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Association between Polymorphisms rs1333040 and rs7865618 of Chromosome 9p21 and Sporadic Brain Arteriovenous Malformations

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Abstract

Background: The chromosomal locus 9p21 is a novel genetic marker for a variety of cardiovascular and cerebrovascular diseases. In a recent study, we have demonstrated an association between the single nucleotide polymorphism (SNP) rs1333040C>T on chromosome 9p21 and sporadic brain arteriovenous malformations (BAVMs). Here, we extended our analysis to an additional SNP on chromosome 9p21 (rs7865618A>G) and increased our sample size including BAVMs from two different Italian neurosurgical centers.

Methods: We studied 206 patients with sporadic BAVMs and 171 unaffected controls. Genomic DNA was isolated from peripheral blood and the rs1333040C>T and rs7865618A>G polymorphisms were assessed by PCR-RFLP using the *Bsm*I and *Msp*I restriction endonucleases, respectively. For each SNP, we performed dominant, recessive, and additive genetic models.

Results: The distribution of the three possible genotypes of rs1333040 (TT, TC and CC) was statistically different between cases and controls ($p = 0.0008$). The TT genotype was significantly associated with BAVMs both in the dominant ($p = 0.013$) and recessive ($p = 0.012$) models. The T allele was significantly associated with BAVMs in the additive model ($p = 0.002$). Also the distribution of the three possible genotypes of rs7865618 (GG, AG and AA) was statistically different between cases and controls ($p = 0.005$), and the GG genotype and G allele were significantly associated with BAVMs in the dominant ($p = 0.032$), recessive ($p = 0.007$), and additive models ($p = 0.009$). We also detected a significant association between BAVMs with large nidus size and the GG genotype and G allele of rs7865618 and the TT genotype of rs1333040. A deep venous drainage was instead associated with the TT genotype of the rs1333040 and the GG genotype of the rs7865618. The occurrence of bleeding was associated with the TT genotype and T allele of rs1333040, while the presence of seizures appeared associated with the GG genotype of rs7865618.

Conclusions: SNPs of the 9p21 region, in addition to be genetic markers for coronary artery disease, stroke, and intracranial aneurysms, are associated with sporadic BAVMs. These results extend and strengthen the role of the 9p21 chromosomal region as a common risk factor for cerebrovascular diseases.

Background

The 9p21 chromosomal locus is the strongest genomic marker for cardiovascular diseases that has thus far been identified in multiple genome-wide association studies [1,2,3,4]. These studies have revealed that the 9p21 locus contains single nucleotide polymorphisms (SNPs) associated with myocardial infarction, coronary artery disease (CAD), peripheral artery disease, abdominal aortic aneurysms (AAA), intracranial aneurysms, and stroke [4,5,6,7,8,9,10].

Intriguingly, we have recently reported that one SNP on chromosome 9p21 - the rs1333040 gene polymorphism - is associated with sporadic brain arteriovenous malformations (BAVMs) [11]. The rs1333040 polymorphism is located in proximity of a noncoding RNA sequence named ANRIL (antisense noncoding RNA in the INK4 locus) and has been consistently associated with both stroke and intracranial aneurysms [12]. Its association with BAVMs thus suggests that SNPs in the 9p21 chromosomal locus may be common genetic markers for a variety of cerebrovascular diseases and that BAVMs, intracranial aneurysms, and stroke may share common, currently unknown, pathogenic mechanisms.

To further investigate this issue, we have extended our analysis to an additional SNP of the 9p21 locus - the rs7865618 gene polymorphism - which is also located in proximity of ANRIL, but is representative of a different linkage block compared to rs1333040, and has been previously identified as an independent predictor of myocardial infarction in multivariable models adjusted for conventional cardiovascular risk factors [13]. We also increased the sample size of our previous study, enrolling additional individuals from our Division of Neurosurgery at the Catholic University School of Medicine in Rome and including BAVMs from an additional Italian tertiary referral neurosurgical center (University of Turin).

Material and Methods

Study Participants

The BAVM group consisted of subjects undergoing neurosurgical treatment or follow-up for BAVMs (n = 206) at two different Italian referral centers: the Division of Neurosurgery at the A. Gemelli University Hospital of Rome and the Division of Neurosurgery at the San Giovanni Battista Hospital of Turin. In all cases, the diagnosis of BAVM was based on the results of brain MRI and digital subtraction angiography. Clinical records and neuroimaging data of all patients were retrospectively analyzed in detail in order to determine the natural history of the disease, in terms of bleeding and/or occurrence of seizures, and BAVM angioarchitectural features (Martin-Spetzler grading). The control group (n = 171) consisted of individuals consecutively admitted to the Department of Neurosurgery of the A. Gemelli University Hospital of Rome, from January 2011 to December 2012, because of a traumatic brain injury. To be included as controls, subjects needed a contrast-enhanced brain CT scan negative for any type of vascular lesion. In addition, a negative history of stroke and other cardiovascular diseases, assessed through the analysis of medical records and physical examination, was required. All patients and controls were Caucasians and unrelated to each other. All participating individuals were adults, except for 16 BAVM patients, who were younger than 18 years. All participating individuals (or their legal representatives) signed an informed consent to participate in this study, which was approved by the Ethics Committees of the Catholic University of Rome and the University of Turin.

Genotyping

Venous blood (2 ml) was collected and stored at -80°C before use. Genomic DNA was isolated from whole blood using a commercially available DNA extraction kit (Illustra Blood GenomicPrep Mini Spin Kit, GE Healthcare). Polymorphism spanning fragments were amplified by a PCR-RFLP assay. PCR reaction was carried out in a total volume of 25 µl using 5 µl of template DNA, 1 × PCR buffer (10 mM Tris-HCl, pH 8.5, 50 mM KCl), 1.5 mM $MgCl_2$, 0.0625 mM of each deoxynucleotide triphosphate (dATP, dTTP, dGTP, dCTP), 0.5 mM of each primer (forward 5′-TCT GGA AGC ACT GGG AAG GAT G-3′ and reverse 5′-TTG ATT TGG GAG CCA CTG TTG-3′) and 1.6 U of Taq DNA polymerase (Roche). Thermal cycling was performed in a PE Applied Biosystems 9700 Sequence Detector with the following program: 4 min at 95°C, 40 cycles of 1 min at 94°C, 1 min at 65°C and 1 min at 72°C, with a final extension step at 72°C for 7 min. Genotyping of the rs1333040C>T polymorphism was identified using restriction endonuclease *Bsm*I. The amplified DNA (5 ml), 10 U of *Bsm*I, and 1 µl of ×10 recommended restriction buffer in a final volume of 10 µl were incubated at 65°C for 1 h. Digested products were separated on 2% agarose gel by electrophoresis. Polymorphic region rs7865618 of gene CDKN2B was PCR amplified using a forward primer: 5′-ATA ACA GGG GAT GGA TTC TTG TGG A-3′ and reverse primers: 5′-TCC ATG AAC CTG CTT TTC TCA TCT TT-3′. The cycling conditions were 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 65°C for 1 min, 72°C for 1 min and final extension at 72°C for 7 min. The PCR products of 579 bp were digested with *Msp*I (Biolabs) for the rs7865618 (A/G) polymorphism. The resulting products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. Positive and negative controls were included in each analysis. In case of missing or defective results, genotyping was repeated. At the end of the analyses, no samples were excluded from the study. Demographic, clinical and angiographic data were available for all studied individuals.

Statistical Analysis

Statistical analysis was performed with SPSS Statistical Package version 12.0 (Chicago, Ill., USA). Hardy-Weinberg equilibrium was calculated by a Pearson's χ^2 test calculator for biallelic markers. The association between rs1333040 and rs7865618 genotypes and BAVMs was assessed using dominant, recessive and additive (trend) genetic models. Associations with the following variables were also considered and calculated using a Pearson's χ^2 test: nidus diameter, deep venous drainage, Spetzler-Martin score, bleeding, and seizures. Results were corrected for number of tested polymorphisms by using Bonferroni test correction (p value/number of SNPs). We also created a comprehensive risk score, ranging from 0 to 2, according to the number of ‘at-risk' genotypes concomitantly carried by a given individual. Categorical variables were compared using Pearson's χ^2 test. A value of $p < 0.05$ was considered statistically significant.

Results

Demographic characteristics of the studied population are shown in table 1. Mean age in the BAVM group was 42.6 ± 18.2 years (age range: 4-85 years), while in the control group it was 52.7 ± 19.9 years (age range: 18-96 years). There was no significant difference in mean age between patients and controls ($p = 0.23$). The cohort of BAVM individuals included 111 men (53.88%), while the number of men in the control group was 109 (63.74%). The male/female ratio was not statistically different between the two groups ($p = 0.053$).

Table 1. Demographic characteristics of the studied population

	BAVM group	Control group	p
Subjects, n	206	171	–
Sex (M/F ratio)	111/95	109/62	0.053
Mean age ± SD, years	42.6±18.2	52.7±19.9	0.23
Age range	4–85	18–96	

SD = Standard deviation.

Differences in demographic, clinical, and angioarchitectural features between the two groups of BAVM patients from Rome and Turin are reported in table 2. Patients of the Turin cohort were significantly older and showed a higher incidence of bleeding. Patients of the Rome cohort had significantly larger BAVMs.

Table 2. Demographic, clinical, and angioarchitectural features of BAVM patients in the two cohorts (Rome and Turin).

	Rome	Turin	p
Patients, n (n = 206)	100	106	–
Mean age ± SD, years (42.6±18.2)	38.6±19.1	46.3±16.6	0.002
Male sex (n = 111; 53.8%)	52	59	0.59
Bleeding (n = 125; 60.6%)	51	74	0.005
Seizures (n = 50; 24.2%)	27	23	0.37
Nidus diameter >4 cm (n = 49; 23.7%)	37	12	<0.001
Deep venous drainage (n = 81; 39.3%)	39	42	0.92
Spetzler-Martin 4–5 (n = 39; 18.9%)	25	14	0.03

Genotype frequencies in the Turin and Rome cohorts are presented in table 3. Genotypes were in Hardy-Weinberg equilibrium ($p = 0.87$ for rs1333040 and $p = 0.5$ for rs7865618). Except for the TC genotype of rs1333040, genotype frequencies were not differently distributed between the two cohorts.

Table 3. Genotype distribution in the two cohorts of BAVM patients (Rome and Turin)

Polymorphism	Genotype	Rome (n = 100)	Turin (n = 106)	p
rs1333040	TT	55	46	0.09
	TC	37	54	0.04
	CC	8	6	0.50
rs7865618	GG	8	18	0.05
	AG	47	52	0.73
	AA	45	36	0.10

Table 4 presents the distribution of the three possible genotypes of rs1333040 (TT, TC and CC), which was statistically different between cases and controls (p = 0.0008). The TT genotype was significantly associated with BAVMs both in the dominant (p = 0.013) and recessive (p = 0.012) models. The T allele was also significantly associated with BAVMs in the additive (trend) model (p = 0.002). The distribution of the three possible genotypes of rs7865618 (GG, AG and AA) is also presented in table 4. Genotypes distributed in a statistically different manner between cases and controls (p = 0.005). The GG genotype and the G allele were significantly associated with BAVMs in the dominant (p = 0.032), recessive (p = 0.007) and additive (trend) models (p = 0.009).

Table 4. Genotype distribution in cases and controls

SNP	Test	Genotypes	Affected	Unaffected	d.f.	p
rs1333040	genotypes	TT/TC/CC	101/91/14	62/84/25	2	0.0008
	dominant	TT+TC/CC	192/14	146/25	1	0.013
	recessive	TT/TC+CC	101/105	62/109	1	0.012
	trend	T/G	293/119	208/134	1	0.002
rs7865618	genotypes	GG/AG/AA	26/99/81	8/77/86	2	0.005
	dominant	GG+AG/AA	125/81	85/86	1	0.032
	recessive	AA/AG+GG	26/180	8/163	1	0.007
	trend	A/G	151/261	93/249	1	0.009

d.f. = Degrees of freedom.

Then, BAVMs patients and controls were stratified according to the number of ‘at-risk' genotypes concomitantly carried by a given individual, with a score ranging from 0 (no ‘at-risk' genotypes) to 2 (two ‘at-risk' genotypes; table 5). Among the 189 subjects with score = 0, 45.5% were patients with BAVMs. Interestingly, this percentage increased significantly - to 63.1% - among subjects with score = 1 (p < 0.01; OR 1.93, 95% CI: 1.28-2.92). It further increased to 77.7% among subjects with score = 2, although this difference did not reach statistical significance (p = 0.15; OR 2.97, 95% CI: 0.61-14.5), probably due to the small sample size.

Table 5. Distribution of BAVM patients and controls among subjects concomitantly carrying 0, 1, or 2 'at-risk' genotypes (rs1333040 TT and rs7865618 GG)

Number of 'at-risk' genotypes	BAVM (n = 206)	Controls (n = 171)	p	OR (95% CI)
0 (n = 189)	86 (45.5%)	103 (54.5%)	reference	reference
1 (n = 179)	113 (63.1%)	66 (26.8%)	<0.01	1.93 (1.28–2.92)
2 (n = 9)	7 (77.7%)	2 (22.2%)	0.15	2.97 (0.61–14.5)

We also evaluated the possible association between different genotypes of the two investigated SNPs and a number of important clinical and angioarchitectural features of BAVMs (table 6). We detected a significant association between nidus size (>4 cm in diameter) and the GG genotype and G allele of rs7865618 in dominant (p = 0.02), recessive (p = 0.039), and additive (p = 0.008) models. We replicated the same association with the TT genotype of rs1333040 in the recessive model (p = 0.022). The presence of a deep venous drainage was instead associated with the TT genotype of rs1333040 in the dominant model (p = 0.01) and the GG genotype of rs7865618 in the recessive (p = 0.007) model. The occurrence of bleeding during the natural history of the disease was associated with the TT genotype of rs1333040 (p = 0.001) and the T allele in the trend model (p = 0.04), while the presence of seizures appeared associated with the GG genotype of rs7865618 (p = 0.01) in the dominant model.

Table 6. Univariate association of rs1333040 and rs7865618 with clinical and angioarchitectural features of BAVMs

SNP	Features	Dominant model		Recessive model		Additive model	
		TT+CT vs. CC	p	TT vs. CT+CC	p	T vs. C	p
rs1333040	nidus diameter ≥4 cm (n = 49)	46	0.83	31	0.022	77/21	0.06
	deep venous drainage (n = 81)	80	0.010	41	0.71	121/41	0.19
	Spetzler-Martin ≥3 (n = 39)	37	0.64	23	0.17	60/18	0.20
	bleeding (n = 125)	122	0.001	65	0.28	187/63	0.040
	seizures (n = 50)	44	0.09	25	0.87	69/31	0.59
		GG+AG vs. AA	p	GG vs. AG+AA	p	G vs. A	p
rs7865618	nidus diameter ≥4 cm (n = 49)	23	0.02	2	0.039	25/73	0.008
	deep venous drainage (n = 81)	48	0.73	4	0.007	52/110	0.12
	Spetzler-Martin ≥3 (n = 39)	201	0.33	0	0.008	21/57	0.047
	bleeding (n = 125)	78	0.53	18	0.33	96/154	0.35
	seizures (n = 50)	38	0.010	6	0.87	44/56	0.079

Discussion

The vast majority of the studies that have so far investigated the genetic base of sporadic BAVMs have focused on SNPs of genes involved in angiogenesis and inflammation [14,15,16]. However, most of these studies have provided controversial and inconsistent results and a recent meta-analysis carried out by our research team has demonstrated a statistically significant association with BAVMs only for the IVS3-35A>G polymorphism of the ALK1 gene (ACVRL1) [14], consistent with the notion that ACVRL1 mutations are involved in the formation of arteriovenous dysplasia in patients with hereditary hemorrhagic telangiectasia [17].

In this study, we investigated the hypothesis that the 9p21 locus, which is the most replicated genetic marker for diseases such as CAD, peripheral artery disease, AAA, stroke, and intracranial aneurysms [4,5,6,7,8,9,10], is also associated with sporadic BAVMs. To test this hypothesis, we studied the distribution of two prototypical SNPs of chromosome 9p21 in two cohorts of BAVM cases and controls from Rome and Turin, Italy. First, we found a statistically significant association between BAVMs and the TT genotype and T allele of rs1333040, consistent with our previous findings in a smaller sample of cases and controls from Rome [11,18]. Then, we found a significant association between BAVMs and the GG genotype and G allele of rs7865618. Finally, we saw significant associations between these 'at-risk' genotypes and alleles and various clinical and angioarchitectural features of BAVMs, such as nidus size, presence of deep venous drainage, history of bleeding, and occurrence of seizures.

Genes mapping in the 9p21 region include the cyclin-dependent kinase inhibitors CDKN2A ($p16^{INK4a}$), including its alternative reading frame (ARF) transcript variant ($p19^{ARF}$), and CDKN2B ($p15^{INK4b}$), along with ANRIL, that undergoes splicing and is transcribed from the opposite strand to CDKN2A/B. These genes are involved in the regulation of cell cycle progression and influence key physiological processes, such as replicative senescence, apoptosis, stem cell self-renewal and repair of aged tissue [11,19,20,21]. The SNPs that we evaluated in this study are on different linkage blocks in close proximity to ANRIL. ANRIL is expressed in atherosclerotic tissues and its transcript, DQ485453, has been detected in primary coronary smooth muscle cells and macrophages and in carotid endarterectomy and AAA tissue samples [22]. Folkersen et al. [23]have identified eight ANRIL transcripts also in lymphoblastic cells and carotid artery, medial aorta, and mammary artery plaque tissue, while Holdt et al. [24] have found differential expression of ANRIL in peripheral blood mononuclear cells of patients with CAD and carotid, aortic and femoral plaques. In addition, there are genome-wide association studies that have associated ANRIL with type 2 diabetes mellitus [25], glioma [26], and basal cell carcinoma [27]. The 5′ portion of ANRIL gives rise to a natural antisense RNA of 34.8 kb, named p15, that has been shown to specifically interfere with transcription of $p15^{INK4b}$ (sense) RNA from CDKN2B in tumor cells [26].

Although our analysis was limited to only 2 SNPs of the 9p21 chromosomal region, our findings provide proof of the concept that this genetic locus may play a role in BAVM pathophysiology and that BAVMs may share common pathogenic mechanisms with a number of other cardiovascular and cerebrovascular diseases. Indeed, rs1333040 and rs7865618 have been associated, to various extent, with CAD, peripheral artery disease, AAA, and, most importantly, stroke and intracranial aneurysms [4,5,6,7,8,9,10,12,13]. It is important to note that the SNPs of chromosome 9p21 lack associations with common cardiovascular risk factors, thus the mechanisms through which they influence the risk of vascular diseases, in addition to being novel and unknown, are potentially amenable to diagnostic and therapeutic interventions.

This study has some potential limitations. First, it is based only on a relatively small patient/control population, thus our findings should be considered as hypothesis-generating and need confirmation in larger prospective studies and in other ethnic groups. In addition, we cannot exclude the fact that the reported associations are caused by genes in linkage disequilibrium with the investigated SNPs. This possibility needs to be further investigated through transmission disequilibrium tests and/or linkage disequilibrium mapping.

Conclusions

This study shows that two SNPs of the 9p21 region - rs1333040 and rs7865618 - in addition to be genetic markers for CAD, stroke, and intracranial aneurysms, are associated with sporadic BAVMs. These findings need confirmation in additional replication cohorts.

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Disclosure Statement

All authors have read and approved the submitted manuscript; it has not been submitted elsewhere nor published elsewhere in whole or in part. There are no competing interests.

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