Title

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Authors

Choksi, F
Weinsheimer, S
Nelson, J
et al.

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ORIGINAL ARTICLE

Assessing the association of common genetic variants in *EPHB4* and *RASA1* with phenotype severity in familial cerebral cavernous malformation

Foram Choksi[1] | **Shantel Weinsheimer**[2,3] | **Jeffrey Nelson**[2] | **Ludmila Pawlikowska**[2,3] | **Christine K. Fox**[4] | **Atif Zafar**[5] | **Marc C. Mabray**[6] | **Joseph Zabramski**[7] | **Amy Akers**[8] | **Blaine L. Hart**[6] | **Leslie Morrison**[5] | **Charles E. McCulloch**[1] | **Helen Kim**[1,2,3]

[1]Department of Epidemiology and Biostatistics, University of California San Francisco, San Francisco, California, USA

[2]Department of Anesthesia and Perioperative Care, Center for Cerebrovascular Research, University of California San Francisco, San Francisco, California, USA

[3]Institute for Human Genetics, University of California San Francisco, San Francisco, California, USA

[4]Department of Neurology, University of California San Francisco, San Francisco, California, USA

[5]Department of Neurology, University of New Mexico, Albquerque, New Mexico, USA

[6]Department of Radiology, University of New Mexico, Albquerque, New Mexico, USA

[7]Department of Neurosurgery, Barrow Neurological Institute, Phoenix, Arizona, USA

[8]Angioma Alliance, Durham, North Carolina, USA

Correspondence
Shantel Weinsheimer, Department of Anesthesia and Perioperative Care, Center for Cerebrovascular Research, University of California San Francisco, 1001 Potrero Avenue, Box 1363, San Francisco, CA 94143, USA.
Email: shantel.weinsheimer@ucsf.edu

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Abstract

Background: To investigate whether common variants in *EPHB4* and *RASA1* are associated with cerebral cavernous malformation (CCM) disease severity phenotypes, including intracranial hemorrhage (ICH), total and large lesion counts.

Methods: Familial CCM cases enrolled in the Brain Vascular Malformation Consortium were included (n = 338). Total lesions and large lesions (≥5 mm) were counted on MRI; clinical history of ICH at enrollment was assessed by medical records. Samples were genotyped on the Affymetrix Axiom Genome-Wide LAT1 Human Array. We tested the association of seven common variants (three in *EPHB4* and four in *RASA1*) using multivariable logistic regression for ICH (odds ratio, OR) and multivariable linear regression for total and large lesion counts (proportional increase, PI), adjusting for age, sex, and three principal components. Significance was based on Bonferroni adjustment for multiple comparisons (0.05/7 variants = 0.007).

Results: *EPHB4* variants were not significantly associated with CCM severity phenotypes. One *RASA1* intronic variant (rs72783711 A>C) was significantly

associated with ICH (OR = 1.82, 95% CI = 1.21–2.37, $p = 0.004$) and nominally associated with large lesion count (PI = 1.17, 95% CI = 1.03–1.32, $p = 0.02$).

Conclusion: A common *RASA1* variant may be associated with ICH and large lesion count in familial CCM. EPHB4 variants were not associated with any of the three CCM severity phenotypes.

KEYWORDS

cerebral cavernous malformation, *EPHB4*, Ras-Erk/Ras-MAPK signaling, *RASA1*, vascular malformation

1 | INTRODUCTION

Cerebral cavernous malformations (CCMs) are collections of thin-walled, dilated capillary channels (caverns) with leaky tight junctions and a propensity for hemorrhage, that are found predominantly in the central nervous system. CCM lesions can lead to intracranial hemorrhage (ICH), seizures, and neurological deficits, and there is currently no approved medical therapy for treatment. CCM affects 0.3%–0.5% of the population (Al-Shahi et al., 2003), may arise sporadically as a single lesion or can be inherited as an autosomal dominant condition with multiple lesions. Familial cases are caused by germline loss-of-function mutations in one of three genes (*CCM1/KRIT1*, *CCM2/MGC4607*, and *CCM3/PDCD10*) (Bergametti et al., 2005; Liquori et al., 2007; Sahoo et al., 1999). The majority of CCM subjects have mutations in *CCM1*/*KRIT1 (53%)*, followed by *CCM2*/*MGC4607 (15%)* and *CCM3*/*PDCD10 (10%)* (Choquet et al., 2015). The CCM proteins belong to a common signaling pathway that regulates various cellular processes, growth in the endothelial layer, and development of the neurovasculature (Li et al., 2015). The three CCM proteins exist in a trimeric complex suggesting various protein–protein interactions might contribute to different severity phenotypes (Draheim et al., 2014).

Prior studies have shown that disease severity varies among subjects with different CCM gene mutations. *PDCD10* carriers show exceptionally aggressive phenotypes (Denier et al., 2006) with greater lesion burden, earlier age of onset, and greater risk of ICH (Shenkar et al., 2015). However, even carriers of the same gene mutation, for example, in *KRIT1*, exhibit a wide range in clinical symptoms and disease severity (Choquet et al., 2015, 2016; Choquet, Pawlikowska, et al., 2014). Previous studies conducted in a cohort of Hispanic subjects all harboring the founder *KRIT1*/*CCM1* Q455X "Common Hispanic Mutation" (CCM1–CHM) have identified common variants in inflammatory and immune response, cytochrome P450, and matrix metalloproteinase genes associated with lesion burden and ICH history (Choquet et al., 2016; Choquet, Pawlikowska, et al., 2014), suggesting that CCM disease severity is influenced by genetic modifiers.

Familial CCM cases also display cavernous malformations outside the brain, including in the bone, spine, adrenal glands, and skin (Campione et al., 2013; Haghighi et al., 2013; Manole et al., 2020; Sirvente et al., 2009; Strickland et al., 2017; Tandberg et al., 2020; Toll et al., 2009). Specifically, among familial cases with *CCM1*/*KRIT1*, cutaneous lesions reported include congenital hyperkeratotic capillary-venous malformations (HCCVMs), punctate capillary malformations (PCMs), and deep blue nodules (DBNs) (Manole et al., 2020; Sirvente et al., 2009). Interestingly, similar appearing cutaneous lesions have been reported in another inherited vascular disease called capillary malformation-arteriovenous malformation (CM-AVM) caused by mutations in *RASA1* (CM-AVM1) and *EPHB4* (CM-AVM2) (Amyere et al., 2017; Eerola et al., 2003). ICH from ruptured brain vascular malformations, albeit of different type, is also common in both diseases.

RASA1 (RAS p21 protein activator 1, also known as p120RasGAP) is a negative regulator of the Ras pathway through its GTPase activating protein (GAP) activity (Kawasaki et al., 2014). p120RasGAP is also implicated in signaling to the cytoskeleton by binding Rap1a (a Ras-family GTPase) and/or p190RhoGAP (RhoGAP) (Eerola et al., 2003; Frech et al., 1990; Hata et al., 1990). RASA1 interacts with receptor tyrosine kinases (RTKs) including EPH family receptors, among which the EPHB4 receptor is involved in regulating arteriovenous morphology (Kawasaki et al., 2014). Furthermore, KRIT1, a membrane bound protein also interacts with Rap1a (Serebriiskii et al., 1997). Murine models have also implicated activated MEKK3-KLF2/4 signaling via RAS/RAF/MAPK pathway in brain endothelial cells as important for CCM lesion formation (Tang et al., 2017). These studies suggest a shared signaling pathway between CCM and CM-AVM that may explain the common vascular phenotypes.

Hence, the purpose of this study was to investigate whether common variants in *EPHB4* and *RASA1* are associated with familial CCM disease severity phenotypes,

including intracranial hemorrhage (ICH), total and large lesion counts.

2 | METHODS

2.1 | Study population

This is a cross-sectional analysis of 338 familial CCM subjects enrolled between June 2010 and February 2018 through the Brain Vascular Malformation Consortium (BVMC). By design, the majority of cases (299/306) were confirmed carriers of the Common Hispanic Mutation (CHM) in *KRIT1* (Q455X, rs267607203). Subjects were eligible for the study if they fulfilled the following requirements: (a) were carriers of a CCM mutation in *KRIT1*, *CCM2*, *or PDCD10*; or (b) without confirmed mutation, meet at least two of three following clinical criteria: diagnosis of CCM, multiple CCMs on magnetic resonance imaging (MRI), or a family history of CCMs. For this study, we excluded subjects if they had missing genotype or phenotype data for ICH. All genetic, clinical, and imaging data were de-identified prior to performing data analysis.

2.2 | Phenotypes

Clinical, medical history, and outcome data were collected from in-person interviews and medical record review (Choquet, Nelson, et al., 2014). Diagnostic or research baseline MRI scans were obtained for all subjects at study enrollment, and at a minimum included T2 gradient-recalled echo (GRE), susceptibility-weighted imaging (SWI), and FLAIR sequences. Images were reviewed and lesions were manually counted by the study neuroradiologists (B.H. and M.M.). Large lesions were defined as ≥5 mm diameter on T2 images. We analyzed three markers as measures of disease severity: (a) clinical history of ICH at baseline, (b) total lesion count, and (c) large lesion count.

2.3 | Genotypes

Genomic DNA was extracted from blood or saliva (Oragene kits, DNA Genotek) using standard protocols. Genotyping was performed at the UCSF Genomics Core Facility using the Affymetrix Axiom Genome-Wide LAT1 Human Array, which includes 817,810 single-nucleotide polymorphisms (SNPs) optimized for genotyping Hispanic populations. The Affymetrix Genotyping Console (GTC) 4.1 software package was used to generate genotype calls and standard quality control (QC) metrics were performed to exclude variants with genotype call rate <99%, minor allele frequency (MAF) <1%, or in Hardy–Weinberg disequilibrium ($p < 0.001$).

2.4 | Gene and variant selection

We selected the two genes mutated in CM-AVM, *EPHB4* (OMIM: 600011, GenBank: NC_000007.14 version GRCh38.p13), and *RASA1* (OMIM: 139150, GenBank: NC_000005.10 version GRCh38.p13), for analysis. We extracted genotype data for 21 SNPs on the Affymetrix Axiom GW LAT Human Array that map to ±5 kb upstream and downstream of the candidate genes (human genome assembly hg18). Of these 21 SNPs, 6 failed QC and 8 SNPs were excluded because MAF was <5%, leaving 7 SNPs for analysis: 3 for *EPHB4* (rs2472559, rs2571607, and rs314316) and 4 for *RASA1* (rs117340098, rs13362486, rs440855, and rs72783711). We compared the MAF of these seven SNPs in the CCM cohort to the MAF reported in public databases including the 1000 Genomes global population (phase 1 genotype data from 1094 worldwide individuals) and the HapMap MEX population (Table S1). The MAFs were similar in all three populations, except for *EPHB4* rs2571607 (CCM MAF: 0.25, HapMap MEX MAF: 0.38) and *RASA1* rs13362486 (CCM MAF: 0.41, HapMap MEX MAF: 0.34).

2.5 | Statistical analysis

To identify genotypes associated with CCM disease severity phenotypes, we performed either multivariable logistic regression (for ICH) or linear regression (for logarithm of total or large lesion counts after adding 1) analyses, assuming an additive genetic model and adjusting for age and sex; standard errors were adjusted to account for familial clustering. Log transformation of lesion counts was used to improve the normality of residuals. Additionally, in order to adjust for population stratification, we included the top three principal components (PCs) into the models. PCs were computed in the total cohort using GCTA software (Yang et al., 2011). We performed a sensitivity analysis for the subset of subjects on whom we had skin lesion data available ($n = 228$); skin lesions were included as a covariate in multivariable models as well as analyzed as a severity phenotype. We report odds ratios (ORs) for ICH, proportional increase (PI) for lesion counts (from exponentiated beta coefficients), 95% confidence intervals (CIs), and nominal p values for all seven variants. PI is interpreted as increase in lesion count if >1 or decrease if <1. The threshold for significance was set based on Bonferroni-adjusted p value which accounts

for the number of variants tested (0.05/7 = 0.007). SNPs with a Bonferroni-adjusted p value ≤0.05 and >0.007 were considered to be nominally significant. Data analysis was performed with Stata 15.1 software (College Station, TX: StataCorp LLC.). To identify SNPs in high linkage disequilibrium (LD) with CCM phenotype associated variants in the MEX population, we used the LDlink LDproxy tool (Machiela & Chanock, 2015). Proxy SNPs in high LD ($r^2 > 0.8$) with associated variants were evaluated bioinformatically for functional potential using the UCSC Genome Browser and RegulomeDB (https://regulomedb.org/).

3 | RESULTS

3.1 | Participant characteristics

Table 1 shows the descriptive statistics of 338 CCM subjects included in this study. The mean age at enrollment was 39 ± 21 years; the majority of subjects were female (62%) and of Hispanic ethnicity (89%). At baseline enrollment, 32% of CCM subjects had a history of intracranial hemorrhage. The median total number of lesions was 13 (range 0–713) and the median number of large lesions (≥5 mm) was 3 (range 0–104). Seventy-five of 228 subjects (33%) also had skin lesions. Most subjects had the *KRIT1* CHM (98%).

TABLE 1 Demographic and clinical characteristics of familial CCM subjects enrolled in the Brain Vascular Malformation Consortium (BVMC) study

	Values	
Characteristics (n = 338)	***n***	**%**
Sex (male)	129	38.2
Ethnicity		
Hispanic, Latino, or Spanish origin	299	88.5
Not Hispanic, Latino, or Spanish origin	35	10.4
Unknown or not reported	4	1.2
Race		
White	315	93.2
Mixed	4	1.2
Asian	1	0.3
Unknown or not reported	18	5.3
Age at enrollment, years		
Mean ± SD	39.4 ± 20.6	
Range	0.44–84.9	
Clinical history of intracerebral hemorrhage (ICH) at enrollment	108	31.9
Total lesion count	309	
Median (IQR)	13 (5–44)	
Range	0–713	
Large lesion count (≥5 mm)	309	
Median (IQR)	3 (1–5)	
Range	0–104	
Skin lesion positive	75/228	32.9
CHM positive	299/306	97.7

Abbreviations: CHM, common hispanic mutation; IQR, interquartile range; SD, standard deviation.

3.2 | Association with CCM severity phenotypes

One intronic variant in *RASA1* (rs72783711) was significantly associated with ICH (OR = 1.82; p = 0.004) (Table 2), and was also nominally, but not statistically significantly associated with large lesion count (PI = 1.17, p = 0.02) but not with total lesion count (Table 3). *EPHB4* variants were not associated with any of the three CCM severity phenotypes (Tables 2 and 3).

We also performed a sensitivity analysis in a subset of subjects with skin lesion data available (n = 228). *RASA1* rs72783711 was significantly associated with ICH (OR = 2.85, $p \leq 0.001$) and also with large lesion count in this subset (PI = 1.23, p = 0.006). Additionally, another *RASA1* variant was significantly associated with ICH (rs13362486; OR = 1.76 p = 0.004). In analysis adjusting for skin lesion as a covariate, the same *RASA1* variants were significantly associated with ICH (rs72783711; OR = 2.79, $p \leq 0.001$ and rs13362486; OR = 1.76, p = 0.003). No association was observed with *RASA1* or *EPHB4* variants and skin lesions as a phenotype (data not shown).

3.3 | Functional evaluation of CCM phenotype-associated and proxy SNPs

We used bioinformatics tools and reference databases to evaluate whether the ICH and total lesion-associated SNP, *RASA1* rs72783711, or the five proxy SNPs in high LD ($r^2 > 0.8$) with it are predicted to have functional effects. There is a modest H3K4me1 histone mark at the *RASA1* rs72783711 position detected in lymphoblastoid cells (GM12878), traditionally associated with enhancers (UCSC Genome Browser, GRCh38/hg38, ENCODE Regulation). There is currently no other evidence that suggests a putative functional effect for any of these variants.

TABLE 2 Genetic variants associated with clinical history of ICH in familial CCM subjects

				Intracranial hemorrhage (ICH)		
Gene	**SNP**	**Minor allele**	**MAF**	**OR[a]**	**95% CI**	***p* value**
EPHB4	rs2472559	T	0.27	1.04	0.70–1.54	0.85
EPHB4	rs2571607	T	0.25	1.04	0.73–1.47	0.84
EPHB4	rs314316	G	0.14	1.31	0.78–2.19	0.31
RASA1	rs117340098	G	0.06	0.89	0.45–1.81	0.76
RASA1	rs13362486	A	0.41	1.32	0.98–1.78	0.07
RASA1	rs440855	C	0.06	0.87	0.45–1.71	0.70
RASA1	rs72783711	C	0.15	1.82	1.21–2.73	0.004*

Note: EPHB4 (GenBank: NC_000007.14 version GRCh38.p13); *RASA1* (GenBank: NC_000005.10 version GRCh38.p13).

Abbreviations: CI, confidence interval; MAF, minor allele frequency; OR, odds ratio; SNP, single-nucleotide polymorphism.

[a]Adjusted for age, sex, and top three principal components.

*Statistically significant *p* value.

TABLE 3 Genetic variants associated with total and large lesion counts in familial CCM subjects

				Total lesion count			Large lesion count		
Gene	**SNP**	**Minor allele**	**MAF**	**PI[a]**	**95% CI**	***p* value**	**PI[a]**	**95% CI**	***p* value**
EPHB4	rs2472559	T	0.27	1.12	0.91–1.38	0.27	1.04	0.91–1.17	0.58
EPHB4	rs2571607	T	0.25	0.95	0.76–1.18	0.63	1.02	0.88–1.17	0.83
EPHB4	rs314316	G	0.14	0.77	0.58–1.04	0.09	0.86	0.74–1.01	0.06
RASA1	rs117340098	G	0.06	0.86	0.61–1.21	0.37	0.87	0.69–1.08	0.21
RASA1	rs13362486	A	0.41	1.09	0.90–1.33	0.37	1.11	0.99–1.23	0.06
RASA1	rs440855	C	0.06	0.88	0.64–1.20	0.41	0.85	0.68–1.06	0.15
RASA1	rs72783711	C	0.15	1.11	0.86–1.43	0.44	1.17	1.03–1.32	0.02*

Note: EPHB4 (GenBank: NC_000007.14 version GRCh38.p13); *RASA1* (GenBank: NC_000005.10 version GRCh38.p13).

Abbreviations: CI, confidence interval; MAF, minor allele frequency; PI, proportional increase; SNP, single-nucleotide polymorphism.

[a]Proportional increase in lesion count if >1 or decrease if <1, adjusted for age, sex, and top three principal components.

*Nominally significant *p* values.

4 | DISCUSSION

Our findings provide the first evidence of association between common variants in *RASA1* and disease severity in familial CCM. Specifically, an intronic variant in *RASA1*, rs72783711, was significantly associated with history of ICH and nominally associated with large lesion count in all 338 CCM subjects. No other genotyped variants in *RASA1* or in *EPHB4* were associated with any of the CCM disease severity phenotypes. Sensitivity analyses restricted to those with skin lesion data revealed an additional *RASA1* variant (rs13362486) associated with ICH and large lesion count.

No prior studies have looked at these candidate genes in CCM, but there have been associations reported for brain AVM, a high-flow vascular malformation which can cause intracranial hemorrhages and a feature of CM-AVM disease. Two *EPHB4* SNPs (rs314313 and rs314308) were associated with risk of ICH in Caucasian subjects with sporadic brain AVM (Weinsheimer et al., 2009). Clinical findings from past studies have identified novel *RASA1* variants and also broader phenotypic spectrum for CM-AVM caused by these variants; one of the phenotypes being brain AVM or AVF (Wooderchak-Donahue et al., 2018). Past studies have shown the significance of *RASA1* and *EPHB4* in the disease severity of many vascular malformations and their involvement in complex signaling cascades; Ras-MEKK-Erk being an important one. *RASA1* protein, Ras GTPase activating protein 1, belongs to a non-catalytic domain; it serves as an effector of Ras by binding to growth factors and cytoplasmic proteins along with

playing a role in cellular differentiation and proliferation, which is indicative of its role in defective angiogenesis, neovascularization, and malignancies. (de Wijn et al., 2012). *RASA1* is also an effective downstream regulator of endothelial receptor EPHB4 and experimental studies on zebrafish have shown that inhibition of either *EPHB4* or *RASA1* caused similar vascular defects. (Amyere et al., 2017). *EPHB4*, which is expressed by venous endothelial cells, is involved in kinase-dependent forward signaling, which regulates diverse endothelial functions and angiogenesis along with concomitant activation of Erk1/2. (You et al., 2017). Thus, it is plausible that common *RASA1* variants could influence disease severity in vascular diseases with involvement in the complex Ras-Erk signaling pathway.

In our study, we found an association between *RASA1* rs72783711 variant and ICH and large lesions in familial CCM. This variant is intronic and located in a potential gene regulatory region, as there is a modest H3K4me1 histone mark traditionally associated with enhancers at this position detected in lymphoblastoid cells (GM12878). Our familial CCM cohort had MAFs that were comparable to those reported for both the 1000 genomes and HapMap MEX (Los Angeles) samples, suggesting that our genotyping methods have not been subjected to biases related to sampling methodology or standard bioinformatic processing steps (Linck & Battey, 2019).

This study has several strengths and limitations. A sample size of 338 subjects is modest for genetic association studies, but large for a rare vascular malformation disease. In addition, the study was conducted in a unique population of familial CCM cases primarily with the same genetic mutation in *KRIT1 (CHM)*, which helps in the evaluation of genotype–phenotype associations. However, we do not know if these results are generalizable to other CCM populations, for example, patients with other CCM gene mutations or sporadic cases. Furthermore, our study only focused on two candidate genes involved in the Ras-Erk pathway, and it is likely that that other genes in this important pathway may be genetic modifiers of CCM disease severity.

5 | CONCLUSION

In conclusion, these results suggest that common genetic variants in *RASA1* influence the disease severity of familial CCM. These findings, if replicated in other cohorts, will improve our understanding of the natural history of CCM, including risk factors for disease severity and phenotype variability and the biological mechanisms of CCM pathogenesis. This improved knowledge may lead to better predictions of disease course and new medical therapies for treatment in familial CCM.

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CONFLICT OF INTEREST

The authors have declared that no competing interests exist.

ETHICAL COMPLIANCE

The study was approved by the local institutional review boards at the University of New Mexico, University of California San Francisco (UCSF), and Barrow Neurological Institute; and Quorum IRB for Angioma Alliance. Written informed consent was obtained from all participants.

AUTHOR CONTRIBUTIONS

Patient recruitment and data collection: HK, AA, LM, AZ, and JZ. Neuroradiological review: MCM and BLH. Genetic analysis: LP, SW, and HK. Statistical analysis: FC, JN, CKF and CEM. Drafting of manuscript: FC, SW, and HK. Critical revision of the manuscript: All co-authors. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available upon request from the corresponding author.

ORCID

Foram Choksi https://orcid.org/0000-0001-7489-0227
Shantel Weinsheimer https://orcid.org/0000-0001-7486-9110
Ludmila Pawlikowska https://orcid.org/0000-0003-2182-9953
Helen Kim https://orcid.org/0000-0002-1937-354X

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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