

## Cerebral cavernous malformations: clinical insights from genetic studies

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✓ Familial disease is responsible for one third to one half of cerebral cavernous malformation (CCM) cases presenting to clinical attention. Much has been learned in the past decade about the genetics of these cases, which are all inherited in an autosomal dominant pattern, at three known chromosome loci. Unique features of inherited CCMs in Hispanic-Americans of Mexican descent have been described. The respective genes for each locus have been identified and preliminary observations on disease pathways and mechanisms are coming to light, including possible explanations for selectivity of neural milieu and relationships to endothelial layer abnormalities. Mechanisms of lesion genesis in cases of genetic predisposition are being investigated, with evidence to support a two-hit model emerging from somatic mutation screening of the lesions themselves and from lesion formation in transgenic murine models of the disease. Other information on potential inflammatory factors has emerged from differential gene expression studies. Unique phenotypic features of solitary versus familial cases have emerged: different associations with venous developmental anomaly and the exceptionally high penetrance rates that are found in inherited cases when high-sensitivity screening is performed with gradient echo magnetic resonance imaging. This information has changed the landscape of screening and counseling for patients and their families, and promises to lead to the development of new tools for predicting, explaining, and modifying disease behavior.

**KEY WORDS** • cavernous malformation • hemangioma • angioma • genetics • genomics

**C**EREBRAL cavernous malformations are discrete multilobed vascular malformations that consist of a cluster of thin-walled vascular sinusoids. Lined by a single layer of endothelium, they lack intervening neural parenchyma or identifiable mature vessel-wall elements.<sup>8,21,34</sup> Histological analysis shows the lesions to lack an arterial wall smooth muscle layer and frequently reveals a peripheral hemosiderin deposition suggestive of chronic hemorrhage, a CCM hallmark.<sup>34,62</sup> Electron microscopy analyses have implicated defective endothelial tight junctions as a potential explanation for the propensity for hemorrhage seen in these lesions.<sup>8,60</sup>

Cavernous malformations are most commonly found in the cerebral cortex, although they may also occur in the brainstem, spinal cord, retina, cranial nerves, and cerebral ventricles.<sup>3,6,11,17</sup> Although CCMs are often clinically silent, patients with these lesions may present with hemorrhage, focal neurological impairment, headache, and/or seizure.<sup>31,42</sup> Cerebral cavernous malformations may also predispose patients to hemorrhagic stroke and epilepsy.

The prevalence of these lesions has not been clearly elucidated. Data gathered from autopsy studies and retro-

spective cohort studies suggest that CCMs comprise from 5 to 13% of all cerebrovascular malformations and that from 0.3 to 0.6% of the general population harbors these lesions.<sup>11,17,24,35,48</sup>

### Molecular Genetics of CCMs

Researchers have made considerable progress in understanding the genetics of CCMs by focusing on familial forms of the disease, although both familial and sporadic cases are recognized. Using a positional cloning strategy founded on linkage analysis, investigators have identified three loci in inherited CCMs: *CCM1* on human chromosome arm 7q; *CCM2* on 7p; and, most recently, *CCM3* on 3q.<sup>9,13,19,24</sup>

In the general population, *CCM1* is thought to be responsible for approximately 40 to 50% of inherited cases of CCM, and *CCM2* and *CCM3* are estimated to account for 10 to 20% and 40% of cases, respectively.<sup>9,56</sup> The prevalence of CCMs is higher in Hispanic-Americans than in other ethnic groups.<sup>20,22</sup> Gunel and colleagues<sup>20</sup> compared the segregation of genetic markers and clinical cases of CCM in Hispanic-American kindreds with familial disease and concluded that virtually all cases of familial and sporadic CCM in this population were due to the inheritance of the same mutation from a common ancestor. The apparent paradox of sporadic cases being due to the same mutation as familial cases can be explained by incomplete penetrance and misdiagnosis of disease, especially in ear-

*Abbreviations used in this paper:* AVM = arteriovenous malformation; CCM = cerebral cavernous malformation; cDNA = complementary DNA; ICAP = integrin cytoplasmic domain-associated protein; Ig = immunoglobulin; KRIT = Krev interaction trapped; mRNA = messenger RNA; VEGF = vascular endothelial growth factor; STA = superficial temporal artery.

lier generations of the same kindred.<sup>9,26,58</sup> The use of gradient echo MR imaging results in higher estimates of prevalence and increases detection of multiple lesions (Fig. 1), many of which may be small and asymptomatic.

Insight into the pathogenesis of CCM has in part been fueled by the recent development of the capacity to clone all three CCM genes, which has enabled researchers to compare the molecular differences between sporadic and inherited lesions.<sup>4,12,27,29,47</sup> Inherited CCMs are typically multiple lesions, whereas sporadic cases are mostly solitary lesions and often occur in association with developmental venous anomalies. Sporadic cases of multiple lesions may be due to unrecognized familiarity (occult germ line mutations as seen in many cases involving Hispanic patients of Mexican descent), multiple CCMs in association with a single developmental venous anomaly (Fig. 2), or multiple CCMs after craniospinal irradiation.<sup>1,20</sup> Several groups of researchers have supported this premise by demonstrating a distinct absence of *CCM1* or *CCM2* mutations in cohorts with sporadic single CCM lesions.<sup>44,57,58</sup>

#### Two-Hit Mechanism

The pathogenesis of CCMs has been proposed to follow the two-hit model proposed by Knudson and colleagues<sup>25</sup> in their description of retinoblastoma formation caused by mutations in the *RB* gene, the first tumor suppressor gene discovered. The two-hit mechanism has also been demonstrated in cyst formation in autosomal dominant polycystic kidney disease.<sup>37,43</sup> In this model, both alleles coding for a particular gene or factors affecting their function must be rendered inactive before lesion formation occurs.

Although germ line mutation of both copies of the CCM gene is embryonic lethal,<sup>38</sup> individuals who are heterozygous for an inherited mutation exhibit a predilection for disease formation but require a second somatic mutation (second hit at the lesion site) for a CCM lesion to develop.

The mechanism of biallelic somatic mutation has recently been confirmed in humans.<sup>16</sup> It is possible that the second hit might arise from mutation of any factor in a related disease pathway or in another disease gene (transheterozygous mutation), or from a genetic cause (for example, a *p53* mutation) or environmental effect predisposing to mutation (for example, radiation exposure). Indeed, mice heterozygous for *CCM1* are more likely to develop CCM lesions if they also have a mutation of a *p53* suppressor gene<sup>38</sup> (Fig. 3).

#### The *CCM1* Locus at 7q

The *CCM1* gene was positionally cloned by linkage, haplotype, and mutation analyses mainly in patients with familial CCM and a Hispanic-American ancestral disease haplotype in the 7q11.2–21 region and a common mutation in *CCM1* that encodes the CCM1 or KRIT1 protein.<sup>13,41,47,49</sup> The *CCM1* gene consists of 20 exons spanning 45,799 base pairs and maps to the 7q11.2–21 region. The start of translation seems to be in exon 5, and approximately 88 different germ line mutations distributed throughout the *CCM1* gene have been described in association with CCM in several different racial groups.<sup>7,10,27</sup> The mutations in *CCM1* described to date all presumably result in premature truncation of the CCM1 protein with loss of function. Germ line *CCM1* mutations have been identified in apparently

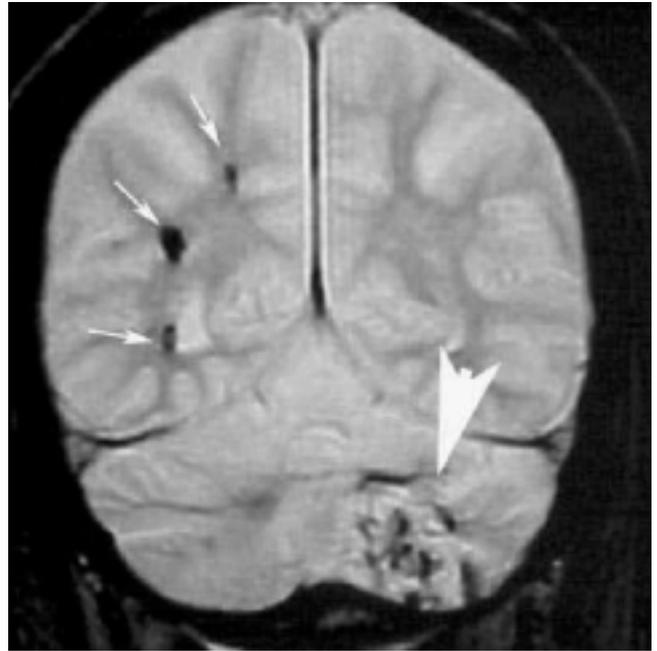


FIG. 1. Axial gradient-echo MR image revealing multiple small lesions (*small arrows*) not seen on conventional T<sub>1</sub>- and T<sub>2</sub>-weighted MR sequences. The larger cerebellar lesion, which was symptomatic (*large arrow*), was seen on T<sub>1</sub>- and T<sub>2</sub>-weighted images and was thought to be solitary. Gradient echo sequences enhance detection for CCM lesions, leading to greater estimates of lesion multiplicity and penetrance in familial disease.

sporadic CCM cases that were caused by unrecognized familial or spontaneous germ line mutations.<sup>28,32</sup> Two different somatic mutations in *CCM1* were identified in DNA isolated from a lesion surgically excised from a patient who did not have a family history of the disease and who harbored a single CCM, supporting a two-hit hypothesis of lesion genesis.<sup>16,23</sup> In the two-hit model, a vascular cell with two mutations (either germ line or somatic), resulting in complete loss of functional CCM1 protein, expands clonally to form a CCM. Presumably, the multiple CCM lesions found in familial cases result from the same germ line mutation of one copy of the gene found in every cell, plus a second somatic mutation in the lesions. Lesions are probably a mosaic of normal cells morphologically disrupted by abnormal cells with two hits. Familial CCM exhibits an autosomal dominant mode of inheritance but is likely to be recessive at the cellular level, and lesion genesis may require two hits to the same gene or a related one. Other mechanisms such as haploinsufficiency or transheterozygous mutations (somatic mutations at *CCM2* or *CCM3* in addition to a *CCM1* somatic mutation) are also possible.

Since the identification of the *CCM1* gene, investigators have focused on functional characterization of the CCM1 protein. Three functional domains have been predicted on the basis of sequence homology with known proteins and protein–protein analysis using yeast two-hybrid screening. The two-hybrid system is set up to detect transcription of a reporter gene either by colorimetric tests or selection for growth. Reporter gene transcription depends on association

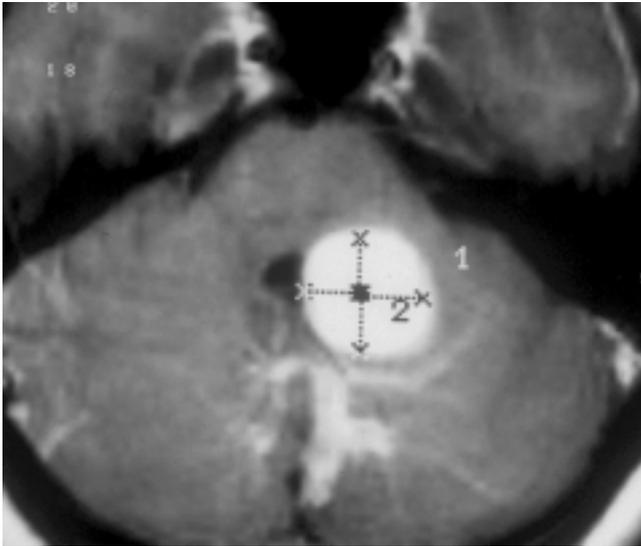


FIG. 2. Axial contrast-enhanced T<sub>1</sub>-weighted MR image showing a venous developmental anomaly associated with CCM lesions. Most cases of CCM associated with gross venous developmental anomaly are not associated with known germ line mutations or genetic disease.

of a DNA-binding domain fused to the gene of interest and a transcriptional activation domain fused to many different genes that may interact with the gene of interest. Protein–protein interactions are identified when a yeast colony grows or turns blue, indicating that the fusion protein containing the gene of interest is interacting with one of the fusion proteins containing the activation domain and allowing transcription of the reporter gene. The NPXY motif in the amino terminal of CCM1 apparently binds ICAP1, suggesting that CCM1 is part of the integrin signaling pathway (specifically in  $\beta 1$  integrin complexes) and cell adhesion to other cells as well as to the extracellular matrix.<sup>50,63,65</sup> At least eight of 22 integrin heterodimers are expressed on angiogenic and quiescent vascular endothelial cells, including five with  $\beta 1$  integrin components.<sup>53</sup> Integrins are activated by VEGF and fibroblast growth factor 2 signaling in angiogenesis.<sup>53</sup> Ankyrin repeats in the middle of the CCM1 protein are thought to be involved in protein–protein interaction. Expression of the band F ezrin-radixin-moesin homology domain found in exons 14 through 18 of *CCM1* has been found in proteins that link cytoplasmic proteins to transmembrane proteins.

The carboxy 1 terminal of CCM1 interacts with Ras-related protein 1A (RAP1A, also known as Krev-1/rap1a), a member of the Ras family of guanosine triphosphatases, with a yeast two-hybrid screen, suggesting a tie to the tumor suppression pathway, possibly at the point where Ras becomes part of the integrin pathway.<sup>5,50,63</sup> The CCM1–RAP1A interaction may not be biologically relevant, because the reciprocal bait-prey swapping with the yeast two-hybrid system does not show interaction.<sup>33</sup> The *CCM1* gene is transcribed as a 350-kb message in brain and additional tissues not known to be affected in patients with CCM (heart and muscle).<sup>14,23,50</sup> In the presence of CCM1 antibodies, CCM1 colocalizes with  $\beta$ -tubulin in endothelial cells in culture and is thought to interact with the cytoskeleton to determine cell shape through cell–cell and cell–matrix

interactions. This functional link appears relevant, as it may result in impaired endothelial cell junctions during a critical phase of angiogenesis, and hence result in the dilated leaky capillaries of CCM lesions.

#### The CCM2 Locus at 7p

Recently, two independent groups identified a novel gene at the *CCM2* locus by means of sequencing of positional candidate genes<sup>12</sup> and loss-of-heterozygosity mapping.<sup>29</sup> This gene, *MGC4607*, encodes a protein similar to the KRIT1 binding partner ICAP-1 $\alpha$ ; this protein, which contains a phosphotyrosine-binding domain, may be part of the complex pathway of integrin signaling that, when perturbed, causes abnormal vascular morphogenesis in the brain, leading to CCM formation.<sup>29</sup>

It is likely that in addition to functioning as an ICAP1 binding partner, CCM1 influences CCM2 function.<sup>40</sup> There is evidence to suggest that KRIT1 and the CCM2 protein (malcaverin) interact and share a common functional pathway.<sup>64</sup> The CCM1/CCM2 association is dependent upon the phosphotyrosine-binding domain of CCM2. This interaction between these two proteins appears to be critical for p38 mitogen-activated protein kinase activation and/or for regulation of integrin-mediated adhesion by ICAP1.<sup>64</sup> This premise is supported by the observation that the familial *CCM2* missense mutation *L198R* disrupts the CCM1–CCM2 interaction, suggesting that this interaction is pertinent to the pathogenesis of CCM.<sup>12</sup> The p38 signaling pathway plays an essential role in the pathogenesis of CCM, and mutations of the *p38 $\alpha$*  gene have led to embryonic death in mice due to placental defects ascribed to decreased vascularity, increased apoptosis, and aberrant angiogenesis.<sup>2,36,61</sup>

Although the molecular functions of the CCM genes have begun to be elucidated, it remains unclear which cells within the brain ultimately result in the formation of CCMs. Uncertainty remains as to whether cavernous malformations are caused by a defect intrinsic to the endothelial cells or by defects in the brain parenchyma that surrounds the vessels. In an attempt to answer this question, Plummer and colleagues<sup>39</sup> used the gene trap allele and in situ hybridization to characterize the expression pattern of *CCM2* in the adult mouse brain. They found that *CCM2* expression in the adult brain is primarily neuronal with additional expression in choroid epithelium and that *CCM2* is not expressed at significant levels in vascular endothelium within the brain. Therefore, cavernous malformations probably arise from abnormalities in surrounding neuronal and glial cells rather than any defect intrinsic to the endothelium.

#### The CCM3 Locus at 3q

Identification of *PDCD10* as the *CCM3* gene has been one of the most recent advances in unveiling the pathogenesis of CCMs.<sup>4,18</sup> Using loss-of-heterozygosity mapping, Bergametti and colleagues<sup>4</sup> found seven distinct mutations in eight unrelated families included on the basis of a negative *KRIT1* (*CCM1*) and *MGC4607* (*CCM2*) mutation screening. The nature of some of these mutations, particularly the deletion of the whole gene observed in one family, strongly suggests that one of the mechanisms that leads to cavernous angiomas might be *PDCD10* haploinsufficiency.

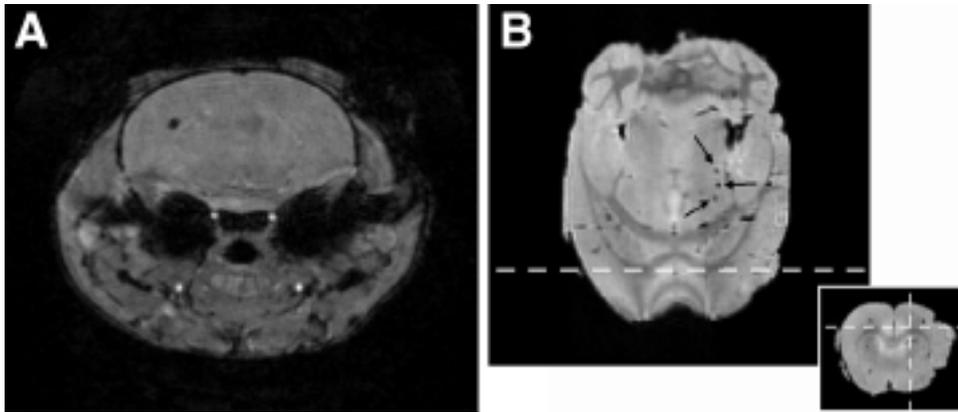


FIG. 3. A: A 4.5-tesla MR image of a CCM lesion in a transgenic mouse ( $ccm^{+/-}; p53^{-/-}$ ). B: An MR image of the brain of a mouse with these mutations revealing numerous CCM lesions not seen in animals without such a mutation or those with  $CCM^{+/-}$  or  $p53^{-/-}$  alone. Unpublished data.

On the basis of multiloci linkage data, investigators have suggested that in 40% of families with CCMs, the CCMs are linked to the *CCM3* locus.<sup>9</sup> Because the families screened in this study were included on the basis of negative results of *KRIT1* (*CCM1*) and *MGC4607* (*CCM2*) mutation screening, a higher proportion of families with a *PDCD10* mutation would have been expected. Several hypotheses have been proposed to explain this paradox, including the possibility that a fourth CCM locus may exist.<sup>4,30,59</sup>

### Differential Gene Expression

Differential gene expression can be measured at the level of transcription (mRNA) or protein and can be used for both confirmation and discovery of gene involvement in disease. Gene transcription is quantitated on cDNA or oligonucleotide arrays allowing simultaneous assessment of the expression levels of thousands of genes. Complementary DNA arrays are generally more sensitive for measuring less abundant mRNA, and oligonucleotide arrays are generally more gene-specific due to less cross-hybridization. Gene-specific cDNA or oligonucleotide arrays are attached to quartz wafers, glass slides, or nylon membranes, and hybridized with cDNA that has been reverse transcribed from mRNA isolated from the tissues of interest. Hybridization can be separate, one chip per isolated mRNA or combined with one color for each cDNA set. Gene expression array results should provide confirmation that the known disease genes may actually be differentially expressed and may implicate previously unsuspected genes, including genes with unknown functions.

Results from gene expression array experiments must be verified independently using Northern Blot analysis and quantitative reverse transcription polymerase chain reaction to confirm gene expression differences and followed up with in situ hybridization using gene-specific probes; hybridizing to tissue sections is necessary to characterize tissue-specific gene expression. Confirmed differential gene expression does not necessarily mean that the protein is present at higher levels, and promising findings should be extended to include quantification of protein expres-

sion. Identifying differential protein expression is more labor intensive than measuring gene expression and depends on optimal separation of proteins. Proteomics allows the identification of hundreds of proteins, and mass spectrometric and other methods are now available for reliable and rapid quantification and identification of differentially expressed proteins in biologic tissues. When antibodies are available for specific proteins, immunoprecipitation, Western blot analysis, and immunohistochemical methods can verify protein quantification and determine tissue-specific expression. Proteomic studies in general would corroborate only a subset of the differential gene expression results (only proteins present in sufficient quantities that they can be resolved using two-dimensional gel electrophoresis), and gene transcription levels do not always correlate with protein levels.<sup>15</sup>

Gene and protein expression data are compiled and analyzed with computer programs, and the statistical analyses take thousands of comparisons into account. Normalization of expression levels per unit of tissue or mRNA, appropriate controls for differential expression, and the problem of tissue heterogeneity must each be considered in analyzing and interpreting the data. Advanced bioinformatics methods are being developed, including complex statistical approaches that can factor groups of functionally related genes and proteins (such as those involving related pathways), rather than assuming independent expression of each gene and protein.

The expectation that some vasculogenesis-, angiogenesis-, and disease-related genes are differentially expressed in cerebral vascular malformations has been partially confirmed.<sup>51,52</sup> Preliminary gene-expression results identified 310 upregulated and 558 downregulated genes in both AVMs and CCMs compared with STAs ( $p = 0.012$ ), including differences in genes involved in growth factor signaling (decreased *ANGPT1*; increased *VEGF* [a trend]; and increased *ENG*, endoglin, a TGF $\beta$  receptor component), decreases in a cell adhesion gene (*PECAMI1*, alias *CD31*), decreases in an endothelium-specific gap junction gene (*GJA4*), and decreases in extracellular matrix genes (*LAMA3* and *SMTN*).<sup>51,52</sup> Increased protein expression measured by immunohistochemical analysis of VEGF<sup>45,46</sup>.

## Clinical insights from genetic studies

<sup>54,55</sup> and decreased expression of *LAMA3* and *SMTN*<sup>54</sup> in both AVM and CCM in comparison to STA is consistent with results of transcript quantitation.

In addition, CCMs showed unique decreased expression of a VEGF receptor (*KDR*), cell adhesion molecules involved in integrin signaling (*ITGB3*, integrin  $\beta 3$ ; *ROCK1*) in comparison to AVMs and STAs. The AVMs showed specific differential gene expression of growth factor signaling molecules (increased *FLT1*, a VEGF receptor; decreased *TIE* and *TEK*, angiopoietin receptors), decreased integrin signaling molecules (*ITGB5*, integrin  $\beta 5$ ; *ITGA6*, integrin  $\beta 6$ ; *CTNNA1*,  $\alpha$ -catenin) and decreased *CCM1* gene expression.<sup>51</sup>

Notably, probe sets for 10 Ig genes and a distinct allele of the major histocompatibility complex (*DQB1\*06011*) were upregulated in CCMs compared with AVMs and STAs, revealing possible evidence of a unique immune response within the CCM lesions. Expression for Ig genes that were upregulated in CCMs included the IgA heavy-chain gene of two accession numbers (AF067420 and S71043, with 26- and 17-fold increases, respectively); the *IGJ* gene (AI660656) for the IgJ chain with a 23-fold change; the *IGHG3* gene (Y14737) for the heavy chain of IgG subclass 3 with a 20-fold change; three Ig light-chain genes, the *IGLJ3* gene (M18645) with a 15-fold change; the *IGL* gene (X57809 and AF058075, with changes of 15- and threefold, respectively); the *IGKC* gene (M63438 and X72475, with changes of 14- and 12-fold, respectively); the *IGHM* gene (AI147237) for the heavy chain of IgM with a ninefold change; the Ig-related *LOC91316* gene (AI932613) with a 22-fold change; and the gene (U80114) for the hypothetical Ig MGC 27165 with a sixfold change. The increases in expression of the Ig genes were among the highest in the microarray analysis. In addition, the gene for the IgG Fc (CD64) receptor was upregulated in CCM with a 2.4-fold change. Among the upregulated genes in CCMs, the greatest degree of upregulation (43-fold) was for a probe set (M16276) for a 3' untranslated region of the *DQB1\*06011* allele of the gene for HLA-DQB.

Numerous observations have bolstered our confidence in the microarray results: 1) significant differential expression was shown in the microarrays for genes coding angiogenesis factors, receptors, and structural proteins that had previously been determined through immunohistochemical analysis to be expressed differentially in CCMs and AVMs; 2) downregulation of the expression of mature vessel wall components in CCMs, such as myosin, actin, calponin, desmin, transgelin, and filamin A endothelial actin binding protein; 3) differential expression of Igs was shown in CCMs but not in AVMs; and 4) constant expression of hemoglobin and leukocyte markers. The mean values  $\pm$  the standard deviations for the expression in AVMs, CCMs, and STAs, respectively, were 109,144  $\pm$  66,212, 62,611  $\pm$  7,520, and 51,385  $\pm$  18,489 for hemoglobin B and 264  $\pm$  28, 246  $\pm$  74, and 207  $\pm$  33 for the B-cell marker CD19.

The CCM phenotype predisposes to vascular leakage and accumulation of blood products in adjacent brain tissue. This situation may create a special milieu for antigenic challenge and immune response, which may in turn contribute to lesion proliferation and clinical manifestations. The extent of inflammatory cell infiltration within lesions could motivate future studies on the imaging of these cells in vivo, with the aim of monitoring and modi-

fying biological activity in the lesions.

## Conclusions

The identification of the three CCM genes has provided significant insight into the molecular basis of CCM formation. Coupled with our knowledge about the mechanisms of vasculogenesis and angiogenesis, our understanding of the pathogenesis of these lesions is steadily expanding. The convergence of clinical and biological information holds promise of a new era in which clinicians will be better able to translate scientific advancement to clinical applications for the treatment of this lesion.

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