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Angiopoietin-Like 4 *(ANGPTL4)* Gene Polymorphisms and Risk of Brain Arteriovenous Malformations

Bahar Mikhak[a] Shantel Weinsheimer[a] Ludmila Pawlikowska[a, e] Annie Poon[f] Pui-Yan Kwok[e, f] Michael T. Lawton[c] Yongmei Chen[a] Jonathan G. Zaroff[g] Stephen Sidney[g] Charles E. McCulloch[b] William L. Young[a, c, d] Helen Kim[a, b, e] for the BAVM Study Project

[a]Center for Cerebrovascular Research, Department of Anesthesia and Perioperative Care, Departments of [b]Epidemiology and Biostatistics, [c]Neurological Surgery, [d]Neurology, [e]Institute for Human Genetics, and [f]Cardiovascular Research Institute, University of California, San Francisco, Calif., and [g]Division of Research, Kaiser Permanente Medical Care Program, Oakland, Calif., USA

Key Words
Arteriovenous malformations · Cerebrovascular disorders · Epidemiology · Genetics · Intracranial hemorrhage

Abstract
Background: Brain arteriovenous malformations (BAVM) are high-flow vascular lesions prone to intracranial hemorrhage (ICH). Abnormal angiogenesis is a key characteristic of BAVM tissue. Angiopoietin-like 4 (ANGPTL4), a secreted glycoprotein, is thought to be involved in angiogenesis and required for proper postnatal blood vessel partitioning. We investigated whether common single nucleotide polymorphisms (SNPs) in *ANGPTL4* were associated with risk of BAVM or ICH. ***Methods and Results:*** We conducted a case-control study of 216 Caucasian BAVM cases and 246 healthy controls, and a secondary case-only analysis, comparing 83 ruptured (ICH) with 133 unruptured BAVM cases at presentation. Four tag-SNPs in *ANGPTL4* captured variation over a 10-kb region (rs2278236, rs1044250, rs11672433, and rs1808536) and were tested for association with BAVM or ICH. The minor allele *(A)* of rs11672433 (exon 6, Pro389Pro) was associated with an increased risk of BAVM ($p = 0.006$), which persisted after adjusting for multiple comparisons ($p = 0.03$). After adjustments for age and sex, carriers of the minor allele *(A)* remained at higher risk for BAVM compared to noncarriers (odds ratio, OR = 1.56; 95% confidence interval, CI = 1.01–2.41; $p = 0.046$) and risk of BAVM was increased with increasing copy of the minor *A* allele (OR = 1.49, 95% CI = 1.03–2.15; $p_{trend} = 0.03$). Five common haplotypes (frequency >1%) were inferred; overall haplotype distribution differed between BAVM cases and controls ($\chi^2 = 12.2$, d.f. = 4, $p = 0.02$). Neither SNPs ($p > 0.05$) nor haplotype distribution ($\chi^2 = 1.1$, d.f. = 4, $p = 0.89$) were associated with risk of ICH among BAVM cases. ***Conclusion:*** A synonymous SNP in *ANGPTL4* and haplotypes carrying it are associated with risk of BAVM but not with ICH presentation in BAVM cases.

BAVM Study Project Collaborators: Achal S. Achrol, MD; Christopher F. Dowd, MD; Van V. Halbach, MD; Randall T. Higashida, MD; S. Claiborne Johnston, MD, PhD; Patricia Leighton; Nerissa U. Ko, MD; April Manns; Michael W. McDermott, MD; Nancy Quinnine, RN; Michael Sorel, MPH; Vineeta Singh, MD.

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William L. Young, MD
Department of Anesthesia and Perioperative Care
University of California, San Francisco, 1001 Potrero Avenue, Room 3C-38
San Francisco, CA 94110 (USA)
Tel. +1 415 206 8906, Fax +1 415 206 8907, E-Mail youngw@anesthesia.ucsf.edu

Introduction

Brain arteriovenous malformations (BAVM) are high-flow vascular lesions with direct shunting of blood from the arterial to venous circulation with no intervening capillary bed. Patients with BAVM are susceptible to intracranial hemorrhage (ICH), and approximately half of all patients present with ICH [1–3]. The pathogenesis of BAVM is unknown, but genetic factors may contribute to BAVM susceptibility and disease progression [4–6]. BAVM tissue is characterized by excessive angiogenesis and inflammation [2, 4]. Pro-inflammatory cytokines such as interleukins (IL-6 [7] and IL-1β [6]), and transcription factors such as homeobox D3 (HOXD3) [8] can induce angiogenic activity that may contribute to BAVM. A highly positive correlation between angiopoietin-2 [9, 10] and vascular endothelial growth factor (VEGF) levels in BAVM surgical specimens has been reported and suggests that angiogenic factors may contribute to vascular instability resulting in BAVM hemorrhage [11–13].

Members of the angiopoietin/angiopoietin-like (ANGPTL) family play important roles in regulating angiogenesis [9, 10]. While ANGPTL4, a secreted glycoprotein encoded by the *ANGPTL4* gene, is well known for its role in lipid metabolism [14–22], it is also thought to mediate angiogenesis with both anti- and pro-angiogenic effects [23–29]. ANGPTL4 has been reported to inhibit vascular permeability, tumor cell motility, invasiveness [30, 31], sprouting [32], tubule-like structure formation [32–35], and vascular leakiness [31, 34]. Under hypoxic conditions, ANGPTL4 is up-regulated at both the protein and mRNA level [24, 25, 36]. More recently, *Angptl4* knockout mice studies demonstrated that Angptl4 was necessary for functional partitioning of postnatal lymphatic and blood vessels in the intestine [17, 37, 38] and to protect against development and progression of atherosclerosis [39]. Thus, we hypothesized that polymorphisms in the *ANGPTL4* gene may be associated with increased risk of BAVM susceptibility or with ICH in BAVM cases.

Methods

Study Population

Our study included 216 Caucasian BAVM cases and 246 healthy controls. BAVM cases were recruited at the University of California, San Francisco (UCSF) or Kaiser Permanente Medical Care Plan of Northern California (KPNC) as part of our larger UCSF-KPNC Brain AVM registry. Details on case identification, enrollment, ascertainment, verification of diagnosis, and data collection have been described previously [40–42] using standardized classification guidelines [43]. Controls were healthy volunteers with no significant medical history recruited from the same clinical catchment area for a pharmacogenetics study conducted at UCSF [44]. Informed consent was obtained on all study participants, and the study was approved by the Institutional Review Boards at UCSF and KPNC. The subset of patients who provided blood or saliva specimens, and self-reported as Caucasian, our largest ethnic subgroup, were eligible for this genetic study. The study population was restricted to Caucasians to reduce the potential for population stratification or confounding by race/ethnicity. Of the 493 eligible participants, 462 individuals were successfully genotyped for all four single nucleotide polymorphisms (SNPs) and were included in this study.

We also performed a secondary case-only analysis, comparing 83 ruptured with 133 unruptured BAVM cases at presentation. New intracranial blood on computed tomography or magnetic resonance imaging was used to define ICH presentation, and coded as 'ruptured' irrespective of clinical presentation. Cases without evidence of new bleeding and presenting with seizure, focal ischemic deficit, headache, apparently unrelated symptoms or asymptomatic were coded as 'unruptured'.

SNP Selection

Tagging SNPs in the *ANGPTL4* gene were selected from HapMap CEU population data (dbSNP build 126 on NCBI human genome build 36), using the Tagger algorithm [45] available in Haploview [46]. We used pairwise tagging to select a minimal set of tagSNPs with a minor allele frequency ≥5% such that all captured alleles are correlated at $r^2 \geq 0.8$ with a marker in that set. Thus, each tagSNP acts as a direct proxy to all other correlated untyped SNPs, and, by definition, is not highly correlated to other tagSNPs selected for genotyping. Four tag SNPs capturing variation over a 10-kb region were selected for genotyping: rs2278236 *C/T* (intron 3), rs1044250 *C/T* (exon 5, missense Thr266Met), rs11672433 *G/A* (exon 6, Pro389Pro), and rs1808536 *A/G* (3′ UTR) (table 1).

Genotyping

Genomic DNA was extracted from peripheral blood lymphocytes using a salt modification method (Gentra Systems, Minneapolis, Minn., USA). Polymorphism-spanning fragments were amplified by polymerase chain reaction and genotyped by Beck-

Table 1. Properties of *ANGPTL4* polymorphisms selected for genotyping

Location	dbSNP ID	Base change	Function
Intron 3	rs2278236[a]	*C>T*	N/A
Exon 5 (Thr266Met)	rs1044250[a]	*C>T*	missense
Exon 6 (Pro389Pro)	rs11672433[b]	*G>A*	synonymous
3′ UTR	rs1808536[b]	*G>A*	N/A

[a] SNP genotyped using SNPstream 48plex technology.
[b] SNP genotyped using singleplex fluorescence-polarization detection using template-directed dye-terminator incorporation assay (FP-TDI).

man Coulter SNPstream 48plex technology. However, two SNPs (rs11672433 and rs1808536) performed poorly on multiplex assay and were redesigned as single genotyping assays using template-directed primer extension with fluorescence polarization detection (AcycloPrime II; Perkin Elmer, Boston, Mass., USA) [47, 48]. For each SNP, all cases and controls were genotyped using the same method with ≥95% genotyping call rate in the combined data set, and did not differ significantly between cases and controls. Duplicate samples (5% controls, 16% cases) for each genotyping assay served as positive controls, with concordance rates ≥99.5%. We did not observe an excess or scarcity of heterozygotes.

Statistical Analysis

Demographic and clinical characteristics of the BAVM cases and healthy controls were compared using t tests for continuous variables (presented as mean ± standard deviation) and χ^2 test for categorical variables.

Allelic Test of Association

Allele frequencies between BAVM cases and controls and between ruptured and unruptured cases were compared using χ^2 tests of association in PLINK version 1.06 [49]. To account for multiple comparisons, we performed 1,000 permutations of case-control status, comparing each observed test statistic against the maximum of all permuted statistics over all four SNPs. The empirical p value thus controls the studywide error rate.

Genotypic Test of Association

Hardy-Weinberg equilibrium was evaluated among controls using the χ^2 goodness-of-fit test [50]. Assuming a co-dominant inheritance model, indicator variables were created for each *ANGPTL4* genotype, with homozygotes for the major allele as the reference category (table 3). Multivariable logistic regression models were used to estimate the odds ratios (OR) and 95% confidence interval (CI) for each genotype compared to the wild-type homozygote, adjusting for age and sex. We also tested a dominant inheritance model, in which genotypes were collapsed into carriers of the minor allele versus noncarriers. A test for trend, assuming an additive inheritance model, was conducted with a single variable entered with values of 0, 1, and 2 corresponding to the number of minor alleles. Genotypic analyses were conducted using Intercooled Stata software version 10 (StataCorp LP, College Station, Tex., USA).

Haplotype Test of Association

Common haplotypes were inferred from unphased genotype data using the expectation-maximization algorithm and a minor haplotype frequency ≥1%. Omnibus χ^2 tests of haplotype association were performed, with degrees of freedom (d.f.) equal to one fewer than the number of haplotypes tested –1, using PLINK version 1.06 [49]. Haplotype-specific tests of association were also performed comparing each haplotype to all other haplotypes combined (i.e. 1 d.f.).

ANGPTL4 Protein Expression Analysis in BAVM Tissue Specimens

We performed Western analysis to determine ANGPTL4 protein expression in 20 frozen BAVM tissue lysates, and evaluated whether expression levels were correlated with ANGPTL4 genotypes for associated SNPs. BAVM tissues were homogenized in standard RIPA buffer (Santa Cruz Biotechnology) with a protease inhibitor cocktail (Sigma, St. Louis, Mo., USA). Equal amounts of proteins were fractionated by gel electrophoresis, and were electroblotted onto a PVDF membrane. The membranes were then probed with goat anti-human ANGPTL4 antibody, 0.2 μg/ml (R&D Systems, Minneapolis, Minn., USA) followed by horseradish peroxidase-conjugated horse anti-goat IgG. Blots were reprobed with mouse anti-β-actin (Sigma) as a loading control. ANGPTL4 bands were quantified by scanning densitometry and analyzed using NIH Image 1.63 software. The optical densities of full-length ANGPTL4 (75 and 50 kDa) and the C-terminal fibrinogen-like domain (35 kDa) were normalized to that of β-actin.

Unpaired t test was used to compare mean ANGPTL4 expression levels for each protein form (75, 50, and 35 kDa) between high-risk versus low-risk (reference) genotype groups for associated SNPs. A two-tailed $\alpha < 0.05$ was considered statistically significant. Data are presented as means ± SD.

Results

Demographic and clinical characteristics of the BAVM cases and healthy controls are shown in table 2. BAVM cases were significantly older than controls (39 ± 17.6 vs. 31 ± 5.7 years, respectively, $p < 0.001$), but did not differ by gender ($p = 0.63$). Among BAVM cases, 38.4% presented with hemorrhage and 46.4% had deep venous drainage with a mean BAVM size of 2.9 ± 1.5 cm. Genotype frequencies for BAVM cases were similar between the two recruitment sites (data not shown).

All four *ANGPTL4* SNPs were in Hardy-Weinberg equilibrium among the controls ($p > 0.05$). In the allelic test, the minor allele *(A)* of rs11672433 was found to be associated with an increased risk of BAVM (19% among cases vs. 13% among controls; $p = 0.006$), and the association persisted after permutation testing to correct for multiple comparisons across all four SNPs ($p = 0.03$).

Genotypic results for the four *ANGPTL4* SNPs are shown in table 3. A greater proportion of BAVM cases (33.8%) were carriers of the minor allele *(A)* of rs11672433 compared to controls (22.8%). After adjusting for age and sex, the risk of BAVM was 56% higher in *A* carriers compared to noncarriers (OR = 1.56; 95% CI = 1.01–2.41; $p = 0.046$; table 3). As a sensitivity analysis, we further adjusted for *ALK-1* genotype, which we and others have previously reported as a significant genetic risk factor for BAVM susceptibility [51, 52]. The *ANGTPL4* rs11672433 *A* carrier association remained (OR = 1.56, 95% CI = 1.00–2.42, $p = 0.051$) after adjustment for age, sex and *ALK1* IVS3–35 any A genotype. No other *ANGPTL4* SNPs were associated with BAVM.

Five common haplotypes with a frequency >1% were inferred from the data and the overall haplotype distribu-

Table 2. Demographic and clinical characteristics of study cohort

Characteristics	BAVM cases		Controls		p value
	n	%	n	%	
Total participants (n = 462)	216	46.8	246	53.3	
Mean age ± SD, years	39 ± 17.6		31 ± 5.7		<0.01
Sex					0.63
Women	119	55.1	141	57.3	
Men	97	44.9	105	42.7	
Initial presentation			N/A	N/A	
Nonhemorrhagic	133	61.6			
Hemorrhagic	83	38.4			
BAVM size ± SD, cm	2.9 ± 1.5		N/A	N/A	
Venous drainage			N/A	N/A	
Superficial only	98	53.6			
Any deep	85	46.4			

N/A = Not applicable. p value: t test for continuous (presented as mean ± standard deviation) and χ^2 test for categorical variables.

Table 3. Association of *ANGPTL4* polymorphisms with risk of brain arteriovenous malformation

Genotype	Case		Controls		OR (95% CI)[a]	OR (95% CI)[b]
	n	%	n	%		
rs2278236						
TT	70	32.4	68	27.6	1.00 (reference)	1.00 (reference)
CT	112	51.9	126	51.2	0.86 (0.57–1.31)	0.96 (0.62–1.51)
CC	34	15.7	52	21.1	0.64 (0.37–1.10)	0.73 (0.41–1.30)
Any C versus TT	146	67.6	178	72.4	0.80 (0.54–1.19)[c]	0.90 (0.58–1.38)[c]
rs1044250						
CC	100	46.3	112	45.5	1.00 (reference)	1.00 (reference)
CT	90	41.7	110	44.7	0.92 (0.62–1.35)	0.85 (0.57–1.29)
TT	26	12.0	24	9.8	1.21 (0.66–2.25)	1.23 (0.63–2.41)
Any T versus CC	116	53.7	134	54.5	1.00 (0.67–1.40)[c]	0.92 (0.62–1.35)[c]
rs11672433						
GG	143	66.2	190	77.2	1.00 (reference)	1.00 (reference)
AG	63	29.2	50	20.3	1.67 (1.09–2.57)	1.48 (0.93–2.33)
AA	10	4.6	6	2.4	2.21 (0.79–6.23)	2.25 (0.77–6.64)
Any A versus GG	73	33.8	56	22.8	1.73 (1.15–2.61)[c]	1.56 (1.01–2.41)[c]
rs1808536						
GG	153	70.8	181	73.6	1.00 (reference)	1.00 (reference)
AG	57	26.4	64	26.0	1.05 (0.70–1.60)	1.31 (0.84–2.04)
AA	6	2.8	1	0.4	N/A[d]	N/A[d]
Any A versus GG	63	29.2	65	26.4	1.15 (0.76–1.72)[c]	1.42 (0.92–2.20)[c]

[a] Estimated from logistic regression model assuming a co-dominant inheritance model.
[b] Additionally adjusted for age at diagnosis and gender.
[c] Estimated from logistic regression model assuming a dominant inheritance model.
[d] N/A due to small sample size.

Table 4. Association of *ANGPTL4* haplotypes with risk of BAVM

Haplotype[a]	Cases, %	Controls, %	χ^2	d.f.	p
Global	NA	NA	12.2	4	0.02
TTGG	0.33	0.32	0.132	1	0.72
CCGA	0.16	0.13	0.876	1	0.35
TCAG	0.19	0.12	6.835	1	0.01
CCGG	0.26	0.33	6.593	1	0.01
TCGG	0.07	0.10	0.976	1	0.32

[a] Haplotype, 5′→3′: rs2278236, rs1044250, rs11672433, rs1808536.

tion differed significantly between BAVM cases and controls ($\chi^2 = 12.2$, d.f. = 4, p = 0.02; table 4). Two haplotypes were associated with risk of BAVM: TCAG (p = 0.01) and CCGG (p = 0.01). Consistent with the single SNP results, the haplotype (TCAG) containing the minor *A* allele of rs11672433 was present at a higher frequency in BAVM cases (19%) compared to controls (12%) whereas the haplotype (CCGG) containing the major allele at rs11672433 was present at a lower frequency in BAVM cases (26%) compared to controls (33%).

In the secondary analyses, we assessed whether any of the *ANGPTL4* SNPs were associated with ICH presentation among BAVM patients. There were 83 ruptured (38%) and 133 unruptured (62%) cases at the time of presentation. Neither *ANGTPL4* SNPs ($p > 0.05$) nor haplotypes ($\chi^2 = 1.1$, d.f. = 4, p = 0.89) were associated with the risk of ICH presentation. Further adjustments for BAVM size or deep venous drainage did not change the results (data not shown).

The results of our protein expression analysis suggest that ANGPTL4 was expressed in all AVM samples, and that patients with the at-risk rs11672433 AA or AG genotype (n = 13) exhibited a trend towards reduced mean expression of the 50-kDa ANGPTL4 protein compared with the GG genotype group (n = 7), respectively (15.1 ± 11.5 vs. 26.4 ± 15.1; p = 0.08). Carriers had no difference in mean expression for the 75- or 35-kDa ANGPTL4 protein forms compared to noncarriers ($p > 0.1$, data not shown).

Discussion

Our study is the first to investigate the role of *ANGPTL4* gene variants for susceptibility to BAVM and ICH in BAVM cases. In this population of Caucasians, we found that carriers of the minor allele *(A)* of rs11672433 were at 56% higher risk for BAVM compared to noncarriers. Consistent with the SNP analysis, the minor allele *A* for rs11672433 was present in only one haplotype (TCAG), which was associated with an increase in BAVM risk. The other significant haplotype (CCGG) contained the major allele *G* for rs11672433 and was associated with a decrease in BAVM risk.

The *ANGPTL4* gene is located on chromosome 19p13.3 with 7 protein-coding exons and 2 noncoding exons and encodes the ANGPTL4 protein that belongs to a superfamily of secreted proteins, including angiopoietins [53]. Out of the four *ANGPTL4* SNPs studied, we found the minor allele *(A)* of synonymous (Pro389Pro) SNP rs11672433 in exon 6 to be associated with increased BAVM risk. No association was observed with the missense (Thr266Met) SNP rs1044250 *C/T* in exon 5 or other genotyped SNPs. The positive association observed with rs11672433 may be due to chance, although the association remained after adjustment for multiple comparisons using permutations. Alternatively, the SNP associated with BAVM risk may not be the causal allele, but instead serve as a surrogate marker in linkage disequilibrium with other putatively functional variants located in its proximity. Indeed, four rare missense SNPs (rs3210981, rs3210982, rs3210983, and rs3210984) and two synonymous SNPs (rs3210980, rs3210985) are located in a conserved region of exon 6 approximately 45 bp away from rs11672433 (dbSNP build 128, March 2006).

Normal angiogenesis is regulated by many angiogenic factors including angiopoietin 1, 2, and 4 [54], VEGF receptor family, and the TIE receptor family (TIE1 and TIE2) in vivo [10]. Although germ line and somatic mutations in TIE2 have been associated with venous malformations [55], common polymorphisms in TIE receptor family genes have not been associated with BAVMs [48]. Furthermore, polymorphisms in key genes involved in the angiogenic pathway (i.e. *ANGPT2, FLT4, KDR,* and *VEGF*) were also not associated with BAVM or ICH presentation in BAVM cases [48].

ANGPTL4 has been implicated in regulation of angiogenesis [23–28]. Unlike angiopoietin 1, 2, and 4, ANGPTL4 exerts its effect independent of the TIE receptor family (TIE1 and TIE2) [54]. Moreover, ischemia models have shown that the angiogenic effects of ANGPTL4 expression are increased at both the mRNA and protein levels in response to hypoxia, and ANGPTL4-induced angiogenesis is independent of VEGF [24, 25, 35]. A study of immature rat brain showed that several vascular genes including *Angptl4* were up-

regulated, and cerebral blood flow was attenuated during a subsequent hypoxic-ischemic insult [56]. Perhaps the most relevant data come from a knockout mouse study demonstrating that the *Angplt4* gene is required for proper functional partitioning of postnatal lymphatic and blood vessels in the intestine [17, 37].

While genetic variation in *ANGPTL4* has been shown to affect protein processing and function [29], the SNP associated with BAVM disease is synonymous and the direct effect of this variant on protein expression or processing is unknown. Our results suggested lower levels of 50-kDa full-length ANGPTL4 in AVM tissues from patients who carry the rs11672433 risk allele. Several studies suggest that ANGPTL4 is a potent anti-angiogenic factor [28, 33], and our data support a potential role for ANGPTL4 in the pathogenesis of BAVM, which involves dysregulated angiogenesis. Furthermore, brain-specific ablation of the *Angptl4* gene in the mouse brain could provide direct evidence for biological plausibility of *Angptl4* gene involvement in BAVM pathogenesis. One study showed that the signaling pathways underlying the anti-angiogenic activities of ANGPTL4 possibly act through inhibition of the Raf/MEK/ERK1/2 MAP kinase pathway in endothelial cells [33]. Thus, a study of the signaling pathways underlying the angiogenic activities of ANGPTL4 in BAVM tissue is warranted.

Our sample size of 216 BAVM cases with DNA available for genetic studies of this rare disease constitutes a strength of the study. Given the assumption of a dominant mode of inheritance or a dose response linear relationship, we observed a significant association with rs11672433 in our primary analysis comparing cases and controls, even after adjusting for multiple testing. However, our secondary analysis comparing ICH (n = 83) to non-ICH cases (n = 133) at presentation was likely underpowered to detect anything but strong effects given the smaller sample size.

Our study also had several important limitations: (1) the analysis was restricted to Caucasians, so our results may not be generalizable to other race/ethnic groups; (2) unrecognized population substructure differences between BAVM cases and controls or between ICH and non-ICH cases may result in false-positive associations, but this is not likely a major concern in US Caucasian populations [57, 58], and (3) given the relatively small size of the cohort, replication in additional cohorts is needed to validate findings and provide a more reliable estimate of the effect size. Upon replication of association findings in other AVM cohorts, in vitro and/or in vivo experiments will be necessary to evaluate the functional consequence of genetic variants in *ANGPTL4*. In addition, future studies should include association testing for functional polymorphisms, including common and rare variants that are located nearby and/or in high linkage disequilibrium with the associated SNP.

In summary, in this population of Caucasians, we observed an association between the minor allele *(A)* for *ANGPTL4* rs11672433 and haplotypes carrying this allele with risk of BAVM but not with ICH presentation. This association appears independent of the *ALK1* genotype, which was previously shown to be associated with BAVM risk in two different BAVM cohorts [51, 52]. These results suggest that *ANGPTL4* polymorphisms may predispose individuals to BAVM and may represent a pathway independent of ALK1 (TGF-β signaling) for further study in BAVM pathogenesis.

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