral hemorrhages	8 (25.8)	10 (19.2)	0	18
			Other symptoms	7 (22.6)	0	0	7
			Asymptomatic	4 (12.9)	4 (7.7)	0	8
				31	52	4	87

[a]The percentages given in parentheses are calculated on the total number of individuals with the same genotype. In this association those patients who had more than one symptom (4 patients) were not taken into account.

tion 53 (p.Val53Ile) and c.358 G>A in exon 4 (rs11552377) leading to the substitution of a valine by isoleucine at amino acid position 120 (p.Val120Ile); the third synonymous, c.915 G>A in exon 8 (rs2289367) leading to a silent alteration at amino acid position 305 (p.Thr305Thr).

Variation numbering is based on the cDNA sequences obtained from GenBank (accession no. NM_031443) with +1 corresponding to the A of the ATG initiation codon. SNP database www.ncb.nlm.org was also utilized.

Genotyping

Primers and polymerase chain reaction (PCR). DNA blood samples were obtained with informed consent from patients and controls. Genomic DNA was isolated from peripheral blood leukocytes by the salting out method (24) and then stored in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) until analysis.

The amplification of *CCM2* gene exons 2, 3, 4, 8, was performed using the pairs of primers designed according to the CCM2 published nucleotide sequence of GenBank (accession no. NG_016295). The nucleotide sequence of primers and relative expected amplicon size is shown in Table II.

PCR reaction was carried out in a 50 μl final volume containing 0.2 μM concentration of each primer, 0.8 μg genomic DNA as template and 1 unit Euro Taq polymerase (EuroClone SpA Life Sciences Division, Italy). PCR was performed with a thermal cycler GeneAmp PCR System 9600 (PE Applied Biosystems, Foster City, CA) samples were subjected to 35 cycles of amplification, comprising 30 sec of denaturation at 95˚C, 1 min of annealing, and

Table IV. Allele and genotype frequencies of CCM2 polymorphisms and risk.

Exon	Sequence variation	Coding change	SNP reference	Genotype	No. (%)[a] in controls	No. (%)[a] in patients	Odds Ratio	95% CI	P-value
2	c.157 G>A	p.Val53Ile	rs2107732	GG	82 (82)	66 (72.5)	1		
				GA	18 (18.0)	25 (27.5)	1.7		0.225
				AA	0	0			
				G	0.91	0.86			
				A	0.09	0.14			
3	IVS2-36 A>G	Intronic	rs2304689	AA	74 (74)	43 (47.25)	1		
				AG	24 (24.0)	43 (47.25)	3.08	1.5-5.9	
				GG	2 (2.0)	5 (5.5)	4.3	1.4-22.6	<0.037
				A	0.86	0.71			
				G	0.14	0.29			
4	c.358 G>A	p.Val120Ile	rs11552377	GG	64 (64.0)	67 (73.6)	1		
				GA	28 (28.0)	21 (23.1)	0.72		
				AA	8 (8.0)	3 (3.3)	0.3		0.462
				G	0.78	0.85			
				A	0.22	0.15			
8	c.915 G>A	p.Thr305Thr	rs2289367	GG	76 (76.0)	33 (36.3)	1		
				GA	20 (20.0)	53 (58.2)	6.1	3.0-12.6	<0.001
				AA	4 (4.0)	5 (5.5)	2.9		
				G	0.86	0.65			
				A	0.14	0.35			
8	IVS8+119 C>T	Intronic	rs2289369	CC	48 (48.0)	33 (36.3	1		
				CT	44 (44.0)	45 (49.4)	1.5		
				TT	8 (8.0)	13 (14.3)	2.4		0.509
				C	0.7	0.61			
				T	0.3	0.39			

[a]The percentages given in parentheses are calculated on the total number of individuals with the same genotype. In this association those patients who had more than one symptom (4 patients) were not taken into account. Odds ratio are calculated relative to subjects with the G/G (polymorphism c.157 G>A), A/A (polymorphism IVS2-36 A>G), G/G (polymorphism c.358 G>A), G/G (polymorphism c.915 G>A) and C/C (polymorphism IVS8 +119 C>T) genotypes, respectively. OR, odds ratio; CI, confidence interval.

30 sec of extension at 72˚C. The final extension step was extended to 10 min. Annealing temperature was optimized for each primer set.

Sequencing. PCR products were analyzed by direct sequencing, nucleotide sequence analysis was performed using both 5' and 3' primers, and the dideoxynucleotide method with the BigDye Terminator Cycle Sequencing kit (vers.1.1, Applied Biosystems), on a 377 ABI PRISM Sequencer Analyzer (Applied Biosystems) (25).

Statistical analysis. Analysis of data was performed using computer software SPSS for Windows (version 6.0.1) and Epi Info (version 6.0.4).

Comparisons between the mean age at onset of symptoms of male versus female in the CCM group and overall CCM age at onset versus overall healthy control age were calculated with the Student's t-test. For each group (control and patients), allele frequencies were calculated by direct gene counting. Associations between gene genotype and risk of CCM disease were assessed by odds ratios (ORs) and 95% confidence intervals (CIs). ORs express relative risk (OR) of CCM patients with a specific genotype and are calculated by dividing the odds of a CCM patient having a specific phenotype by the odds of a control subject having the same genotype. Estimates of statistical significance were calculated by standard χ^2 analysis for one degree of freedom.

Descriptive analysis included Student's t-test of means and the respective standard deviation (SD) for cases and controls. A two-sided probability value of <0.05 was considered to indicate statistical significance. Tests for associations using multi-marker haplotypes were performed using the statistics environment 'R' package 'haplo.score' (http://www.R-project.org)

Results

There are scarce data in literature on possible associations of SNPs and CCM. We determined the frequencies and odds ratio of *CCM2* polymorphisms in a group of CCM sporadic patients and in a control sample.

Table V. Haplotype frequencies c.358 G>A, c.915 G>A and IVS2-36 A>G polymorphisms in CCM patients and in controls.

Haplotype[a]	Patients (%)	Controls
G-G-A (+++)	49.88	63.26
G-G-G (++-)	1.43	3.10
G-A-A (+-+)	6.69	2.24
G-A-G (+--)	27.51	9.39
A-G-A (-++)	14.47	18.63
A-G-G (-+-)	14.47	18.63
A-A-A (--+)	14.47	1.85
A-A-G (---)	14.47	1.50

[a]Base position haplotypes.

With regard to age and gender, there were no significant differences between the two groups, and no significant association was observed between the CCM patients (male and female) and age at onset of symptoms.

No significant relation between lesion sites (supratentorial, subtentorial and spinal-cerebellar) and age at onset of symptoms, nor between the type of symptom and age at onset of symptoms was found. None of the allelic variants of *CCM2* gene examined appear to have any influence on the age at onset of symptoms (P>0.05) or on lesion site.

Polymorphisms c.358 G>A in exon 4 (χ^2, 15.977; P< 0.04) and c.915 G>A in exon 8 (χ^2, 18.109; P<0.02) showed, instead, a significant association with different types of symptoms (Table III). In this type of association, patients who had more than one symptom were not taken into account (4 patients).

In reference to polymorphism c.358 G>A (p.Val120Ile), approximately 60% of patients with genotype G/A suffered from headache while the remaining 40% was distributed equally among patients with epilepsy, cerebral haemorrhages and other symptoms. No patient with this genotype was asymptomatic.

Only 31.2% of patients with genotype G/G suffered from headache, while the percentages of those who suffered from epilepsy, cerebral haemorrhages and other symptoms was 29.7, 23.4 and 3.12%, respectively. The proportion of asymptomatic patients with this genotype was 12.5 vs. 0% of patients with genotype G/A. All patients with genotype A/A had symptoms other than headache, epilepsy or cerebral haemorrhage.

Regarding polymorphism c.915 G>A (p.Thr305Thr), approximately 56.7% of patients with genotype G/A suffered from headache and 15.4 and 19.2% suffered from epilepsy and cerebral haemorrhages, respectively; 7.7% of patients G/A were asymptomatic. Only 6.4% of patients with genotype G/G suffered from headache, while the percentages of those who suffered from epilepsy, cerebral haemorrhages and other symptoms were 32.2, 25.8 and 22.5%, respectively. The percentage of asymptomatic patients with genotype G/G was 15.38%. All patients with genotype A/A were affected by epilepsy.

The possible association between *CCM2* polymorphisms and risk of CCM was checked and the result is shown in Table IV.

Among the polymorphisms examined, IVS2-36A>G and c.915 G>A, showed statistically significant differences in frequencies between patients and controls [(χ^2, 6.583; P<0.037); (χ^2, 14.205; P<0.001)]. With regard to polymorphism IVS2-36A>G, the prevalence of the wild-type *CCM2* A/A genotype was significantly lower in CCM group (47.4%) than in the control sample (74%). We also found that the frequency of patients carrying the A/G genotype among the patient group (47.25%) was 2-fold higher than among the controls (24%).

In the same manner, the frequency of G/G genotype was found to be higher among CCM patients (5.5%) compared with control group (2%). Patients with the A/G and G/G genotypes had a significant increase for CCM risk (OR, 3.08; 95% CI, 1.5-5.9 and OR, 4.3; 95% CI, 1.4-22.6). Similarly, for c.915 G> A polymorphism, the frequency of genotype G/G in patients was 36.3% compared with 76% of controls. The frequency of patients carrying the G/A genotype among the patient group (58.2%) was about 3-fold higher than among the controls (20%); however the frequency of genotype A/A was

Table VI. Prediction of the effects of CCM2 polymorphisms on malcavernin protein structure and splicing.

CCM2 gene polymorphisms	Effects on protein PolyPhen prediction/SIFT prediction	Effects on splicing SpliceAid
c.157 G>A p.Val53Ile		No effects
IVS2-36 A>G Intronic	Benign	No effects
c.358 G>A p.Val120Ile	Benign	No effects
c.915 G>A p.Thr305Thr	Benign	No effects
IVS8+119 C>T Intronic		No effects

only slightly higher in patients (5.5%) compared to controls. Patients with the G/A and A/A genotypes had a significant increase for CCM risk (OR, 6.1; 95% CI, 3.0-12.6 and OR, 2.9). No statistically significant differences were detected between patients and controls for the other three polymorphisms (P>0.05 in all cases).

Therefore, haplotype analysis was performed only on polymorphisms c.358 G>A (p.Val120Ile), c.915 G>A (p.Thr305 Thr) and IVS2-36A>G. Naturally, the complete wild-type haplotype, G-G-A (+++) was the most frequent condition: 63.26% in the controls and 49.88% in CCM patients; the frequency of haplotype G-A-G (+--) was significantly higher in CCM patients than in the control group (27.51 vs. 9.39%, respectively; hap-score = 3.07845; P=0.001) (Table V). No significant difference in the remaining haplotype frequencies was observed between CCM patients and control group.

SIFT, PolyPhen and SpliceAid predictions. With the aim of understanding the possible impact of c.157 G>A p.Val53Ile, c.358 G>A p.Val120Ile and c.915 G>A p.Thr305Thr polymorphisms in the *CCM2* gene protein SIFT and PolyPhen algorithms were used. Predictions analysis indicated that these polymorphisms not are damaging and are predicted to be benign and not to affect the protein function.

SpliceAid prediction analysis indicated that all five polymorphisms examined have no effects on splicing (Table VI).

Discussion

One of the characteristics of sporadic CCMs is that it is more likely to have a benign course (26) than familial CCMs that are more likely to haemorrhage, grow, and form new lesions; moreover, CCM patients with mutations in *CCM2* have a lower number of gradient-echo sequence lesions than those with mutations in *CCM1* or *CCM3*, and the number of lesions increases more slowly with age than in patients with *CCM1* mutations (27).

In the present study, a possible relationship between five known polymorphisms of *CCM2* gene and sporadic CCMs was evaluated, starting from the ascertainment that in the cohort of patients, both sporadic and familial previously examined by us, only the sporadic patients showed these polymorphisms and were negative for CCM mutations (unpublished data) with the exception of two carrying two mutations in *CCM2* gene (22).

Previously, in literature, some of these polymorphisms were described only in families with CCM, resulting negative to molecular screening for *CCM1* mutation (17) or in the affected members of a family harbouring a *CCM1* mutation and carrying genetic variations in *CCM1, CCM2* and *CCM3* genes (23): no correlation analysis has so far been conducted.

Our statistical data showed that the frequencies of CCM2 IVS2-36A>G (rs2304689) and c.915 G>A (rs2289367) polymorphisms were significantly different between CCM patients and control group; in particular, genotypes AG and GG (for IVS2-36A>G polymorphism) and GA and AA (for c.915 G> A polymorphism) were more frequent in patients than in the control group. Patients with these genotypes had a significant increase in risk for CCM than those with wild-type genotype.

In addition, we found a significant association between *CCM2* c.358 G>A (rs11552377) and c.915 G>A (rs2289367) polymorphisms and the different types of symptoms related to CCM.

With regard to c.358 G>A polymorphism, it would appear that the presence of genotype G/A predisposes to a higher occurrence of a 'potentially disabling' symptomatology (such as headache) rather than to a 'possibly life-threatening' symptomatology (such as epilepsy, cerebral haemorrhage, and other symptoms). The latter, in fact, were less frequent in patients with this genotype. Only 2.3% of our patients presented genotype A/A and symptoms other than headache, epilepsy or cerebral haemorrhage.

The same trend was observed for the c.915 G>A polymorphism: heterozygous genotype G/A, again, seems to be associated with a 'potentially disabling' symptomatology. It is possible, therefore, to assume that the finding of these two polymorphisms in patients with sporadic CCMs may be associated with a more benign course of the disease rather than in patients with wild-type genotype. This hypothesis is supported by the results of prediction studies conducted using SIFT, PolyPhen and SpliceAid algorithms, which show that the five polymorphisms are not damaging and do not affect the protein function. This is particularly significant for polymorphism c.358 G>A (rs11552377) (p.Val120Ile) that resides in the malcavernin PTB domain of protein and for which we have identified the above indicated association.

An amino acid substitution that alters the structure of the malcavernin protein PTB domain may be able to interfere with Krit1/malcavernin/PDCD10 complex formation and with subsequent signalling cascade (2). Only 4/91 of our patients had more than one lesion, and, neuroimaging data were insufficient to assess the existence of a correlation between these polymorphisms and the number or size of lesions. Based on the sample of our population examined, it appears that among the five polymorphisms analyzed, only IVS2-36A>G and c.915 G>A were significantly associated with sporadic CCMs and an increased risk of CCM. In addition, polymorphisms c.915 G>A and c.358 G>A were associated with a more benign course of the disease. In agreement with this, haplotype analysis shows that haplotype GAG (+--) was significantly increased among sporadic CCM patients compared to the control group.

It would be of interest to perform the same analysis in patients with sporadic CCM belonging to non-Italian populations in order to determine whether this haplotype has the same trend. If this is confirmed, the examination of this haplotype in CCM patients may represent a potential biomarker, and a useful tool for doctors. We believe it is very important to use molecular screening and genetic counseling to identify, as early as possible, unaffected mutation carriers and sporadic subjects with apparent negative family history.

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