**PDCD10 Gene Mutations in Multiple Cerebral Cavernous Malformations**

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**Abstract**

Cerebral cavernous malformations (CCMs) are vascular abnormalities that may cause seizures, intracerebral haemorrhages, and focal neurological deficits. Familial form shows an autosomal dominant pattern of inheritance with incomplete penetrance and variable clinical expression. Three genes have been identified causing familial CCM: KRIT1/CCM1, MGC4607/CCM2, and PDCD10/CCM3. Aim of this study is to report additional PDCD10/CCM3 families poorly described so far which account for 10-15% of hereditary cerebral cavernous malformations. Our group investigated 87 consecutive Italian affected individuals (i.e. positive Magnetic Resonance Imaging) with multiple/familial CCM through direct sequencing and Multiplex Ligation-Dependent Probe Amplification (MLPA) analysis. We identified mutations in over 97.7% of cases, and PDCD10/CCM3 accounts for 13.1%. PDCD10/CCM3 molecular screening revealed four already known mutations and four novel ones. The mutated patients show an earlier onset of clinical manifestations as compared to CCM1/CCM2 mutated patients. The study of further families carrying mutations in PDCD10/CCM3 may help define a possible correlation between genotype and phenotype; an accurate clinical follow up of the subjects would help define more precisely whether mutations in PDCD10/CCM3 lead to a characteristic phenotype.

**Introduction**

Cerebral cavernous malformation (CCM; OMIM 116860) is one of the most common types of vascular malformations characterized by “blackberry-like” aggregation of grossly enlarged capillary cavities consisting of a single layer of endothelium without involving neuronal tissue [1].

Cavernous malformations can occur anywhere in the body - brainstem, cerebellum, spinal cord, cranial nerves, cerebral ventricles, retina, skin and liver - but are most commonly found in the forebrain [2]. They occur as single or multiple lesions and, depending on size and location, can be clinically silent or show clinical symptoms ranging from headache to focal neurological deficits, seizures and fatal intra-cerebral haemorrhage [3–5].

CCM can arise in a sporadic form, with a single lesion, or in a familial form, with multiple cavernous malformations [6]. The familial form shows an autosomal dominant pattern of inheritance with incomplete penetrance and variable clinical expression [7]. However, multiple lesions have been found in patients with no positive family history [8] and combined clinical and genetic tests have recently revealed that the vast majority of these ‘sporadic cases’ with multiple lesions have indeed a genetic origin: a de novo mutation or a mutation inherited from an asymptomatic parent [2,9–11].

Genetic studies identified three CCM genes in different loci: KRIT1/CCM1 at 7q21–22 [12,13], MGC4607/CCM2 at 7p13–15 [14,15] and PDCD10/CCM3 at 3q25.2–27 [16,17]. Several mutations have been identified so far in the Italian population, all of them appearing to cause a loss of function [15,18–26].Starting from 2004 we have investigated 87 consecutive index cases, with the presence of multiple angiomas and/or with a positive family history [PCCM]; we identified mutations in over 97.7% of FCCM cases. Among the positive cases, KRIT1/
CCM1 accounts for 68.9%; MGC4607/CCM2 for 18.0% and PDCD10/CCM3 for 13.1%.

Our group identified mutations in over 97.7% of FCCM cases, this high score of mutation detection being due to the selection of index case according to the presence of multiple angiomas and/or to a positive family history (FCCM). Not surprisingly, high prevalence of causative mutation has been identified in KRIT1/CCM1 followed by MGC4607/CCM2 and PDCD10/CCM3, these last two with the second similar mutation rate.

According to recent data PDCD10/CCM3 mutations cause 10–15% of FCCM [5] and less than 40 CCM3 families have been reported so far [15,17,19,27–31]. This limited number of patients harbouring a mutation in PDCD10/CCM3 gene hampered establishing the genotype-phenotype correlations. It has been recently reported that PDCD10/CCM3 mutation carriers display earlier symptoms’ onset, usually before 15 years of age, and higher risk of cerebral haemorrhage during childhood; multiple meningiomas are frequently reported too. However the mechanisms leading from CCM3 mutations to meningiomas are still unknown [5,28,29].

We report here the results related to PDCD10/CCM3 gene molecular screening carried out on eleven unrelated Italian CCM affected patients, all of them were found to harbour mutations, four already known and four novel ones.

**Results**

We analyzed 87 Italian cases with multiple lesions and/or positive family history; 11 index patients (13.1%) resulted to be mutated in PDCD10/CCM3 in [Fig. 1]. When relatives’ DNA samples were available, we observed a complete cosegregation of the mutational event with the clinically affected status (i.e., positive MRI).

The eight identified mutations are summarized in Table 1: R35X, R95X, R108X, and a whole gene deletion were already reported in literature [5,15,17,27,30], while four more mutations are described here for the first time: c.367_387dup; [c.376_380del; R35X, R95X, R108X, and a whole gene deletion were already reported in literature [5,15,17,27,30], while four more mutations are described here for the first time: c.367_387dup; c.392_393insGACAGAGTGTCTGCAGACTTGATTGTCTGCA]; c.159dup and c.160G

Among the seven familial cases, inheritance appears to be paternally derived four times (321CCM, 352CCM, 344CCM and 118CCM) while we observed maternal origin only in one case (446CCM); in two cases (482CCM and 415CCM), we were not able to assess the inheritance pattern.

Prenatal diagnosis was requested from 118CCM’s family: after an accurate genetic counselling it has been performed through MLPA with flanking exon probes, giving a negative result. This has been confirmed later on the newborn DNA (II,2).

In all the tested subjects no associations were found with other cerebral vascular malformations, such as meningiomas or venous cavernomas or arterovenous malformations. The mean age of the first onset is 7.3 years (range 0.33–35 years) and in 5 out of 11 patients we found extra-axial cavernous angiomas. Clinical characteristics of patients are summarized in Table 2, while MRI/TC imaging of patients 321CCM, 344CCM and 454CCM are reported in Figure 2.

**Discussion**

The CCM3 gene [Programmed cell death 10 (PDCD10)] is highly conserved in both vertebrates and invertebrates and is the most recently discovered compared with CCM1 and CCM2 [16,17]. It has been shown that PDCD10 interacts in vitro with the other two proteins involved in genesis of cavernomas: K-Rev interaction trapped 1 (KRIT1) and Malcavernin, which participates in CCM1-dependent modulation of β1-integrin-mediated signalling and CCM2-mediated p38 MAPK signalling in response to cellular stress [34].

The roles of these three proteins in the formation and maintenance of cerebral vessels, the genetic mechanism leading to CCMs and factors that may influence their number and growth are still to be clarified. Actually, PDCD10 is involved in many cellular pathways including apoptosis, cellular proliferation and cell survival/resistance to apoptosis [34–39].

PDCD10 protein contains an N-Terminal dimerization Domain and a C-Terminal Focal Adhesion Targeting (FAT) Domain resembling the one of the Focal Adhesion Kinase (FAK) [40,41] (Fig. 3).

Li et al. demonstrated that the presence of a fully folded CCM3 FAT-Homology Domain is important for the stabilization of the expressed protein in the in vivo setting since an example of a truncation mutation (CCM3-1-117) was found to be poorly expressed [41].

The protein can homodimerize and heterodimerize with a variety of proteins including cell adhesion molecule Paxillin [41] and Malcavernin (CCM2) [12,42] through its FAT Domain. The N-Terminal domain is important for the interaction with GCKIII kinases (Germinal Centre Kinase III), a family of protein kinases, and this heterodimerization may be the preferred conformation [42]. In particular, PDCD10-GCKIII signalling facilitates lumen formation by endothelial cells, which is important during the progression of cerebral lesions [43,44] and these kinases are important for the regulation of apoptosis, cell proliferation, polarity, migration, and cytoskeleton remodelling [42,43,45].

Different animal models have been used to draw these conclusions. In mice model, Pdcld10 is required for the control of venous size and integrity, yet it is not required in the embryonic establishment of circulation as Cem2 is. [44]. Other animal models seem to confirm these findings: tracheal tubes of the respiratory system in Drosophila melanogaster, lacking Pdc10l, grow and branch normally, but fail to lumenzize [44] and inhibition of Ccm3a/b in Zebrafish leads to dilations of the embryonic cranial vasculature [46]. Similarly, in vitro studies showed that PDCD10-
depleted HUVECs cells failed to organize themselves into a lumenized network [44].

Finally, loss of PDCD10 has been reported to increase cell survival and proliferation, possibly through reduced Notch signalling, enhanced VEGF signalling, or increased ERK activity [39,47,48].

These literature data show that PDCD10 interacts with a wide variety of proteins through its different domains, in addition to...
<table>
<thead>
<tr>
<th>FAMILY NUMBER</th>
<th>EXON</th>
<th>MUTATION (NM_007217)</th>
<th>THEORETICAL EFFECT ON THE PROTEIN</th>
<th>FAMILY HISTORY</th>
<th>REF</th>
<th>ITALIAN GEOGRAPHICAL ORIGIN</th>
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<td>5</td>
<td>c.103C&gt;T</td>
<td>R35X</td>
<td>N</td>
<td>Lee et al., 2008</td>
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<td>5</td>
<td>c.103C&gt;T</td>
<td>R35X</td>
<td>Y</td>
<td>Lee et al., 2008</td>
<td>Centre</td>
</tr>
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<td>321 CCM’s mother</td>
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<td>c.103C&gt;T</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>5</td>
<td>c.103C&gt;T</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
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<td>5</td>
<td>c.103C&gt;T</td>
<td>R35X</td>
<td>Y</td>
<td>Lee et al., 2008</td>
<td>North</td>
</tr>
<tr>
<td>446 CCM’s mother</td>
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<td>R35X</td>
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<td>7</td>
<td>c.283C&gt;T</td>
<td>R95X</td>
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<td>Guclu et al., 2005</td>
<td>North/South</td>
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<td>7</td>
<td>c.322C&gt;T</td>
<td>R108X</td>
<td>de novo</td>
<td>Riant et al., 2013</td>
<td>North</td>
</tr>
<tr>
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<td>7</td>
<td>c.367_387dup</td>
<td>D123_Q129dup</td>
<td>Y</td>
<td>novel</td>
<td>Centre</td>
</tr>
<tr>
<td>415 CCM</td>
<td>7</td>
<td>c.376_390del: 392_393insGACAGAGTG-</td>
<td>P.?</td>
<td>?</td>
<td>novel</td>
<td>South</td>
</tr>
<tr>
<td>454 CCM</td>
<td>6</td>
<td>c.159dup</td>
<td>E54Rfs*22</td>
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<tr>
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<td>c.160G&gt;T</td>
<td>E54X</td>
<td>Y</td>
<td>novel</td>
<td>South</td>
</tr>
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<td>6</td>
<td>c.160G&gt;T</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>118 CCM</td>
<td>whole gene deletion</td>
<td>whole gene deletion</td>
<td>Y</td>
<td></td>
<td>Liquori et al., 2008</td>
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</tr>
<tr>
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<td></td>
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Y = Yes; N = No. Nomenclature according to HGVS.
doi:10.1371/journal.pone.0110438.t001
<table>
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<th>FAMILY NUMBER</th>
<th>CEREBRAL MR IMAGING</th>
<th>AGE AT ONSET (years)</th>
<th>AGE AT FIRST VISIT (years)</th>
<th>INAUGURAL MANIFESTATION</th>
<th>BLEEDING EVENTS UP TODAY (n*)</th>
<th>NEUROSURGERY</th>
<th>ASSOCIATED CAVERNOMAS</th>
<th>OTHER</th>
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<td>multiple lesions</td>
<td>13</td>
<td>32</td>
<td>involuntary movements at right upper limb</td>
<td>1 N 0</td>
<td>angiokeratomas</td>
<td>N N</td>
<td>right vocal cord paralysis</td>
</tr>
<tr>
<td>321 CCM</td>
<td>multiple lesions</td>
<td>1.2</td>
<td>4</td>
<td>mild psychomotor retardation with lack of independent ambulation</td>
<td>nr N 0</td>
<td>plain angioma on the posterior part of neck</td>
<td>N N</td>
<td>left facial hypoplasia with asymmetric palate and dental arcsides</td>
</tr>
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<td>multiple lesions</td>
<td>36</td>
<td>nr</td>
<td>asymptomatic</td>
<td>nr N 0</td>
<td>2 plain angiomas in the lumbar and abdominal areas, respectively + 2 pinkie hyperkeratotic cutaneous capillary venous malformations (HCCVM) in relief and hairy</td>
<td>N N</td>
<td>mild left facial hypoplasia</td>
</tr>
<tr>
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<td>multiple lesions</td>
<td>30</td>
<td>76</td>
<td>headache</td>
<td>nr N 0</td>
<td>plain angioma in the neck</td>
<td>N N</td>
<td>mild facial asymmetry</td>
</tr>
<tr>
<td>446 CCM</td>
<td>multiple lesions</td>
<td>4 months</td>
<td>31</td>
<td>strabismus, exophthalmos, ptosis, at 1.5 years acute palsy of right 3rd cranial nerve</td>
<td>1 N 0</td>
<td>N N N N nr</td>
<td>one brother with CCM lesions underwent surgical intervention</td>
<td></td>
</tr>
<tr>
<td>446 CCM's mother</td>
<td>not performed</td>
<td>48</td>
<td>nr</td>
<td>N N N N N</td>
<td>359 CCM multiple lesions</td>
<td>6</td>
<td>13</td>
<td>attention disorder and seizures</td>
</tr>
<tr>
<td>410 CCM</td>
<td>multiple lesions</td>
<td>8</td>
<td>13</td>
<td>headache</td>
<td>N N 0</td>
<td>three median cervico-dorsal angiomas</td>
<td>N isolated medullar cavernous angioma</td>
<td>nr</td>
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<tr>
<td>338 CCM</td>
<td>multiple lesions</td>
<td>4</td>
<td>4</td>
<td>rigor nucalis, pain during flexion of the neck, hypotonia, difficulties in speech</td>
<td>nr N 0</td>
<td>N N N N</td>
<td>occipital scale elevated with crowning of the foramen magnum and cerebellar tonsillar hernia (Chiari I anomaly)</td>
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<tr>
<td>FAMILY NUMBER</td>
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<td>AGE AT FIRST VISIT (years)</td>
<td>INAUGURAL MANIFESTATION</td>
<td>BLEEDING EVENTS UP TODAY (n*)</td>
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<td>INTERVENTION N</td>
<td>ASSOCIATED CAVERNOMAS CUTANEOUS</td>
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<tr>
<td>352 CCM</td>
<td>multiple lesions</td>
<td>35</td>
<td>39</td>
<td>2 episodes of dysarthria and paresthesias in the right upper limb and ipsilateral hemiface</td>
<td>N</td>
<td>N 0</td>
<td>N</td>
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<tr>
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<td>1</td>
<td>11</td>
<td>seizures</td>
<td>4</td>
<td>Y 1</td>
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<tr>
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<td>1.4</td>
<td>5</td>
<td>palsy of 7th cranial nerve and left hemiplegia</td>
<td>4</td>
<td>Y 1</td>
<td>N</td>
<td>N</td>
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<tr>
<td>344 CCM</td>
<td>multiple lesions</td>
<td>8</td>
<td>13</td>
<td>left-sided focal sensory-motor seizures, transient hemiparesis and dysarthria</td>
<td>several</td>
<td>Y 1</td>
<td>N</td>
<td>N</td>
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<td>42</td>
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<td></td>
<td>nr</td>
<td>N 0</td>
<td>N</td>
<td>N</td>
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<tr>
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<td>multiple lesions</td>
<td>2</td>
<td>11</td>
<td>bleeding cavernous angioma at the pontine site</td>
<td>3</td>
<td>Y 2</td>
<td>N</td>
<td>N</td>
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<tr>
<td>118 CCM’s father</td>
<td>multiple lesions</td>
<td>15</td>
<td>42</td>
<td>seizures</td>
<td>N</td>
<td>N 0</td>
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Y = Yes; N = No; nr = not reported; ni = not investigated.

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those involved in CCM pathology (KRIT1, Malcavernin), and participates in several different molecular pathways. So mutations affecting the integrity and stability of CCM3 may disrupt not only the ternary complex with the CCMs proteins but also the interactions with the proteins described above, which act in such different pathways. This pleiotropy of PDCD10 may explain the

Figure 2. 1–3 MRI/TC scans from subjects: 1) 454CCM patient harbouring the de novo and novel mutation p.E54Rfs*22. A) T1 sagittal image at 16 months showing a cavernous malformation with recent bleeding in the pons; B) T1 axial image at 25 months showing increased size of the pontine cavernous malformation with compression on the mesencephalon, the cisterna interpeduncularis and the cisterna pontis. 2) 321CCM patient harbouring the mutation p.R35X. A) Imaging characteristics of the CCM lesion (in the white circle) located at the left anterior temporal lobe: at CT scan the lesion is inhomogeneous due to haemorrhagic components, B and D) the haemorrhagic component is hyperintense both in T1 and in T2 sequences, C) contrast enhancement is absent, E) and at GET2* the lesion is hypointense due to the paramagnetic characteristics of the haemosiderin ring and of the clotted lesion content. F) Finally, other two lesions can be detected at other sites (arrows) in the same patient. 3) 344CCM patient harbouring the novel mutation p.R54X. A) MRI showed a right cortical and subcortical parietal hemorrhagic CCM lesion (arrow) and other non-haemorrhagic CCM lesions at different sites: bilateral temporal polar (not shown), B) left superior temporal sulcus (arrow) and right parietal (arrowhead), left insular and fronto-insular (not shown), C) left frontal parasagittal (arrow), subcortical frontal with small areas of vacuolization and microcalcification, left posteromedial thalamic (not shown).

doi:10.1371/journal.pone.0110438.g002
earlier age at onset we observed in patients mutated in this gene compared to those mutated in KRIT1/CCM1 or MGC4607/CCM2.

Our data appear to indicate that PDCD10 may play a major role in the ternary complex formed by KRIT1/MALCAVER-NIN/PDCD10 proteins and in driving the FCCM associated disorder.

A PDCD10/CCM3 gene mutation [40,41] has to be considered a very rare cause of CCMs, since this mutation have been diagnosed to fewer than 100 people worldwide. Herein we describe 11 additional new cases with a mutation in PDCD10/CCM3 gene which are negative for KRIT1/CCM1 and MGC4607/CCM2 genes. Among the eight mutations identified, four changes have been already reported while other four are described here for the first time; furthermore, we also identified de novo mutations in four different patients. In our cohort de novo mutations seem to be more frequent in PDCD10/CCM3 gene, since no de novo mutations have been found in KRIT1/CCM1 and only a single de novo mutation has been identified in MGC4607/CCM2 mutated cases [11].

We tried to establish a common geographic origin between the patients, but the mutations distribution turned out to be rather heterogeneous.

The majority of the detected mutations generates a stop codon (R35X, R108X, E54Rfs*22, E54X), that leads to the predicted formation of a truncated protein lacking the original function, however further studies are needed to define the effect on the protein of the two duplications, the complex rearrangement and the loss of the entire allele on the final protein.

We observe that the majority of mutations especially occur in exons five and seven (8/11 CCM cases), with a prevalence of the R35X mutation (5/11 CCM cases). Thus, we suggest that in an analytical procedure it would be appropriate to first investigate these exons.

All the described mutations in PDCD10/CCM3 lead to a stop codon, [15,17,19,27–31] that cause the loss of a variable portion of the protein, that generally is the C-Terminal FAT-Homology Domain. Only in a single case (352 CCM) a mutation did not lead to a premature stop codon, but to a in frame duplication of six aminoacids in the FAT-Homology Domain instead. Furthermore this patient showed a late onset disease (35 years) with a milder phenotype compared to other cases. We can hypothesize that this mutation leads to the malfunctioning of the protein but preserves its overall structural integrity and its ability to take interactions with its other partners.

Wide phenotypic variability is present among the reported patients, even within those sharing the same mutation and within the same family. Symptoms range from headache, psychomotor retardation, to attention disorder, haemorrhage and seizures.

Together with Denier’s group findings [28] we observed an earlier onset in symptomatic PDCD10/CCM3 mutation carriers compared to symptomatic patients with a mutation in KRIT1/CCM1 and MGC4607/CCM2 genes. Conversely, we are unable to confirm the association with multiple meningiomas and early onset haemorrhage reported by Riant F. et al., 2013 [5].

The causes of this variability are unknown but are likely associated with other genetic factors, environment or lifestyle (physical exercise and nutrition), as reported by Choquet et al. for a wide cohort of KRIT1/CCM1 patients sharing the Common Hispanic Mutation [49].

Functional characterization of the identified mutations, further in vitro studies and cellular models may help understand the complex mechanisms through which PDCD10 is involved in the CCMs pathology.

Indeed, the functional heterogeneity of PDCD10 makes it difficult to define its role in the pathogenesis of the disease and further studies on wider cohorts of patients, as well as prospective clinical follow up studies, will help to define whether PDCD10/CCM3 mutations are associated to specific clinical features.

Material and Methods

Subjects

Clinically affected CCM probands (index patients) were consecutively enrolled on the basis of one of the two following criteria: each proband had at least one affected relative and/or had multiple cerebral cavernous angiomias. Diagnosis was based on brain magnetic resonance imaging (MRI) features and, when possible, post-surgery histopathological analysis findings: 5/11 patients underwent neurosurgical intervention. Detailed clinical and brain MR imaging data were collected for all patients with symptomatic CCM through direct interview and review of medical records. Clinical assessment focused on the occurrence of seizures, cerebral haemorrhage, focal neurological symptoms, and headache. All the analyzed subjects gave written informed consent and they underwent to a review of their medical records, brain MR imaging, and blood sampling for genetic analysis; their medical records were reviewed as well. Niguarda Ca’ Granda Ethic Committee approved this study. Subjects with cavernomas seen on MR images were considered affected and those with no abnormalities seen on MR images were considered unaffected; those who did not undergo MR imaging were classified as “unknown”.

DNA Extraction, Polymerase Chain Reaction and Sequencing

Genomic DNA from each proband and all consenting relatives was extracted from peripheral blood leukocytes using the salting
out method [30]. All coding exons and the corresponding intron/exon boundaries of KRIT1/CCM1, MGC4607/CCM2 and PDCD10/CCM3 genes were amplified by PCR with a specific subset of primers described elsewhere [51].

Direct sequence analysis was performed using BigDye Terminator Cycle Sequencing kit Version 1.1 (Applied Biosystems) on 3730 DNA automated analyzer (Applied Biosystems). The nucleotide position of variants present in the coding regions refers to the mRNA sequence (NM_007217) with +1 corresponding to the A of the ATG initiation codon.

The novel mutations were not found upon screening by direct sequencing 300 normal control chromosomes; moreover they were not reported in different online genetic databases of control subjects, such as HGMD [52], NHLBI ESP [53] and the 1000 Genome project [54].

Multiplex Ligation-Dependent Probe Amplification Assay

Multiplex ligation-dependent probe amplification (MLPA) was performed on patients who were negative for direct sequencing analysis for mutation in KRIT1/CCM1, MGC4607/CCM2 and PDCD10/CCM3 by using two MLPA kits (SALSA MLPA Kits P130 & P131 CCM, MRCHolland). The P130 probe mix contains probes for part of KRIT1/CCM1 exons and for all MGC4607/CCM2 exons. The P131 probe mix contains probes for the remaining KRIT1/CCM1 exons and for all exons of PDCD10/CCM3 gene. MLPA was performed according to the protocol supplied, by use of 100 ng of DNA sample per reaction, using FAM labelled primers. Samples were run on a 3730 DNA automated analyzer (Applied Biosystems), and data were analyzed with the GeneMapper software version 4.0 (Applied Biosystems) to size the PCR products and to obtain peak areas.

For the visual inspection, peak heights were compared between the samples and the controls, to find any alteration in relative peak heights within the test sample. For the normalized peak area calculations, each peak area was normalized by dividing the individual peak area by the total peak area of all peaks for that sample. See Penco et al., 2009 for details [24].

Short Tandem Repeat Multiplex Assay

STR (Short Tandem Repeat) multiplex assay was performed by using the AmpFISTR Identifier Kit (Applied Biosystems) according to the manufacturer’s instructions. The kit has been designed to amplify 15 tetranucleotide repeat loci and the amelogenin gender-determining marker in a single PCR amplification; a five-dye fluorescent system was used for automated DNA fragment analysis.

Samples were run on a 3730 DNA automated analyzer (Applied Biosystems), and data were analyzed with the Gene Mapper software version 4.0 (Applied Biosystems); allele peaks were interpreted when the peak heights were greater than or equal to 50 relative fluorescence units.

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