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Somatic Second Hit Mutation of *RASA1* in Vascular Endothelial Cells in Capillary Malformation-Arteriovenous Malformation

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Abstract

Capillary malformation-arteriovenous malformation (CM-AVM) is an autosomal dominant vascular disorder that is associated with inherited inactivating mutations of the *RASA1* gene in the majority of cases. Characteristically, patients exhibit one or more focal cutaneous CM that may occur alone or together with AVM, arteriovenous fistulas or lymphatic vessel abnormalities. The focal nature and varying presentation of lesions has led to the hypothesis that somatic “second hit” inactivating mutations of *RASA1* are necessary for disease development. In this study, we examined CM from four different CM-AVM patients for the presence of somatically acquired *RASA1* mutations. All four patients were shown to possess inactivating heterozygous germline *RASA1* mutations. In one of the patients, a somatic inactivating *RASA1* mutation (c.1534C>T, p.Arg512*) was additionally identified in CM lesion tissue. The somatic *RASA1* mutation was detected within endothelial cells specifically and was in trans with the germline *RASA1* mutation. Together with the germline *RASA1* mutation (c.2125C>T, p.Arg709*) in the same patient, the endothelial cell somatic *RASA1* mutation likely contributed to lesion development. These studies provide the first clear evidence of the second hit model of CM-AVM pathogenesis.

Keywords

capillary malformation; arteriovenous malformation; endothelial cell; *RASA1*; somatic mutation

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Competing interests

The authors declare that they have no competing interests

1. Introduction

Capillary malformation-arteriovenous malformation (CM-AVM) is an autosomal dominant vascular disorder that affects at least 1:100,000 individuals in Northern European populations (Eerola et al., 2003; Revencu et al., 2013; Revencu et al., 2008). The pathognomonic feature of CM-AVM is the presence of one or more small randomly-distributed pink to red cutaneous CM, often with a pale halo. In one third of patients, there are additional AVM and arteriovenous fistulas (AVF) that can occur at different sites including skin, muscle bone and brain. Some CM-AVM patients also exhibit Parkes-Weber syndrome in which AVF/AVM and CM are associated with overgrowth of a single limb. Lymphatic vessel abnormalities, although rare, have also been described in CM-AVM, particularly in patients with the Parkes-Weber form of the disease (Burrows et al., 2013; Revencu et al., 2008).

CM-AVM is caused by germline inactivating mutations of the *RASA1* gene in the majority of cases (Eerola et al., 2003; Revencu et al., 2013; Revencu et al., 2008). In affected families, 95 percent of individuals that inherit a mutated *RASA1* gene develop lesions, attesting to the high penetrance of *RASA1* mutations in this disease. *RASA1* is a Ras GTPase-activating protein (RasGAP) that converts the Ras small GTP-binding protein from an active GTP-bound to an inactive GDP-bound state during growth factor receptor signal transduction (King et al., 2013). In addition, *RASA1* has been reported to perform Ras-independent functions in intracellular signal transduction (King et al., 2013). In CM-AVM, germline *RASA1* mutations are located throughout the gene and essentially all are nonsense, frameshift or splice site mutations that would result in rapid nonsense-mediated RNA decay and loss of *RASA1* protein (Conti and Izaurralde, 2005; Eerola et al., 2003; Revencu et al., 2013; Revencu et al., 2008).

Consistent with an important role for *RASA1* in the vasculature, genetically engineered *RASA1*-deficient mice also develop blood and lymphatic vessel abnormalities (Henkemeyer et al., 1995; Lapinski et al., 2007; Lapinski et al., 2012; Lapinski et al., 2017). Furthermore, similar abnormalities are observed in mice that express a mutant form of *RASA1* that carries a point mutation (R780Q) that inactivates its catalytic activity specifically (Lubeck et al., 2014). This indicates that vascular abnormalities in CM-AVM result from loss of a Ras-regulating function of *RASA1* rather than loss of a Ras-independent function for this molecule.

The focal nature of lesions in CM-AVM and the variable expressivity of disease has led to the notion that lesion development is additionally dependent upon the acquisition of a somatic “second hit” mutation in the intact wild type *RASA1* allele (Revencu et al., 2013; Revencu et al., 2008). In this hypothesis, the somatic mutation would result in *RASA1* null cells that would then give rise to lesions. Given that CM-AVM is a vascular disease, it is reasonable to assume that such *RASA1* somatic mutations would occur and reside within lesional endothelial cells. Consistent with this, endothelial cell-specific disruption of the *Rasa1* gene in mice is sufficient for the development of vascular abnormalities (Lapinski et al., 2012).

Thus far, two independent studies have addressed the possibility of somatic mutation of *RASA1* in CM-AVM. Revencu et al reported somatic mosaic deletion of a stretch of chromosome 5 encompassing the wild type *RASA1* allele in a Parkes-Weber syndrome patient with a germline *RASA1* mutation (Revencu et al., 2013). However, the genomic DNA used in that study was derived from a neurofibroma that had developed within the Parkes-Weber syndrome lesion rather than the vascular component of the lesion itself. Whether the *RASA1* somatic deletion detected in the neurofibroma contributed to the development of the vascular abnormalities in the patient is uncertain. In a more recent study, MacMurdo et al used next generation sequencing (NGS) to identify a nonsense *RASA1* somatic mutation within a CM of CM-AVM patient with a germline *RASA1* mutation (Macmurdo et al., 2016). However, whether the somatic mutation resided within vascular endothelial cells of the lesion was also not determined in this study. Moreover, whether the somatic *RASA1* mutation was in cis or trans with the germline *RASA1* mutation was not addressed.

In the current study, we examined CM lesions from four additional CM-AVM patients for the occurrence of somatic *RASA1* mutations. In one of the patients, we discovered an inactivating somatic *RASA1* mutation that is present specifically within lesional endothelial cells. The somatic *RASA1* mutation was in trans with the germline *RASA1* mutation and thus likely contributed to lesion development. This study provides the first solid evidence for the somatic second hit model of CM-AVM pathogenesis.

2. Patients and methods

2.1 Patients

All studies were approved by the Institutional Review Boards of the Children's Hospital of Wisconsin and the University of Michigan. Patient clinical characteristics are provided in Table 1.

All four patients had multiple cutaneous CM that were oval or punctate in shape with a pink to dark red color, some with a pink halo (Fig. 1). Ultrasonography performed on cutaneous CM of three of the patients revealed at least one lesion in each patient with dilated cutaneous or subcutaneous blood vessels. All four patients also presented with symptomatic AVM or AVF that was shown by conventional angiography. Two patients had additional asymptomatic AVMs confirmed by clinical examination and sonographic imaging. Areas affected by AVM included the neck, face, maxilla and digits. All of the AVM were small vessel infiltrating AVM with multiple feeding arteries and draining veins (Type IIIa malformations) and all had associated tissue overgrowth that was confirmed by ultrasonography and/or MRI. In three of the patients, there was a confluent pink to dark red cutaneous or mucosal stain of the skin or mucosa overlying the AVMs. The AVF was located in the spine and involved the anterior spinal artery and a medullary vein. Skin overlying the AVF was normal in appearance and there was no associated soft tissue or bone overgrowth.

2.2 Methods

2.2.1 Tissue procurement—Biopsies of a single CM containing cutaneous and subcutaneous tissue were obtained from each patient using a 4 mm punch biopsy tool. Peripheral blood was obtained by venipuncture of an antecubital vein.

2.2.2 Endothelial cell isolation and culture—Biopsy specimens were finely minced with a sterile scalpel blade and digested with 0.1 % Collagenase II and 0.25 U/ml Dispase (Sigma) in Hanks Balanced Salts Solution at 37°C for 1 h with constant agitation. Digested tissue was pushed through a 70 µm cell strainer, collected cells were pelleted, resuspended in 10 ml of Endothelial Cell Growth Medium MV (Promocell), and cultured in wells of a gelatin-coated 24-well tissue culture plate to allow endothelial cell expansion. After 2–3 weeks, cells were detached from the plate, stained with a fluorochrome-conjugated anti-CD31 monoclonal antibody (BD Biosciences), and separated into endothelial cell (CD31+) and non-endothelial cell (CD31–) populations by flow cytometric sorting using a BD FACS Synergy.

2.2.3 DNA sequencing—Genomic DNA was prepared from peripheral blood, frozen bulk lesional tissue and sorted lesional CD31+ and CD31– cells using a DNEasy Blood and Tissue kit (Qiagen) and from fresh bulk lesional tissue using a Gentra Puregene kit (Qiagen). To identify germline *RASA1* mutations, all 25 exons of *RASA1* were PCR-amplified from peripheral blood genomic DNA using forward and reverse primers located approximately 100 bp upstream and downstream of exons respectively. PCR products were then subject to Sanger sequencing. To identify any somatic second hit mutations, all 25 exons of *RASA1* were PCR-amplified from genomic DNA prepared from sorted lesional CD31+ and CD31– cells using the same primers. PCR products were then pooled and analyzed by Single Molecule Real Time (SMRT) sequencing (Pacific Biosciences). The sequencing data was analyzed using SeqMan NGen software (DNASTAR). To verify somatic mutation in patient 2, separate PCRs of the relevant exon (exon 11) were performed using genomic DNA prepared from frozen bulk lesional tissue and sorted lesional CD31+ and CD31– cells and products were analyzed by Sanger sequencing. To determine if the identified *RASA1* somatic mutation was in cis or trans with the germline *RASA1* mutation, a PCR was performed upon genomic DNA from fresh lesional tissue using forward and reverse primers located approximately 450 bp upstream and downstream of the exon 11 somatic mutation and exon 16 germline mutation respectively. The 14 kb product was then cloned into the pCR-XL-2-Topo vector (ThermoFisher) and 5′ and 3′ ends of inserts of individual clones were analyzed by Sanger sequencing.

2.2.3 Digital droplet PCR—Multiplex digital droplet PCR (ddPCR) was performed upon genomic DNA isolated from whole lesion tissue of patient 2 or normal tonsil (Tissue DNA kit, Promega) using primer/probe sets (IDT) that distinguish germline and somatic *RASA1* mutations from the respective wild type *RASA1* sequences. The mutant probes contained a FAM fluorophore and the wild type probes each were labeled with HEX. All four probes included a terminal IBFQ quencher. Droplets were made using the BioRad QX100 Droplet Generator (BioRad), and PCR was performed in a BioRad QX100 (BioRad). Data was analyzed using the QuantaSoft Analysis Pro software version 1.0.596 (BioRad). Analysis of

positive and negative droplets was done separately for each reaction by gating on droplets that were clustered together in the 2D amplitude plot. The concentration of mutant or wild type positive DNA was determined by the software in copies/μl and was used to calculate the percent fractional abundance of each mutant.

3. Results

3.1 Detection of germline *RASA1* mutations in CM-AVM patients

All four patients examined in this study had multiple cutaneous CM that were pathognomonic for CM-AVM (Table 1, Fig. 1). In addition, all four patients had additional AVM or AF or both (Table 1). To establish if patients carried germline *RASA1* mutations we performed Sanger sequencing of genomic DNA isolated from peripheral blood leukocytes. A single heterozygous *RASA1* mutation was identified in each patient (Table 2). Three of the mutations were novel (patient 1, c.1063A>T, p.Lys355*; patient 3, c.1269_1270insTA, p.Ile423*; patient 4, c.1703G>A, p.Trp568*) and one had been reported previously (patient 2, c.2125C>T, p.Arg709*) (Grillner et al., 2016; Revencu et al., 2013; Revencu et al., 2008). All four mutations result in premature stop codons. Therefore, mutations would be expected to result in null *RASA1* alleles as a consequence of nonsense-mediated RNA decay of transcripts (Conti and Izaurralde, 2005).

3.2. Detection of somatic *RASA1* mutations in endothelial cells of CM lesions

We obtained punch biopsy specimens of single CM from each patient to detect somatic *RASA1* mutations in lesional vascular endothelial cells. Following preparation of single cell suspensions from biopsies, selective endothelial cell expansion was encouraged by culture in endothelial cell growth medium. After 2–3 weeks of culture, cells were stained with a CD31 antibody and sorted into CD31+ endothelial cell and CD31– non-endothelial cell (mostly fibroblasts) populations by flow cytometry. Genomic DNA was then isolated from populations and any somatic *RASA1* mutations in CD31+ endothelial cells were detected by SMRT sequencing. Using this approach we discovered a nonsense somatic mutation in exon 11 of patient 2 (c.1534C>T, p.Arg512*) (Table 2). This mutation had been described previously as a germline mutation in another patient and is predicted to be inactivating (Revencu et al., 2013). The somatic mutation was observed in approximately 60 percent of exon 11 PCR amplicons that were sequenced (out of 1364 total), suggesting high representation among endothelial cells derived from the biopsied CM lesion in this patient. In contrast to patient 2, no somatic mutations of the *RASA1* gene were detected in any *RASA1* exon in CM endothelial cells of the other three CM-AVM patients (Table 2). For patient 4, we also performed SMRT sequencing of *RASA1* exons using genomic DNA isolated from sorted lesional CD31– cells. However, no second hit mutations were identified.

To confirm the somatic *RASA1* mutation in endothelial cells of patient 2, we performed separate PCR amplifications of genomic DNA exon 11 followed by direct analysis of the PCR products by Sanger sequencing. In CD31+ lesional endothelial cells, a clear A nucleotide peak was detected at position 1534, together with the dominant wild type G nucleotide peak at this position (Fig. 2A, sequence of the reverse strand is shown). In

contrast, an A nucleotide peak was not detected in PCR products generated from CD31+ cells from the lesion (Fig. 2A). The somatic mutation in patient 2 was not an artifact of endothelial cell culture. Thus, Sanger sequencing revealed the presence of the mutation at low frequency in genomic DNA prepared from frozen bulk lesional tissue but not peripheral blood DNA (Fig. 2B). In addition, ddPCR of genomic DNA from frozen bulk lesional tissue also revealed the presence of the somatic mutation at low frequency (approximately 4 percent abundance) (Fig. 2C).

To address if the somatic *RASA1* mutation was in cis or trans with the germline *RASA1* mutation, we obtained an additional biopsy specimen from the same CM lesion of patient 2. Genomic DNA was prepared from fresh bulk tissue and used as a template for a long range PCR using a forward primer located upstream of the somatic mutation site and a reverse primer located downstream of the germline mutation site. The 14 kb PCR product was then ligated into a cloning vector and inserts of individual clones were analyzed by Sanger sequencing to detect somatic and germline mutations (Fig. 3). Of 39 sequenced inserts, 2 contained the somatic *RASA1* mutation, consistent with the expected frequency of the mutation in bulk lesional tissue. Most importantly, the germline mutation was not present in either insert, although was present in approximately 50 percent (20/39) of clones overall. Thus, the somatic *RASA1* mutation occurs in trans with the germline *RASA1* mutation within endothelial cells of this particular lesion of patient 2.

4. Discussion

The clinical features of the CM-AVM patients examined in this study conformed with previously described patterns. All four patients exhibited CM and soft-tissue AVMs, often multiple, deeper lesions of which were comprised of infiltrating small vessel lesions with arteriovenous shunting, classified as type IIIa in the angiographic classification proposed by Cho et al (Cho et al., 2006). The one CNS lesion in our series consisted of a simple AVF, also conforming to the previously published pattern. High-resolution ultrasound imaging of a CM in CM-AVM has only been reported once (Kim et al., 2015). In that study, the authors were able to demonstrate a vessel with an arterial Doppler signal and an echogenic area deep to a CM, leading to the conclusion that the cutaneous lesion is a reflection of an AVM in the subdermal tissue. In our patients, none of the CM had such a focal echogenic area in the subcutaneous tissue. The degree of vascularity as shown on color Doppler imaging varied from one lesion to another, with some having no visible vessels, and others marked dilation of superficial arteries and veins extending to the skin surface. Histological features of punch biopsies from clinically evident CM of all four of the patients in this study were subtle, but suggestive of increased arterial flow through slightly prominent, but otherwise architecturally normally cutaneous arterial feeders. Overall, our findings support the presence of micro AVMs in the skin and/or underlying subcutaneous tissue.

Consistent with the clinical findings, all four of our CM-AVM patients were found to carry germline inactivating mutations of the *RASA1* gene. Moreover, in one of the patients, an inactivating somatic *RASA1* mutation was detected specifically within endothelial cells derived from a CM. SMRT and Sanger sequencing revealed that the somatic mutation was present at high frequency within lesional endothelial cells. In addition, Sanger sequencing

and ddPCR showed that the mutation could be detected at lower frequency in bulk lesional tissue and was thus not a consequence of endothelial cell culture. Importantly, the somatic mutation was shown to be in trans with the germline *RASA1* mutation in the CM lesion. Taken together with the known effect of biallelic disruption of *Rasa1* with endothelial cells in mice, these findings constitute strong evidence for the second hit origin of CM-AVM.

Studies of inducible *RASA1*-deficient mice have indicated that postnatal loss of *RASA1* does not result in blood vascular abnormalities (Lapinski et al., 2012). Therefore, in order for *RASA1* somatic mutations to contribute to lesions, it is likely that they would have to be acquired by endothelial cells during blood vascular development in the embryo. Presumably, dysregulated Ras signal transduction in *RASA1* null endothelial cells in developing vessels accounts for the development of lesions. The finding that homozygous *RASA1* R780Q knockin mice develop similar blood vascular abnormalities as *RASA1* null mice supports this conclusion (Lubeck et al., 2014). Furthermore, the Ras effectors, mitogen-activated protein kinases and phosphatidylinositol 3-kinase, are known critical regulators of blood vascular development in mice and other species (Fish and Wythe, 2015).

We were unable to detect somatic *RASA1* mutations in endothelial cells derived from CM of the other three CM-AVM patients in this study nor in CD31+ non-endothelial cells derived from the CM of one of these patients. It is conceivable that such mutations were present at very low frequency that precluded detection by the NGS method employed, a possibility supported by the subtlety of microvascular abnormalities observed in the cutaneous punch biopsies of these patients. The possibility of loss of heterozygosity of *RASA1* within endothelial cells in these lesions is largely ruled out with the finding that in SMRT sequencing analyses of lesional endothelial cell DNA from each patient, germline *RASA1* mutations were present at almost exactly 50 percent of the respective exon amplicons. Furthermore, SMRT sequencing analysis revealed that patients 1 and 3 were heterozygous for a SNP based in exon 5 and that patient 1 was additionally heterozygous for a SNP based in exon 21. For these SNPs, the two allelic variants were present in almost exactly 50:50 ratios in endothelial cell-derived DNA. Other theoretical possibilities that might account for lesion development in these patients include random somatic inactivation of the wild type *RASA1* gene in endothelial cells or acquisition of somatic mutations in genes other than *RASA1* in endothelial cells that act in concert with the germline *RASA1* mutation to drive lesion development.

In summary, in this study we identify an inactivating *RASA1* somatic mutation in a CM of CM-AVM patient that is in trans with a germline *RASA1* mutation in that patient. In addition, we show that the somatic *RASA1* mutation resides specifically within lesion endothelial cells. The findings provide the strongest support to date for the second hit hypothesis of CM-AVM etiology.

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Fig. 1.

Clinical and imaging features of CM-AVM (Patients 1 and 2). A: Multiple CM's involving the hand (patient 1). B: T2-weighted axial MR image of the face (patient 1) shows soft tissue overgrowth of the left facial structures with vascular flow voids. C: Color Doppler ultrasonography of the upper lip (patient 1) shows diffuse dilation of small arteries and veins. D and E: Arterial and early venous phases of left facial angiogram (patient 1) shows the characteristic small vessel AVM throughout the upper and lower lips and surrounding tissue. Note enlarged arteries and draining veins. F: High-resolution color Doppler ultrasound of a cutaneous CM that shows abnormal dilated blood vessels in the skin (patient 2).

Fig. 2.

A somatic mutation in exon 11 of the *RASA1* locus in CM endothelial cells of patient 2 shown by Sanger sequencing and ddPCR. A and B: Electropherograms show the sequence of part of exon 11 of the *RASA1* gene (reverse strand) in the indicated cell and tissue types. A: Cultured CM lesional CD31+ endothelial cells and CD31- non-endothelial cells. B: Bulk lesion and peripheral blood. In CM CD31+ endothelial cells and bulk lesion, an A nucleotide peak at position 1534 (wild type G) represents a nonsense somatic mutation, c. 1534C>T of the plus strand, p. Arg512*. C: Shown is the mean abundance + 1 SEM of somatic and germline *RASA1* mutations in whole bulk lesional tissue from patient 2 and normal tonsil tissue determined by ddPCR (n=4 repeats). Mutation abundance is defined as: (concentration of mutant target/concentration of mutant target + concentration of wild type target) × 100.

Fig. 3.

Somatic and germline *RASA1* mutations are in trans in patient 2. A: Part of *RASA1* locus showing locations of somatic and germline mutations (asterisks, SM and GM respectively) in patient 2. Positions of primers used to generate a 14 kb PCR product encompassing both putative mutation sites are shown. B: PCR products were inserted into the Topo vector and DNA sequences of inserts of individual clones, at sites of prospective somatic and germline mutations, were determined by Sanger sequencing. Shown are electropherograms of one of two identified clones (clone 21) that contained the somatic mutation. The sequence of the reverse and plus strands are shown for the somatic and germline mutation sites respectively. Note the absence of the germline *RASA1* mutation in clone 21. The germline mutation was also absent in the second clone with a *RASA1* somatic mutation.

Table 1

Clinical Characteristics of CM-AVM patients

Patient	Gender	Ethnicity	Age	CM	AVM [/]	AVF	Inheritance
1	F	Caucasian	18	Multiple	Face	No	CM mother; AVM fetus
2	M	Caucasian	29	Multiple	Neck	No	Parents not affected
3	F	Caucasian	27	Multiple	Maxilla Digit	No	CM and AVM in son
4	F	Hispanic	10	Multiple	Face	Spine	Not known

[/] All AVM were type IIIa

Table 2

RASA1 mutations in CM-AVM patients

Patient	Germline <i>RASA1</i> mutations			CM somatic <i>RASA1</i> mutations		
	Exon	Nucleotide	Protein	Exon	Nucleotide	Protein
1	7	c.1063A>T	p.Lys355*	ND/	ND	ND
2	16	c.2125C>T	p.Arg709*	11	c.1534C>T	p.Arg512*
3	9	c.1269_1270insTA	p.Ile423*	ND	ND	ND
4	13	c.1703G>A	p.Trp568*	ND	ND	ND

/ ND, not detected