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

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Background: Brain arteriovenous malformations (BAVMs) are formed by hypertrophied arterial vessels (afferents, feeders), a large number of arteriovenous shunts which become tangled to form a body (nidus) of malformation, which then expands draining proximal veins. The aim of this study was a replication of single nucleotide polymorphism (SNP) rs11672433 association with BAVM development with the subsequent meta-analysis of published data. *Methods:* A total of 252 Russian patients with brain BAVMs and 480 control subjects were included in the present study. Genotyping was performed using real-time polymerase chain reaction with competitive hydrolysis probes. *Results:* In our case–control study, we found no significant association with brain arteriovenous malformation for the SNP rs11672433 of *ANGPTL4* gene (odds ratio .82, 95% confidence interval = .57-1.17 *P* value = .27) as well as in meta-analysis (odds ratio 1.18, 95% confidence interval = .81-1.73, *P* value = .39). *Conclusions:* Our data showed that SNP rs11672433 was not associated with the BAVM Russian population and the following meta-analysis did not detect an association in total. Thus, in spite of the fact that ANGPTL4 (protein) participates in the angiogenesis regulation processes, we consider that SNP rs11672433, a high-frequency locus in the *ANGPTL4* gene, does not influence the predisposition to BAVM or its effect is too small to be detected in the present size sample set. **Key Words:** Arteriovenous malformations—angiopoietin-like proteins 4 (ANGPTL4)—single nucleotide polymorphisms—association—gene.

Introduction

Brain arteriovenous malformations (BAVMs) are formed by hypertrophied arterial vessels (afferents, feeders), a large number of arteriovenous shunts which become tangled to form a body (nidus) of malformation, which then expands draining proximal veins.[1] The BAVM and cerebral cavernous malformations are the most common vascular malformations with an incidence of approximately 1.1 and .6 per 100,000 adult population per year, respectively.[2]

The estimated BAVM detection rate is 1.1-1.34 per 100,000 patient-year.[3] The survival rate of patients with arteriovenous malformation is 85% in the first 10 years and 65%

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in 30 years after diagnosis. In the treatment of BAVM, the main objective of any kind of intervention is the full obliteration of a malformation for prevention of intracranial hemorrhages (ICHs). There are some debates at the ARUBA study (A Randomized Trial of Unruptured Brain Arteriovenous Malformations [AVMs]), which describes treatment methods between vascular lesions. BAVMs are treated by endovascular embolization, stereotactic radiosurgery, and microsurgical resection.[4]

According to published data, various factors could be involved in initiating pathological processes of malformation, including genetic factors, and may predispose individuals to BAVM. A key role in the development of BAVM is played by angiogenesis: (1) BAVMs are formed as a result of angiogenesis disorders in the primary capillary reduction stage, and (2) neovascularization contributes to AVM progression.[5] The angiogenesis genes belong to several families such as the cyclin-dependent-kinase inhibitors (*CDKN2A*, *CDKN2B*), the family of vascular endothelial growth factors (*VEGF*), their receptors (*VEGFR*), fibroblast growth factors (*FGF-2*), angiopoietins (*Angpt-1,2*), TEK receptor tyrosine kinase (*Tie*) 1 and 2, interleukin-8 (*IL-8*), platelet-derived growth factor (*PDGF*), transforming growth factor-beta (*TGF beta*), and angiopoietin-like proteins (*ANGPTL*).[6] The last one represents a good candidate for regulating angiogenesis, so the *ANGPTL4* gene encodes an important angiogenesis factor involved in the postnatal formation of vessels.

For the first time, the association between the polymorphisms of *ANGPTL4* gene and BAVM was established in 2011 by Mikhak et al, in Caucasian individuals in California (216 Caucasian BAVM cases and 246 healthy controls). They studied 4 SNPs: rs2278236, rs1044250, rs11672433, and rs1808536 of *ANGPTL4* gene for association with risk of BAVM or ICH. They showed that the allele A rs11672433 of the *ANGPTL4* gene (odds ratio [OR] = 1.56; 95% confidence interval = 1.01-2.41; $P = .046$) was associated with the risk of BAVM, but not with ICH. Their data confirmed a potential role of the *ANGPTL4* gene in the pathogenesis of BAVM, which could be linked to an angiogenesis dysregulation. The remaining polymorphic variants did not show any association. As the authors specify, the research was limited to a single Caucasian ethnic group. In addition, the sample size was small.[7]

Four years later, Kremer et al conducted a replicative research of SNPs of *ANGPTL4* genes, *IL-1b*, *GPR124*, *VEGFA*, and *MMP-3* with risk of BAVM in Caucasians from the Netherlands (167 Caucasian BAVM cases and 1038 healthy controls) with meta-analysis. Meta-analysis for rs11672433 of *ANGPTL4* gene has shown a significant association with BAVM (OR 1.39; 95% CI 1.10-1.75, P value = .005). The authors concluded that previous studies of the function of the protein ANGPTL4 and association between BAVM and SNP of *ANGPTL4* could demonstrate contribution of protein to the pathogenesis of BAVM.[8] Nevertheless, the number of cases and controls was disproportional—167 patients versus 1038 healthy controls. It is possible that the results could have been overestimated and further replications are necessary to prove the role of *ANGPTL4* in BAVM predisposition. Thus, the purpose of our research was to carry out a replication of association for SNP rs11672433 in a Russian population with the subsequent meta-analysis of all published research.

Materials and Methods

Patients

The study included 252 patients (mean age: 37.1 ± 15.0, female/male: 111/141) with brain BAVMs. Magnetic resonance imaging and cerebral angiography were performed in the clinical centers in Novosibirsk: Federal Neurosurgical Center, Novosibirsk, Russia, and Novosibirsk Research Institute of Blood Circulation Pathology named after E. N. Meshalkin, Novosibirsk, Russia. Each patient completed a specially developed questionnaire including demographic data (age, gender, nationality) and medical information (age, debut, current type). The average age of patients at the period of manifestation was 33.8 ± 13.5 years. The control group consisted of 480 individuals (mean age: 33.0 ± 11.1, female/male: 199/281) from Novosibirsk without BAVM. The study was approved by the Local Ethics Committee of Center of New Medical Technologies of Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences.

SNP Selection

Polymorphism in the *ANGPTL4* gene region was selected from the analysis based on published data and subsequent sequence database searches (dbSNP). There are 6 SNPs in ANGPTL4 previously studied[7-12] with metabolic traits,[12] lipid metabolism and adiposity,[10] coronary disease,[11] BAVM,[7,8] and severity of post-transplant proteinuria in kidney allograft recipients[9] (Table 1). The set of SNPs was formed by previous investigators based on (1) HapMap genotype and linkage disequilibrium (LD) data (rs4076317, rs2278236, rs1044250, rs11672433, rs1808536) and (2) the previously described effect on the ANGPTL4 function,[13] plasma lipid levels, and cardiovascular risk.[11]

These SNPs covered 100% of the common variants (minor allele frequency [MAF] ≥.05) within the *ANGPTL4* gene, with an $r^2 \geq .8$ according to TAGGER analysis (http://www.broad.mit.edu/mpg/tagger). According to HapMap (data release 21, July 2006 and release 23a/phase II, March 2008) and previously published data,[10,12] the 4 SNPs (rs4076317, rs2278236, rs1044250, rs11672433) were in weak LD with the others based on r^2 ($r^2 < .50$), but they all lie within high-LD block based on D' ($D' > .945$) (Table 2).

Table 1. *Previously studied SNPs reported in association research with diseases. Variant's position is given according to 1000 Genomes Homo sapiens: GRCh37.p13 (GCF_000001405.25).*

Variant	Consequence	Annotation or trivial name	MAF	Disease
19: 8428999 C/G (rs4076317)	12 nucleotides upstream of the transcription initiation site	c.207C>G	G: —* 0.2323† 0.311‡ 0.161§ 0.28‖ 0.29¶ 0.2041#	Metabolic traits,[12] lipid metabolism and adiposity,[10] proteinuria[9]
19: 8429323 A/G (rs116843064)	E40K (exon 1)	c.118G>A	A: .01868 (n = 7013)* 0.0455† 0.02**	Coronary disease,[11] proteinuria[9]
19: 8431581 C/T (rs2278236)	Intron 3	c.547, 378G>A	C: —* 0.3939† 0.485‡ 0.375§ 0.46‖ 0.48¶ 0.4209#	Metabolic traits,[12] lipid metabolism and adiposity,[10] proteinuria,[9] BAVM[7]
19:8436164 C/T (rs1044250)	p.Thr266Met (exon 5)	Missense, c.797C>T; T266M	T: .3042 (n = 60232)* 0.3485† 0.289‡ 0.358§ 0.30‖ 0.29¶ 0.2396#	Metabolic traits,[12] lipid metabolism and adiposity,[10] proteinuria,[9] BAVM[7]
19:8438716 G/A (rs11672433)	p.Pro389Pro (exon 6)	Synonymous, c.1167G>A; P389P	A: .1037 (n = 60271)* 0.1414† 0.159‡ 0.172§ 0.16‖ 0.14¶ 0.0555#	Metabolic traits,[12] lipid metabolism and adiposity,[10] proteinuria,[9] BAVM[7,8]
19:8441857 G/A (rs1808536)	3′-UTR		A: —* 0.1667† —‡ —§	BAVM[7]

Abbreviations: BAVM, brain arteriovenous malformation; SNP, single nucleotide polymorphism.
*ExAC Browser.
†1000 Genomes CEU (n = 198).
‡Staiger et al (N = 629 overweight nondiabetic subjects from the southwest of Germany).[12]
§HapMap.
‖Legry et al (participants were recruited as part of the World Health Organization [WHO]-MONICA population survey performed from 1995 to 1997 in the Lille Urban Community in Northern France [N = 1195]).[10]
¶Participants (N = 1155) were recruited as part of the HELENA study (http://www.helenastudy.com) performed from 2006 to 2007 in 9 European countries (Greece, Germany, Belgium, France, Hungary, Italy, Sweden, Austria, and Spain).
#The National Center for Biotechnology Information (NCBI) dbSNP.
**Muendlein et al (N = 470 from sample of 245 with and 245 without significant coronary stenosis with lumen narrowing >50%, matched for gender).[11]

From previous association study of BAVM by Mikhak et al, only rs11672433 of *ANGTPL4* gene was associated with BAVM.[7] Kremer et al. replicated this association with follow-up meta-analysis.[8] We selected rs11672433 for our replication study at the residents of the West Siberian region.

Genotyping

DNA was extracted from venous blood using a standard method including isolation and lysis of blood cells, hydrolysis of proteins with proteinase K, and purification of DNA by extraction with a phenol–chloroform

Table 2. *Linkage disequilibrium statistics (D′, r^2) among the 4 previously genotyped SNPs of the 17.75-kb genomic region harboring the* ANGPTL4 *gene.*

SNP	rs4076317	rs2278236	rs1044250	rs11672433
rs4076317	—	1.0*	1.0*	1.0*
		0.945†	0.985†	1.0†
		+1.0‡	−1.0‡	−1.0‡
rs2278236	0.33*	—	1.0*	1.0*
	0.429†		0.993†	0.951†
	0.44‡		−1.0‡	−1.0‡
rs1044250	0.11*	0.33*	—	1.0*
	0.178†	0.377†		1.0†
	0.17‡	0.37‡		−1.0‡
rs11672433	0.04*	0.11*	0.11*	—
	0.086†	0.161†	0.077†	
	0.07‡	0.15‡	0.08‡	

Abbreviation: SNP, single nucleotide polymorphism.
*HapMap (CEU, N = 120 subjects).
†Staiger et al (N = 629 overweight nondiabetic subjects from the southwest of Germany).[12]
‡Legry et al (participants were recruited as part of the World Health Organization [WHO]-MONICA population survey performed from 1995 to 1997 in the Lille Urban Community in Northern France [N = 1195]).[10]

mixture, and precipitation of DNA with ethanol. Genotyping of SNP in the *ANGPTL4* gene (rs11672433) was performed using a real-time PCR with competitive hydrolysis probes complementary to the polymorphic DNA sites. Amplification was performed using a CFX-96 cycler (Bio-Rad, Hercules, California, USA). The polymerase chain reaction mixture (25 µL) contained 40-100 ng DNA; 300 nM of each primer (F: 5′-AAGGGAATCTTCTGGAAGACCT-3′, R: 5′-TGCTATGGGCTGGATCAACA-3′); 100-200 nM TaqMan probe (HEX-5′-CTACTACCCGCTGCAGGC-BHQ-3′, FAM-5′-CTACTACCCACTGCAGGCC-BHQ-3′); 200 µM dNTP, buffer for amplification, 1 U of Taq-polymerase. The genotyping success rate was 95%.

Statistical Analysis

The Hardy–Weinberg equilibrium was evaluated using an exact test of Hardy–Weinberg equilibrium for 2-Allele markers in the R package "genetics." Possible associations between SNPs and disease development were found using logistic regression analysis, as implemented in the "glm" function of the R package for statistical analysis (www.r-project.org). Meta-analysis and estimated heterogeneity were carried out using the "rmeta" package for R (http://cran.r-project.org/web/packages/rmeta/rmeta.pdf). Pooled ORs were computed by the random-effect model for data combined under heterogeneity between studies. Results were considered statistically significant for all statistical calculations if $P < .05$.

Results

We performed genotyping SNP rs11672433 in patients with BAVM and controls. The distribution of SNP genotype corresponded to a Hardy–Weinberg distribution in the BAVM patients ($P = 1.00$) and controls ($P = .15$). Association between *ANGPTL4* and the genotype of SNP was estimated using logistic regression. We did not find a significant association between the SNP rs11672433 *ANGPTL4* gene and the BAVM (OR .82, 95% CI = .57-1.17, $P = .27$), which was not consistent with previous data on residents of California (Table 3). The MAF in our control (.11) did not differ significantly from those described by Kremer et al (.15) and Mikhak et al (.13), but we had differences between MAF in cases (.09, .19, .173; our study, Kremer et al, and Mikhak et al, respectively). No association between BAVM and rs11672433 was obtained in our study.

We included all previously published data in the meta-analysis (Mikhak et al[7]) and showed that rs11672433 was not significantly associated with BAVM (OR 1.18, 95% CI = .81-1.72, P value = .39, Phet = .024) (Table 3, Fig 1).

Discussion

Vasculogenesis and angiogenesis are important stages in the development of blood vessels during embryogenesis. One of the most important stimulators of angiogenesis is angiopoietin. There are 4 types of angiopoietins—Ang1, Ang2, Ang3 and Ang4. Angiopoietin-like proteins (ANGPTL) are a family of proteins structurally similar to angiopoietins—which are also involved in angiogenesis.[14] The family of angiopoietin-like proteins consists of 8 proteins, which render a pleiotropic effect on angiogenesis and lipid metabolism stimulates development of hematopoietic stem cells in the culture.[15] Angiopoietin-like proteins (ANGPTL)—being regulators of development of

Table 3. *Association of SNP rs11672433 with BAVM.*

Study ID	Race/Ethnicity	OR	95% CI	*P*	Case/Control*	MAF control	MAF case
Mikhak et al[7] 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. Controls were healthy volunteers with no significant medical history recruited from the same clinical catchment area.	Caucasian	1.65	1.15-2.36	.006	216(143 + 63 + 10)/ 246(190 + 50 + 6)	.13	.19
Kremer et al[8] 167 Caucasian patients (mean age 46 years, SD 14 years; 56% males) with a brain BAVM who had presented to the University Medical Center Utrecht, the Netherlands, between 1985 and 2010. Controls were 1038 healthy Dutch volunteers (mean age 62 years, SD 10 years; 59% male) recruited for a previous genome-wide association study (GWAS).	Caucasian	1.21	.89-1.65	.23	167/1038	.15	.173
Present study	Mixed	.82	.57-1.17	.27	247 (203 + 42 + 2)/ 448 (356 + 83 + 9)	.11	.09
Meta-analysis of all test of heterogeneity: χ^2=7.42, df = 2 (*P* = .024)		1.18	.81-1.72	.39	630/1732		

Abbreviations: BAVM, brain arteriovenous malformation; CI, confidence interval; MAF, minor allele frequency; OR, odds ratio; SD, standard deviation; SNP, single nucleotide polymorphism.

*In the column's cells, the distribution by genotypes is given: total in the AVM group (GG carriers + GA carriers + AA carriers)/total in the control group (GG carriers + GA carriers + AA carriers).

a vascular wall—are not bound to TIE-2 or TIE-1 receptors, unlike angiopoietins.[16] The human *ANGPTL4* gene belongs to the ANGPTL family and is located on chromosome 19p13.3. The gene has 8 exons and 7 introns and encodes the ANGPTL4 protein.[12] ANGPTL4 is a secreted glycoprotein expressed by liver, adipose tissue, pericytes, and smooth myocytes of a vascular wall. ANGPTL4 modulates vascular permeability, inflammatory signaling, angiogenesis, etc.[17] It has been shown that vascular permeability and myocardial infarction severity are increased in ANGPTL4-deficient mice. ANGPTL4-deficient mice also suffer a disorganization of endothelial adherent's junctions, suggesting that ANGPTL4 could promote the endothelial barrier function at multiple levels.

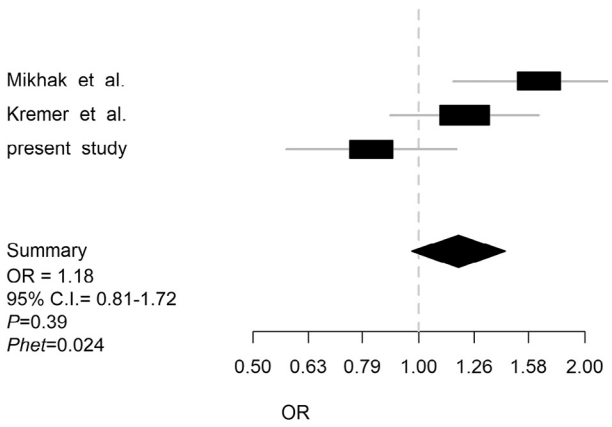

Figure 1. *Meta-analysis of the association between rs11672433 and brain arteriovenous malformations. CI, confidence interval; OR, odds ratio.*

We had no luck to replicate the association of *ANGPTL4* gene polymorphism with BAVM development. This can have a number of explanations. The ANGPTL4 protein is cleaved at the N-terminal-coiled-coil domain (CCD) and the C-terminal fragment—the fibrinogen-like domain (FLD) by several types of pro-protein convertases in the cellular matrix or in blood plasma.[11] The N-terminal domain (CCD) is functionally active, binds heparan and dermatan sulfates, and inhibits endothelial cell adhesion, motility, and tubule-like formation. The SNP rs11672433 (p.Pro389Pro) is localized in the not functionally active C-terminal domain.[18] Moreover, SNP rs11672433 does not change the amino acid sequence and therefore does not

change the protein function. Also, according to the database Blood eQTL browser (http://genenetwork.nl/bloodeqtlbrowser/), Single nucletide polymorphism rs11672433 is annotated like expression quantitative trait locus (eQTL) for the MARCH2, ELAVL1 genes. So it would be unlikely that SNP rs11672433 has a direct functional effect on ANGPTL4.

There are few high-frequency SNPs in *ANGPTL4* gene (MAF > .05). Practically all of them have been previously investigated and an association with BAVM was not detected, except for SNP rs11672433 in Mikhak et al. study. The results of our study support no significant association of SNP rs11672433 of the *ANGPTL4* gene with BAVM in the residents of the West Siberian region of Russia. The meta-analysis has also not revealed an association with BAVMs for rs11672433 in *ANGPTL4* (OR 1.18, 95% CI = .81-1.73, P = .3). Taking into account that we and Kremer et al have not found a significant association, and as it has been found only in a pilot research, perhaps we had deal with the phenomenon "winner's curse."[19]

The limitation of all *ANGPTL4* association studies is the small sample size. The statistical power to detect association in the provided meta-analysis with OR = 1.18 and P = .05 is only 29%. For 80% statistical power, it would be necessary to examine about 2200 person cases and 2200 controls.

Finally, our data showed that SNP rs11672433 was not associated with BAVM in the residents of the West Siberian region of Russia, and the following meta-analysis also did not detect an association. Thus, in spite of the fact that the ANGPTL4 (protein) participates in the angiogenesis regulation processes, we conclude that the high-frequency SNP rs11672433 in *ANGPTL4* gene does not influence the predisposition to BAVM and processes of angiogenesis.

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