

Minireview

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Non-autonomous effects of CCM genes loss

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Abstract

Cerebral cavernous malformation (CCM) is a rare disease of proven genetic origin characterized by vascular lesions affecting capillaries and small vessels of the central nervous system. CCM lesions occur in a range of different phenotypes, including wide differences in lesion number, size, and susceptibility to intracerebral hemorrhage. CCM lesion genesis requires loss of function of any of three genes, namely *KRIT1* (*CCM1*), *MGC4607* (*CCM2*), and *PDCD10* (*CCM3*). These genes exert pleiotropic effects regulating multiple mechanisms involved in angiogenesis, cellular response, cell-cell and cell-matrix adhesion, cytoskeleton dynamics, and oxidative damage protection. Familial CCM is an autosomal-dominantly inherited disease in which the loss of any of the three CCM genes follows a two-hit mechanism. The heterozygous loss-of-function germline variants in one of the involved genes seems to be associated with a second postzygotic mutation, according to Knudson's two-hit model of tumor suppressor genes. This review is an overview of very recent literature on CCM onset and progression focused on the novel concept that the loss of a CCM gene in a single cell is sufficient to induce vascular lesions. Mutated cells undergo clonal expansion and become able to promote the recruitment of non-mutated cells and to induce their angiogenic switch through the increased production of angiogenic factors and downregulation of antiangiogenic factors. A deep understanding of this process and the knowledge of unbalanced secreted factors will be useful to design new pharmacological strategies for CCM patients.

Keywords: Cerebrovascular disease, cerebral cavernous malformation, KRIT1, CCM1, CCM2, CCM3, endothelial cells, mosaicism



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INTRODUCTION

Cerebral cavernous malformation (CCM), is a rare disease characterized by “mulberry-like” vascular lesions present in the brain (caverns) with abnormal ultrastructural features of vessels. The thin vessel wall is enlarged and leaky and composed of amorphous material lacking endothelial support and devoid of normal and intact tight junctions and vessel structural components, such as pericytes and astrocyte foot processes^[1].

CCM lesions are predominantly found in the central nervous system (CNS) but can be present also in the retina, skin, and liver. In the brain, CCM lesions can occur as single or multiple (even hundreds) lesions, with sizes varying from a few millimeters to a few centimeters, depending on the pathogenesis^[2].

Despite the high prevalence of CCM lesions, the development of clinical symptoms is extremely variable, and approximately only 30% of affected people show headaches, focal neurological deficits (25%), seizures (50%), and symptomatic hemorrhage (25%)^[3]. Symptoms may occur at any age, although the typical age of onset is between the second and fifth decade of life and without sex predominance, even though the available data are conflicting. Moreover, the majority of CCM lesions remain clinically and biologically quiescent during the most of the host’s lifetime^[4-6]. To date, diagnosis is made only by magnetic resonance imaging (MRI), and it is mainly possible only when lesions become symptomatic^[3]. The most commonly used classification of CCMs in the literature was proposed by Zabramski *et al.*^[7]. Individual cavernous malformations are divided into four categories on the basis of pathological correlations and magnetic resonance signal characteristics: Type I, characterized by the presence of subacute hemorrhage; Type II, with loculated areas of hemorrhage and thrombosis of varying age; Type III, with chronic areas of hemorrhage; and Type IV, with small multiple punctate microhemorrhages^[7].

CCM is a disease of proven genetic origin (OMIM 116860) that may arise sporadically or can be inherited as an autosomal dominant condition, with incomplete penetrance and variable expressivity. The sporadic form accounts for up to 80% of cases and is characterized by a lack of family history of the disease and the presence of a single lesion on MRI. The familial form accounts for at least 20% of cases and is generally characterized by the presence of multiple CCM lesions in the brain associated with cutaneous and retinal vascular lesions in 9% and 5% of familial CCM cases^[8]. Human genetic studies have so far identified three genes whose heterozygous loss-of-function mutations are associated with CCM disease: *KRIT1* (*CCM1*), *MGC4607* (*CCM2*), and *PDCD10* (*CCM3*). The mutation rates for each gene range between 53% and 65% for *KRIT1/CCM1*, 15% and 19% for *CCM2*, and 10% and 16% for *CCM3*^[9,10]. However, genotype does not entirely explain the large clinical variability of CCM disease, even among family members carrying the same mutation, suggesting that genetic and environmental modifiers can contribute to CCM disease^[11]. Despite the differences between families, some genotype-phenotype correlations are delineated. While *CCM1* and *CCM2* induce similar characteristics with later manifestation, mutations on *CCM3* drive an early age, aggressive phenotype, with higher risk for cerebral hemorrhage and propensity to multiple meningiomas^[2].

At the molecular level, *CCM* gene loss induces the activation of different signaling pathways^[12-14]. In particular *CCM* deficiency have been associated with hyperactivation of the MEKK3-MEK5-ERK5 kinase cascade, that leads to KLF2 and KLF4 upregulation and subsequent altered expression of bone morphogenic protein 6 (BMP6), thrombomodulin (TM), and thrombospondin 1^[15-18], with activation of RhoA-ROCK signaling, and with alteration of the junctional signaling including VE-cadherin and β -catenin^[19,20]. A significant alteration of reactive oxygen species balance has been also extensively described^[13,21-23].

At present, the pharmaceutical therapy available for the treatment of CCM is very restricted and limited to the treatment of clinical symptoms, as headaches, seizures and epilepsy. The clinical approach to CCM

disease depends on the position and size of the lesions and its association with hemorrhages or intractable seizures. When possible, the microsurgical resection of lesions is the eligible route^[11,24]. Therefore, the development of new pharmacological strategies is necessary to prevent lesion formation and for treating patients with severe inoperable disease or with multiple lesions. The knowledge of molecular mechanisms at the basis of disease onset appears in this context fundamental to identify new drugs able to inhibit the dysregulation of specific signaling pathways and aberrant immune responses. Recent studies focus on different molecules that are under investigation due to their ability to revert the *CCM* gene loss effects, such as Vitamin D3 and Tempol (reactive oxygen species inhibitors), atorvastatin and fasudil (Rho/Rock signaling inhibitors), propranolol and bevacizumab [angiogenesis and vascular endothelial growth factor, vascular endothelial growth factor (VEGF), inhibitors], sulindac, LY-364947, and SB-431542 (endothelial-mesenchymal transition inhibitors), TM, and endothelial protein C receptor (EPCR) inhibitors. However, at the present there are no therapies that have demonstrated an effective activity on reducing *CCM* lesion formation^[3,14,24].

CEREBRAL CAVERNOUS MALFORMATIONS ONSET AND DEVELOPMENT

As mentioned, *CCM* lesions are a consequence of a complete loss-of-function of any of the three *CCM* genes. In familial cases, mutation is inherited as a constitutional germline mutation with incomplete penetrance, and is associated with a biallelic somatic mutation that occurs in the same cell. In particular, *CCM* genesis may follow a “two-hit” mechanism in which loss of one of the two alleles (first hit) would be the result of the germline mutation and loss of the second allele (second hit) will occur somatically^[25-28].

CCM onset might be explained with the Knudson’s two-hit model of tumor suppressor genes. According to this model, tumors derive from two mutations, one for each of the two alleles of the gene of interest. At the present, several cancer genes exhibit biallelic disruption and truncating point mutations, revealing the validity of the model^[29]. According to Knudson hypothesis, vascular lesion formation derives from two mutational events in both hereditary (familial) and non-hereditary (sporadic) cases. In familial *CCM* the first mutation occurs before conception (germline mutation) and the second after conception (somatic mutation). In sporadic *CCM* only somatic mutations are present.

However, the mechanisms at the basis of these mutations are unknown and some questions still remain open. It is not completely understood if a single second somatic mutation for familial *CCM* is sufficient to induce vascular lesion formation, where the mutant cells reside within the cellular architecture of the vascular wall and how this second mutation can promote vascular malformation.

In familial *CCM*, the identification of the somatic mutation remains technically challenging also by using very sensitive approaches as next-generation sequencing techniques. The expected allele frequency of somatic mutation within vascular lesions is low due to the tissue nature, characterized by a vascular structure with a single monolayer of endothelial cells. However, some reports describe a genetic two-step inactivation of all the three genes involved in *CCM* onset^[25-27,30-32].

In the last three years new experimental evidences, supported by mouse models and sophisticated cellular and molecular biology techniques, sustained the idea that the loss of a *CCM* gene in a single cell may be responsible for *CCM* development^[30,33,34].

Detter *et al.*^[33], by using a *CCM3 KO* mouse model, showed for the first time that vascular lesions are composed of both mutated and non-mutated endothelial cells. The authors propose a model in which endothelial cells acquire a somatic mutation promoted by a second hit, and undergo clonal expansion

subsequent to the acquisition of a high proliferative capability. This clonal expansion is followed by a massive recruitment of non-mutated endothelial cells that become part of the lesion [Figure 1]^[33].

These first observations for *CCM3* dependent cavernomas have been confirmed by Malinverno *et al.*^[34]. In both mice and humans, the endothelial cells lining the lesion appear as a mosaic, formed by both mutated and non-mutated endothelial cells. Only a very small part of this mosaic is made up of *CCM3*^{-/-} endothelial cells (about 15% of total endothelial cells of the lesion). Malinverno *et al.*^[34] report interesting data demonstrating that *CCM3*^{-/-} cells are able to recruit surrounding *CCM3*^{+/+} cells and to induce them to undergo endothelial-mesenchymal transition (EndMT) through BMP6. Therefore, an important event in the evolution of the cavernomas appears to be the attraction of *CCM3*^{+/+} cells by *CCM3*^{-/-} cells, which, in turn, strongly contribute to the growth of the malformation^[34].

These new findings on *CCM3* loss dependent vascular lesions have been confirmed also for *CCM1/KRIT1*. In the paper by Rath *et al.*^[30], immunohistochemical analysis of samples of a patient with double *KRIT1* mutation revealed scattered immunopositivity and immunonegativity of *CCM1* staining in endothelial of caverns, while *CCM2* and *CCM3* were uniformly immunopositive, indicating that the second *KRIT1* postzygotic mutation occurs only in a little number of endothelial cell present in the lesion.

In the light of the above observations, it is evident that CCM vascular lesions are not composed of a homogenous layer of mutated endothelial cells, but may be defined as a mosaic of CCM mutated and non-mutated endothelial cells [Figure 1]. These observations could also explain the difficulties in the identification of the second postzygotic mutations, as the reduced number of mutated endothelial cells in vascular lesions imposes the use of extremely sensitive techniques. Moreover, the mechanism by which wild type endothelial cells become incorporated in the growing malformation is unknown even under investigation. The identification of specific signal molecules able to promote the recruitment of wild type endothelial cells into growing lesions may represent a challenge for the identification of new therapeutic strategies.

NON-AUTONOMOUS EFFECTS OF CCM GENE LOSS

The hypothesis that *CCM* mutated cells become able to recruit non-mutated cells was initially formulated for *CCM3* by Louvi *et al.*^[35]. The authors showed that loss of *CCM3* in astrocytes induces both cell autonomous and non-autonomous effects. *Ccm3*^{-/-} astrocytes showed increased proliferation and survival through the activation of Akt signaling, but were also able to induce vascular lesion formation in mouse models, with diffusely dilated and simplified vascular tree, similar to human cavernomas^[35]. Similarly, Wang *et al.*^[36], demonstrated in a mouse model that *Ccm3* deletion in mural cells (pericytes and smooth muscle cells) induces CCM lesions throughout the whole brain in mice. Vascular lesions are characterized by a reduction in pericyte coverage and reduction of tight and adherens junctions of endothelial cells. In addition, *Ccm3* loss in brain pericytes reduced their migration and association with endothelial cells^[36]. Finally, very recently, Lopez-Ramirez *et al.*^[37] proposed an interesting model in which the crosstalk between astrocytes and endothelial cells drives vascular lesion formations. They showed that astrocytes are the major source of VEGF during CCM development. The increased amount of VEGF production in astrocytes is linked to increased hypoxia-inducible factor-1 α (HIF-1 α) activity in normoxic conditions promoted by the augmented amount of nitric oxide (NO) produced by neighboring endothelial cells. The elevation of NO production by endothelium is due to endothelial nitric oxide increasing after the elevation of the transcription factors KLF2 and KLF4, previously implicated in the CCM pathogenesis. In addition, they showed that the inhibition of cyclooxygenase-2 (COX-2), a *HIF-1 α* regulated gene, ameliorates the development of CCM lesions in animal models^[37]. These data confirm that *CCM3* deficient stromal cells

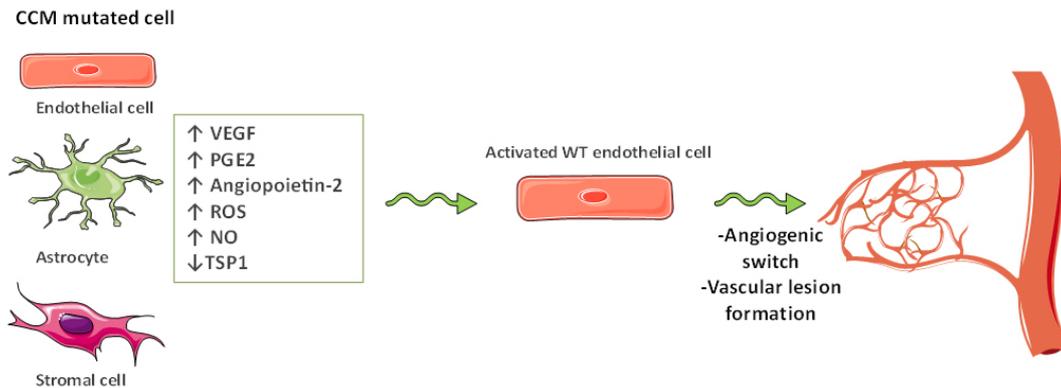


Figure 2. The model by which endothelial cells, astrocytes, or stromal cells lacking one of cerebral cavernous malformations (CCM) genes induce the activation of non-mutated endothelial cells and the consequent formation of vascular lesions. This figure was created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License; <https://smart.servier.com>. VEGF: vascular endothelial growth factor; PGE2: prostaglandin E2; ROS: reactive oxygen species; NO: nitric oxide; TSP1: thrombospondin-1.

increased release of angiopoietin-2, linked to decreased endothelial cell-cell adhesion and pericytes coverage leading to blood vessel disorganization^[46]. In addition *CCM3* loss promotes increased VEGFR and epidermal growth factor receptor expression in both endothelial and non-endothelial cells^[47]. In this scenario, VEGF and PGE2 appear as good candidates to promote the angiogenic switch of quiescent endothelial cells, and VEGF or PGE2 production or signaling cascade inhibition may represent good candidates for CCM therapy^[37,38,48].

Another important connection between CCM and angiogenesis is the evidence that *CCM* gene loss results in impaired DLL-4/NOTCH signaling^[40,49,50]. The earliest report on Notch signaling in CCM pathogenesis came from the studies of Whitehead *et al.*^[52] that demonstrated that vascular defects in mice embryos deficient of *Krit1* gene resulted from the downregulation of Notch-related genes including *Dll4* and *Notch4*. These data were further confirmed by Wüsthube *et al.*^[40] which reported that *CCM1* acts as an anti-angiogenic protein and inhibits endothelial proliferation, migration, and sprouting angiogenesis in human endothelial cells through the induction of DLL4-Notch signaling and the increasing of AKT protein phosphorylation. Consistently, *KRIT1* silencing further led to diminished *DLL4*, *HEY1*, and *HEY2* expression^[40].

Moreover, Maddaluno *et al.*^[15], demonstrated, in *Ccm1* deficient models, that endothelial cells lining the cavernomas undergo an EndMT induced by a strong activation of the phospho-SMAD-dependent signaling pathway linked to an increased endogenous production of the BMP6 ligand^[15]. Endothelial cells that acquire the mesenchymal phenotype are characterized by loss of correct polarization, increased migration rate, and alteration in cell-cell and cell-matrix interactions^[52,53]. Loss-of-function of CCM proteins has also been shown to trigger MEKK3 signaling and to increase the expression of the mechanosensitive transcription factors KLF2 and KLF4 that are considered hallmarks of CCM-related EndMT^[18,24,54,55]. In addition, it has been recently demonstrated that KLF2 and KLF4 upregulation linked to *CCM* loss is also responsible for increased levels of anticoagulant endothelial receptors TM and EPCR, as well as of enhanced generation of activated protein C in endothelial cells that are associated to central nervous system hemorrhage in CCM disease^[56].

In the light of the above reported data, CCM proteins appear to be key regulators of angiogenesis. The capability to promote endothelial cell proliferation and migration, pseudocapillary formation, and activation of angiogenic signal, may be consistent with the new evidence of the presence of mosaicism in CCM vascular lesions. Loss of one of CCM genes appears sufficient to promote endothelial cell mobilization through autocrine and paracrine mechanisms of action [Figure 2].

CONCLUSIONS

In this manuscript we overviewed the very recent advances in understanding the molecular and cellular mechanisms underlying the onset and progression of CCM disease. In the last few years new experimental evidences proposed a new perspective in the study of CCM and a new model for the CCM lesions formation and development. According to these recent studies vascular lesions appear to be composed by a mosaicism of mutated and non-mutated endothelial cells, in which mutated cells, with high angiogenic features, promote the activation of non-mutated endothelial cells that move from quiescent to active state. CCM mutated cells become able to secrete angiogenic factors and to reduce the production of antiangiogenic factors, thus creating an imbalance between pro- and anti-angiogenic molecules that induces the formation of a new defective vascular network.

Despite the few numbers of papers published so far, the data are robust and very promising. Deeper studies on the mechanisms at the basis of mosaicism observed in CCM will lead to the identification of molecular mediators and signaling pathways that contribute to the recruitment of non-mutated endothelial cells into growing vascular network forming the cavernoma. These findings will permit to identify new targets for the development of novel therapies or for evaluating drug repurposing aimed to prevent CCM onset and progression. The possibility of designing therapeutic approaches aimed both at preventing growth of mutated cells and recruitment of non-mutated cells will represent the new era for the treatment of CCM.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Finetti F, Trabalzini L

Availability of data and materials

Not applicable.

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Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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