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Invited Mini Review

Introduction to cerebral cavernous malformation: a brief review

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The disease known as cerebral cavernous malformations mostly occurs in the central nervous system, and their typical histological presentations are multiple lumen formation and vascular leakage at the brain capillary level, resulting in disruption of the blood-brain barrier. These abnormalities result in severe neurological symptoms such as seizures, focal neurological deficits and hemorrhagic strokes. CCM research has identified 'loss of function' mutations of three ccm genes responsible for the disease and also complex regulation of multiple signaling pathways including the WNT/β-catenin pathway, TGF-β and Notch signaling by the ccm genes. Although CCM research is a relatively new and small scientific field, as CCM research has the potential to regulate systemic blood vessel permeability and angiogenesis including that of the blood-brain barrier, this field is growing rapidly. In this review, I will provide a brief overview of CCM pathogenesis and function of ccm genes based on recent progress in CCM research. [BMB Reports 2016; 49(5): 255-262]

INTRODUCTION

The vascular malformations characterizing the disease known as cerebral cavernous malformations (CCMs; OMIM #116860, 603284, 603285) mostly occur in the central nervous system (CNS) and their typical histological presentations are single or multiple lumen formation and vascular leakage at the brain capillary level, aka disruption of the blood- brain barrier (BBB) (1). These abnormalities result in severe neurological symptoms such as hemorrhagic stroke (30-40%), seizures (40-70%), headache (10-30%) and focal neurological symptoms (35-50%) (2). Together with arteriovenous malformation (AVM), CCM is a major cerebral vascular disease entity, albeit showing milder phenotypes than AVM (around 50-80% of CCM cases are asymptomatic) (3, 4). Prevalence of both sporadic and familial type CCMs is estimated to be 0.1-0.5% in the general population and the proportion of familial cases in total CCM cases has been estimated to be as high as 50% in Hispanic-American patients and close to 10-40% in other populations (5, 6).

So far, CCM research has been a small scientific field. However, as CCM research has a good potential to regulate systemic blood vessel permeability and angiogenesis (7-9), importantly those of the BBB and possibly tumor vasculature, the field is now rapidly growing (Fig. 1). Indeed, both *in vivo* and *in vitro* studies revealed that perturbation of the WNT/β-catenin pathway (10, 11), TGF-β/BMP (10, 12, 13) and Notch signaling (14), cytoskeletal regulation (8, 15) and anti-oxidant signaling (16-18) are responsible for CCM pathogenesis and several proteomic studies elegantly showed that all three ccm genes encode CCM proteins comprising distinct macromolecular complexes, implying complex regulation of multiple signaling pathways due to various interactions with many signaling molecules by each CCM protein (19-21). As individual proteins comprising the distinct macromolecular CCM complexes are still not fully characterized, our understanding of the composition of the CCM macromolecular complexes and associated functional networks is still in its infancy. The important unresolved questions in this field are as follows: 1) Why are the phenotypes almost exclusively seen in the CNS, although all the three ccm genes are ubiquitously expressed? 2) How do ccm genes act in formation and maintenance of neurovascular units? 3) What are the functions of ccm genes in non-endothelial cells and extra-CNS endothelial cells? and 4) How to identify the genetic or environmental modifiers that will address incomplete clinical penetrance of CCMs?

MUTATIONS OF CCM GENES

ccm1, ccm2 and ccm3 genes were identified in 1999 (22), 2003 (23) and 2005 (24), respectively. The three genes: ccm1 (Krit1; Krev interaction trapped 1), ccm2 (MGC4607, Malcavernin) and ccm3 (PDCD10), respectively, which are located on chromosomes 7q21.2, 7p13 and 3q25.2-q27 (25, 26), are known to be responsible for familial cases of CCMs and for more than half of the sporadic cases of CCM with multiple lesions (27, 28). Relative frequency of mutations of ccm genes in familial cases is about 53-65%, 15-19% and 10-22% for ccm1, ccm2 and ccm3, respectively (29-31) and familial CCM is an

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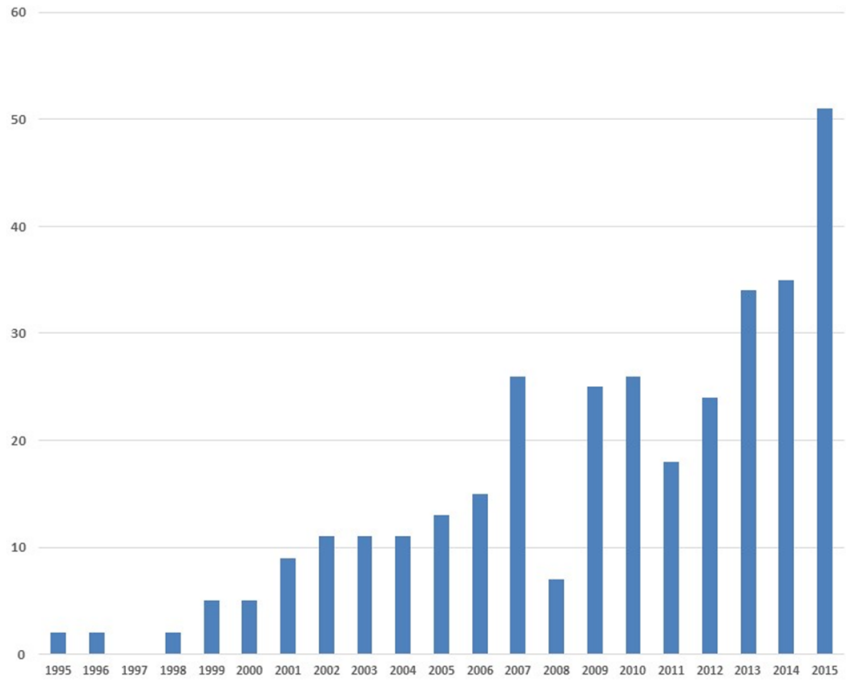

Fig. 1. Annual publication records of CCM from 1995 to 2015. PubMed search using keywords 'Krit1 or ccm1 or ccm2 or ccm3 or cerebral cavernous malformation' yielded 440 publications.

autosomal dominant disease with incomplete clinical and radiological penetrance (1, 3, 32). The existence of additional CCM loci has been suggested as 5-15% of familial cases cannot be explained by the three known ccm genes (31, 33). ccm mutations are also found in sporadic cases (33, 34) and sporadic cases with a single lesion, and not multiple CCM lesions appear to harbor far less ccm mutations (35, 36). Of note, the phenotypes of CCM3 patients or animal models are more severe than those of CCM1 or CCM2 patients or animal models (37-39).

So far, more than 100 distinct CCM1 mutations, 30 CCM2 mutations and 20 CCM3 mutations have been identified and most of the ccm mutations lead to either a premature termination codon or large deletions, strongly suggesting that most of the ccm mutations are 'loss of function' mutations (2, 28).

MECHANISMS OF CCM PATHOGENESIS

It is becoming important to understand how CCM1, CCM2 and CCM3 function, what roles they play in signal transduction, and where do their signaling pathways overlap. The strong interaction between CCM1 and CCM2 appears to be important for the regulation of CCM signaling (40, 41) and evidences imply that the two CCM proteins participate in common signaling pathways (38). CCM3 appears to act in different signaling pathways (37-39, 42). Pathogenesis of CCM follows the Knudsonian two-hit mechanism, in which loss of one allele due to a germline mutation of one of the three known CCM genes in an affected cell (first hit) is accompanied with somatic mutation in the other (second hit) (27, 43-46). Increased vascular permeability was observed both in haplo-insufficient CCM1(+/−) and CCM2 (+/−) mouse endothelial cells in vitro and in lung and liver tissues of CCM1(+/−) and CCM2 (+/−) animals *in vivo* (8), indicating the asymptomatic extra-CNS manifestations. Because only about 30% of humans with CCM lesions will eventually develop clinical symptoms, the existence of a modifier is suggested for the incomplete clinical penetrance.

Activation of RhoA and its effector, Rho kinase (ROCK), induces stress fiber formation, resultant decreased stability of adherens junction and abnormal extracellular matrix (ECM) remodeling, and increases endothelial permeability (47). Ablation of ccm1, ccm2 or ccm3 in endothelial cells has been shown to increase Rho activation (48). CCM1 and CCM2 loss resulted in destabilization of another CCM1 interacting protein, integrin cytoplasmic domain-associated protein-1 (ICAP-1), which increased β1 integrin activation and led to increased RhoA-dependent contractility (49, 50) and commonly activated p38, Akt, and ERK1/2 in endothelial cells (42). Ras-related protein (Rap1)-dependent association of CCM1 with vascular endothelial cadherin at adherens junctions (AJs), with CCM1 dependent cortical cytoskeletal remodeling leads to EC barrier enhancement (7, 8, 50). Aberrant Rho activation was also found in sporadic CCM patients (8, 43). In the early stage of CCM research, the reversal of Rho activation due to inhibition of ROCK in ccm-ablated endothelial cells suggested that Rho ac-

tivation is a major mechanism in CCM pathogenesis (48). However, further studies revealed that Rho/ROCK signaling is not a unique target for CCM disease.

Recent studies elegantly demonstrated endothelial-mesenchymal transition (EndMT) in endothelial cells lining CCMs in tamoxifen-inducible CCM1 loss of function mice (12, 13). EndMT has been previously implicated in cardiac fibrosis and cancer progression and it leads to a modification of the endothelial cell phenotype, resulting in a loss of cellular junctions, acquisition of migratory properties, loss of endothelial-specific markers, and gain of mesenchymal markers. EndMT may occur as a result of upregulation of endogenous bone morphogenetic protein 6 (BMP6) and activation of the transforming growth factor (TGF)-β and bone morphogenetic protein (BMP) signaling pathways. CCM1 is also a Notch activator (14) and loss of CCM1, ICAP1 and CCM3 has been shown to cause downregulation of Notch signaling, leading to increased angiogenesis (51-53). In line with these findings, overexpression of CCM1 caused Notch activation and decreased sprouting angiogenesis after stimulation with VEGF (51). Studies have shown that loss of CCM1-mediated Notch inhibition and Kruppel-like factor 4 (KLF4) induction result in upregulation of BMP6 and resultant EndMT (13, 51). Autophagy appears to be another important mechanism of CCM pathogenesis because ablation of ccm1, ccm2 and ccm3 commonly causes mTOR-ULK1 pathway mediated suppression of autophagy and resultant EndMT (54). Ablation of ccm1 causes increased nuclear β-catenin localization and WNT signaling (15, 48) and Wnt-independent stimulation of β-catenin transcriptional activity precedes TGF/BMP signaling for EndMT (10). Another study revealed that an increase in nuclear β-catenin and VEGF signaling is observed when ccm1 and ccm3, but not ccm2, are ablated (53, 55). Involvement of CCM proteins in VEGF and Notch signaling suggests that the paracrine effect modulated by CCM may also affect non-endothelial cells in the lesion. Indeed, recent reports suggested that ccm3 ablation induced VEGF secretion activated Erk1/2 and AKT in endothelial cells (42, 56), and in a GBM xenograft mouse model, endothelial ccm3 ablation increased tumor progression due to increased proliferation of GBM cells, which indicate autonomous and non-autonomous roles of CCM proteins in tumor progression (56). Also, another report, which suggested that ccm1 knockdown in endothelial cells deregulated Notch signaling in adjacent pericytes, supports the notion (14).

Combinational effects and genetic modifiers may explain radiological and clinical incomplete penetrance of CCM. Combinational effects due to reduced expression or disturbed function of other proteins in CCM signaling have been shown in zebra fish (57), and it has been suggested that genetic susceptibility is related to oxidative stress (3, 58). CCM1 has been shown to modulate the expression level of the antioxidant protein SOD2, indicating a potential contribution of the oxidant pathway to CCM pathogenesis (18, 59). A recent report showed that inducible knockout of ccm2 gene after vessel development did not develop CCM lesions in a mouse model, and this suggests that the time window for genetic changes and also possibly, resultant specific changes in microvascular environment may be essential for the CCM phenotypes (60).

Various animal model systems including zebra fish, drosophila and mouse models are available for CCM studies (8, 38, 60-65). In brief, CCM1(+/−)Msh2(−/−) (61) and CCM1(+/−)p53(−/−) (66) mice were used to prove the Knudsonian two-hit mechanism. The CCM3(+/−) mouse model showed different pathogenetic mechanisms underlying CCM lesion genesis and echoing differences in severity between CCM1 or CCM2 and CCM3 disease (42, 67). Most significant phenotypes are observed due to ccm3 mutation (30). Many animal studies have been performed to identify the cellular component of the BBB; endothelial cells, neuroglial cells and smooth muscle cells, which is responsible for CCM pathogenesis. Inducible knockout experiments of ccm1, ccm2 and ccm3 genes showed that perturbed homeostasis of endothelial cells appears to be the most important for CCM phenotypes, albeit mice with Emx1-Cre, Gfap-Cre and Nestin-Cre induced neuronal cell specific knockout of ccm3 showed considerable CCM phenotypes.

FORMATION OF A HETEROTRIMERIC CCM1-CCM2-CCM3 'CCM COMPLEX SIGNALING PLATFORM'

CCM proteins directly interact with each another to form a CCM1-CCM2-CCM3 based signaling platform (68, 69) with interacting proteins rather distinct for each CCM protein (19-21, 70-72). Interaction between CCM2 and CCM3 is necessary for stability of the two proteins (73) and CCM2 dependent stabilization of CCM1 has also been reported (49). CCM2 appears to act as the central hub in the formation of CCM complex by using its PTB domain and a conserved motif C-terminal of the PTB domain to interact with the 2nd and 3rd NPxY/F motifs of CCM1 and the focal adhesion targeting homology (FAT-H) domain of CCM3, respectively (74). A meticulous phosphomapping study has revealed that CCM2 has fourteen Ser/Thr phosphorylation sites with three sites on its PTB domain, suggesting that phosphorylation events may potentially influence formation of the CCM signaling complex (Fig. 2) (21). Although these three CCM proteins together can form the CCM signaling platform, the function of CCM3 appears to be somewhat different from that of the other CCM proteins. Proteomic studies showed that the interaction of CCM3 with members of the GCKIII family is more frequently detected than that of CCM3 with CCM2 (39, 75). In summary, the interaction between CCM1 and CCM2 appears to be intrinsic to CCM complex function; however, the role of CCM3 in the CCM complex remains to be determined. Precise identification of proteins that interact with all three CCM proteins or either of the three CCM proteins is necessary to acquire a better understanding of the CCM complex signaling platforms.

CCM1 is a 736 amino acid protein, which was originally de-

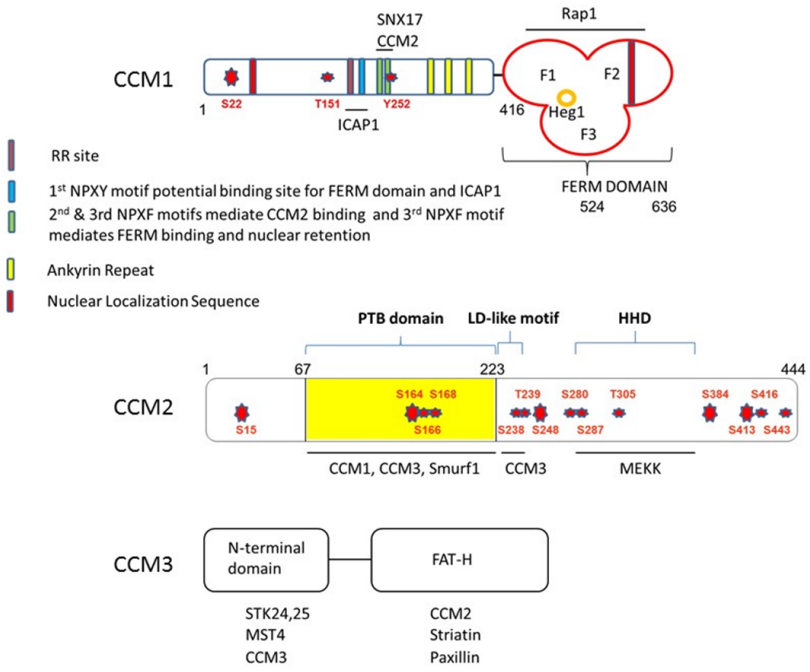

Fig. 2. Representative protein interaction and phosphorylation sites in CCM proteins. Number indicates the location of specific amino acid residue from N-terminus. Size of red stars indicates relative abundance of phosphorylation on serine (S), threonine (T) or tyrosine (Y) residues. Based on (18).

scribed to contain a c-terminal FERM (band 4.1, ezrin, radixin, moesin) domain that interacts with the small GTPase Krev-1 (Rap1) and Nd1-L (59), three PTB binding NPxY/F motifs and an ankyrin repeat domain (ARD) N-terminal to the FERM domain consisting of 4 ankyrin repeats. NPxY/F motifs (^{192}NPAY, ^{231}NPLF, ^{250}NPYF) are important for the protein-protein interactions including intermolecular interactions with CCM2, Heg1 and ICAP1, and intramolecular CCM1 conformational changes and resultant functional outputs (75). CCM1 interaction with microtubules determines subcellular localization of CCM1 in the cytoplasm (76-78). After release from microtubules, CCM1 seems to localize to cell membranes driven predominantly by interaction with Rap1 (78, 79). The FERM domain of CCM1 is comprised of F1, F2 and F3 lobes. F1 and F2 lobes interact with Switch I and II regions of Rap1, and F1 and F3 lobes interact with the c-terminal cytoplasmic region of Heg1 (80, 81). ^{231}NPLF and ^{250}NPYF sequences, the second and third of the three CCM1 NPX (Y/F) motifs, are known to interact with the PTB domain of CCM2, and a recent study showed that the third motif is crucial for the interaction with a single binding site on the CCM2 PTB domain (41). The 2nd NPX (Y/F) motif in CCM1 interacts with the FERM domain of SNX17 (82).

CCM2, a 444 amino acid protein, has a N-terminal PTB domain, LD-like motif and C-terminal harmonin homology domain (HHD) (73). The α-helical LD like motif within CCM2 binds the highly conserved HP1 pocket of the CCM3 FAT-H domain (73). CCM2 interacts with MEKK3 (64), CCM1 and CCM3 (69), and CCM2 either mediates the activation of MEKK3 signaling in response to osmotic stress or negatively regulates MEKK3 signaling. Depletion of CCM2 phosphorylates MEKK3 and ERK5 and activates the transcriptional program downstream of MEKK3 (64). The CCM2-MEKK3 interaction is also known to be partially responsible for Rho-ROCK signaling (83). The PTB domain of CCM2 interacts with Smurf1, a ubiquitin ligase (E3), and the CCM2-Smurf1 interaction was shown to localize Smurf1 for degradation of RhoA (84).

The best identified role of CCM3 would be as a bridging factor within the striatin-interacting phosphatase and kinase (STRIPAK) complex that is essential for cell polarity and migration (73). A recent report, which showed that the CCM2-CCM3 interaction is required for endothelial cell network formation and that CCM3 in the absence of CCM2 is sufficient for endothelial cell growth, indicates a complex function of CCM3, both dependent and independent of CCM2 (73). CCM3 is a 212 amino acid protein with an N-terminal dimerization domain and a C-terminal FAT-H domain (Fig. 2). A flexible hinge region links CCM3's N-terminal dimerization and C-terminal FAT-H domains. The FAT-H domain contains an exquisitely conserved hydrophobic patch 1 (HP1) site, and this site is important for interacting with LD-like motif of CCM2, the striatins (75) and paxillin (85). CCM3 can either homodimerize (86) or directly heterodimerize with each of the three GCKIII serine/threonine kinases: STK24 (MST3), STK25 (Ysk1; Sok1) and MST4 (MASK) (86-89). It has been suggested that the interaction of CCM3 with GCKIII kinases and with striatin, a regulatory subunit of the PP2A phosphatase holoenzyme, may cause CCM3 to act as a hub within the STRIPAK complex,

bringing the GCKIII kinases to the STRIPAK phosphatase for the regulation of cell polarity, further linking CCM3 with vascular development (70, 90). CCM3 is localized to the cell membrane upon VEGF stimulation where it protects VEGFR2 from endocytosis (91), and CCM3 interaction with Phosphatidylinositol (3,4,5)-trisphosphate may play a role in CCM3 localization to the plasma membrane (92).

CCM THERAPEUTICS

Currently, there is no approved medical therapy for treating CCM other than surgical resection (3). Readers are advised to refer to a recent review that provides detailed information about CCM management including diagnosis and surgical and conservative treatment (93). Recent studies including whole genome sequencing studies have suggested that both sporadic cases with multiple lesions and familial cases of CCM have a common genetic underpinning of the two-hit mutation mechanism in the ccm genes and that the majority, if not all, of these sporadic cases with multiple lesions are really genetic cases (43). These findings imply that both familial and part of sporadic cases of CCM may be amenable to the same medical therapy.

Chemical inhibition of Rho activity in endothelial cells rescued CCM phenotypes *in vitro* (48) and administration of fasudil, a Rho-kinase inhibitor, resulted in fewer, smaller, and less hemorrhagic lesions in mice with CCM1 mutations. This was the first report of successful pharmacologic therapy in a CCM animal model (8) and the results were reproduced in a separate report (94). Statin therapy was suggested for CCM and it showed symptomatic improvement in a mouse model (50). Inhibition of HMG-CoA reductase by statin not only decreases cholesterol production, but also reduces geranyl-geranyl-pyrophosphate (GGPP), necessary for the isoprenylation of RhoA, critical for tethering RhoA to the cell membrane and activation of the small GTPase. However, statin administration was associated with an increased risk of intracerebral hemorrhage (95) and CCM patients receiving statin medications for routine cardiovascular indications showed lower permeability in brain white matter, but not in lesion (96). These findings indicate that the clinical application of statin does not appear to be feasible at this moment. It is worthwhile to mention about the inhibitors of TGF signaling. In the CCM1 mouse model, LY-364947, an inhibitor of TGF-β type I receptors and phosphorylation of SMAD signaling, significantly reduced phosphorylated SMAD1 levels and inhibited the EndMT switch (12). The combination of this inhibitor and SB-431542 (another inhibitor of SMAD phosphorylation) reduced the number of vascular malformations and prevented vascular "leakage". Sulindac, a FDA approved, non-steroidal and anti-inflammatory drug, can control the development of CCM lesions in CCM3 knockout mice through suppression of beta-catenin activity (10).

CONCLUSIONS AND PERSPECTIVE

Because functional manipulation of CCM signaling has a good potential for regulating systemic blood vessel permeability and angiogenesis (7-9), importantly that of the BBB and possibly tumor vasculature, the research field of CCM is now growing rapidly. However, our understanding of the composition of the CCM macromolecular complexes and associated functional networks is still in its infancy. Also, we have no understanding of the phenotypes that may arise from increased expression of CCM proteins. In our laboratory, we observed increased expression of ccm genes during the progression of prostate cancer, potentially implicating the involvement of ccm genes in cancer signaling(data not shown). I expect that further studies will reveal how the formation of the CCM signaling platform is regulated and also provide answers to important unresolved questions in this field.

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