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EXPERIMENTAL RESEARCH - VASCULAR

Vascular endothelial growth factor gene polymorphisms and intracranial aneurysms

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Abstract
Background The exact pathophysiology of the development and rupture of saccular aneurysms is still controversial. Several lines of evidence indicate a role for inflammatory processes. Similarly, abnormal angiogenesis might be related to aneurysm growth. Expression of angiogenesis factors is higher in patients harboring aneurysms. The aim of this study was to verify the association of two functionally active polymorphisms (+ 396 C>T and 18 bp microdeletion) in the vascular endothelial growth factor (VEGF) gene with both susceptibility to and clinical features of aneurysmal subarachnoid hemorrhage (SAH) in an Italian population.
Method Allelic and genotypic frequencies of the+396 C>T and the 18 bp microdeletion of the VEGF gene were determined in 200 patients and 200 healthy controls.
Results Both allelic and genotypic frequencies of the examined polymorphisms in the VEGF gene were not significantly different between cases and controls. Furthermore, the different VEGF genotypes did not seem to significantly modify the main clinical features of the disease.
Conclusions Our data suggest that the VEGF gene is not a major genetic risk factor for aneurysmal subarachnoid hemorrhage.

Keywords Vascular endothelial growth factor · Polymorphisms · Genotype · Angiogenesis · Aneurysmal subarachnoid hemorrhage

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Introduction

Non-traumatic subarachnoid hemorrhage (SAH) is mostly due to intracranial aneurysm (IA) rupture. The estimated annual incidence of aneurysmal SAH in most western populations is six to ten new cases per 100,000 people, with a peak age of 55–60 years. Despite recent great improvements in diagnosis, medical care, endovascular procedures and surgical treatment, the related morbidity and mortality still remain elevated, with an overall mortality rate between 32 % and 67 % [2, 3].

The exact pathophysiology of the development and rupture of saccular aneurysms is still controversial. Several factors can predispose to aneurysm formation and growth, including familiar clustering, vascular diseases, arterial hypertension, female gender, increasing age, and cigarette and excessive alcohol consumption [15, 16]. A correlation between particular genetic variants and polymorphisms, and aneurysmal SAH has already been demonstrated in previous studies conducted in the last decade [1, 8–10, 18, 22]. The International Study Group of Unruptured Intracranial Aneurysms pointed out a close correlation between aneurysm size and the risk of aneurysm rupture [14]. Therefore, there is also a great interest in the study of mechanisms contributing to aneurysm growth.

Abnormal angiogenesis may play a role in the development and rupture of cerebral aneurysms [17]. Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen and is one of the most important angiogenic factors involved in

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Table 1 VEGF 18 bp microdeletion in the promoter region and allele frequencies in aneurysmal subarachnoid hemorrhage patients and healthy controls

		Genotype			Allele	
	No.	N/N (%)	N/D (%)	D/D (%)	N %	D %
SAH patients	200	50 (25)	88 (44)	62 (31)	47	53
Controls	200	54 (27)	101 (50.5)	45 (22.5)	52.25	47.75

both physiological and pathological angiogenesis via induction of endothelial cell proliferation. Immunostaining studies on specimens of intracranial aneurysms also found increased local VEGF expression on aneurysm walls, suggesting that this this protein plays a role in the pathogenesis and enlargement of cerebral aneurysms [26]. Furthermore, the expression of VEGF-R1, one of the two high-affinity receptor tyrosine kinases of VEGF, was associated with remodeling and rupture in saccular cerebral artery aneurysm walls [12].

The VEGF gene is located on 6p21.3 [27], and it is unusually polymorphic. In fact, several functional polymorphisms in both the promoter region of VEGF gene, which can regulate VEGF plasma levels, and in the 5′ untranslated region, have been identified [5, 24]. In particular, a single nucleotide polymorphism located at position +936 and a 18 bp microdeletion in the promoter region conditioned the gene expression and the consequent VEGF plasmatic levels. Interestingly, a study investigating VEGF plasmatic levels in patients with non-ruptured intracranial aneurysms showed a trend in expressing higher VEGF levels among patients compared to healthy controls [25].

The aim of our study was to evaluate whether a particular allele or genotype of VEGF gene would modify the occurrence of aneurysmal SAH and the related clinical features. To test this hypothesis, we performed an association study in a cohort of Italian aneurysmal SAH patients who were recruited from a University-based Neurosurgical Division, and in a healthy control group.

Patients and methods

Patients

A total of 200 consecutive unrelated patients (69 men, 131 women; mean age±standard deviation=55.3±12.0 years) with cerebral aneurysms, admitted to the Division of Neurosurgery of the University of Torino (Italy) between January 2003 and December 2007, were involved in the study. The diagnosis of aneurysmal SAH was made according to the presence of symptoms suggestive of SAH combined with subarachnoid blood on computed tomographic (CT) scans and a proven aneurysm on conventional four-vessels angiography. The CT findings were classified according to the grading system of Fisher et al. [7]. Patients with genetic defects (i.e. Turner syndrome, Marfan syndrome, Ehlerss-Danlos syndrome Type IV, Autosomal Polycystic Kidney Disease) known to be associated with an increased risk of IAs were excluded from this study. The clinical conditions on admission were rated according to the Hunt and Hess (H&H) grading scale [13].

Patients were divided into the following subgroups: 1) Single versus multiple aneurysms, according to the results of conventional angiography; 2) Size of aneurysm; 3) Anterior or posterior circulation aneurysms; 4) Development of cerebral ischemia secondary to vasospasm confirmed as a new hypodensity on CT scans in patients with angiographic vasospasm (Transcranial Doppler was performed daily in all patients. The presence of vasospasm was detected on CT perfusion scans and confirmed on digital subtraction angiography studies [11]. It occurred between 3 and 12 days after SAH, and was associated with a gradual decline of the level of consciousness or a gradual development of new focal deficit); 5) Smokers before SAH versus non-smokers; 6) History of hypertension before SAH versus normotensive patients; 7) Posthemorrhagic hydrocephalus requiring permanent shunts.

Outcome was assessed by a neurosurgeon blinded to genetic analyses 6 months after SAH using the Glasgow Outcome Scale (GOS). A standardized record of all the clinical characteristics of the patients, suitable for computer analysis, was obtained.

A group of 200 geographically matched healthy subjects (69 men, 131 women; mean age±standard deviation=54.2±13.8 years) were used as controls. The study was approved by the Hospital Ethics Committee and informed consent was obtained from all participants before the assessment.

Table 2 VEGF+936 C>T genotype and allele frequencies in aneurysmal subarachnoid hemorrhage patients and healthy controls

		Genotype			Allele	
	No.	C/C (%)	C/T (%)	T/T (%)	C %	T %
SAH patients	200	153 (76.5)	44 (22)	3 (1.5)	87.5	12.5
Controls	200	151 (75.5)	46 (23)	3 (1.5)	87	13

Table 3 VEGF deletion and+936 C>T genotype distribution and allelic frequencies in aneurysmal subarachnoid hemorrhage patients and healthy controls

	Genotype		Allele	
VEGF polymorphisms	$\chi 2$	*P* value	$\chi 2$	*P* value
18 bp deletion	3.749	0.153	2.000	0.157
+ 936 C>T	2.269	0.322	0.958	0.328

Genetic analysis

We examined two functional polymorphisms: the biallelic polymorphism (C>T) at position +936 C/T (rs3025039) in 3′ and a 18 base pair (bp) insertion/deletion (I/D) polymorphism at −2549 (rs34357231) in the promoter region of VEGF gene, according to previously described methods [19].

Peripheral blood was collected from the subjects in 2-mL EDTA tubes, and genomic DNA was isolated using the QIAamp DNA miniKit (Qiagen S.p.A., Milan, Italy).
Primers were as follows:

- for rs3025039 F: AAGGAAGAGGAGACTCTGCGCAGAGC, R: CCTGTAGACACACCCACCCACATACATACATTTA;
- for rs34357231 F: AGGATGGGGCTGACTAGGTAAG, R: GTTGGAGGAAAAGGGGGCT.

PCR reactions were performed with 90 ng of genomic DNA, 0.3 unit of TaqGold DNA polymerase, 250 nM of each primer, 1.5 mM MgCl2 and 50 mM dNTPs. The PCR conditions were as follows: an initial denaturation at 95 °C 10 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing specific temperature at 60 °C for 40 s for each couple of primers, then extension at 72 °C for 1 min and a final extension at 72 °C for 5 min. PCR products were detected on a 2 % agarose TBE 1X gel and stained with ethidium bromide. Subsequently, the PCR product for rs3025039 polymorphism were digested with the restriction enzyme NlaIII. Conversely, the PCR product for rs34357231 resulted in three amplicons, acccording to the presence of insertion or deletion, respectively.

Statistics

A $\chi 2$ test was used to verify Hardy-Weinberg equilibrium. Statistical analyses were performed using Genepop version 4.0 (http://wbiomed. curtin.edu.au/genepop) and SigmaStat-version 3.1 (Jandel Corp., San Rafael, California). The distribution of alleles and genotypes was compared using the Fisher exact test and $\chi 2$ test. Analysis of variance was used to compare the clinical characteristics of cases and controls. Genetic Power Calculator (http://statgen.iop.kcl.ac.uk/gpc) was used to calculate the power of the association study.

According to recent guidelines for genetic association studies, the level of statistical significance was set at $P< 0.01$ [4]. In the comparisons of the clinical features, the level of statistical significance was set at $P<0.05$.

Results

Hardy-Weinberg equilibrium was verified for all tested populations. The genotypic frequency (GF) and allelic frequency (AF) are remarkably similar to what is reported in other populations (http://www.ncbi.nlm.nih.gov/SNP). The observed GF and AF of rs3025039 and rs34357231 in patients with cerebral aneurysms and in controls are listed in Tables 1 and 2. No significant differences were found in the distribution of either genotypic or allelic frequencies between cases and controls (Table 3). No gender difference was found in cases and controls.

When aneurysmal SAH patients were stratified into subgroups (age at onset, Hunt and Hess grade, Fisher grade, GOS, anterior versus posterior IAs, single versus multiple IAs, aneurysms size≤6 or>6 mm, smoke, history of hypertension,

Table 4 Aneurysmal SAH patients stratified into subgroups

	P value	
Clinical features	18 bp deletion[a]	+ 936 C>T[a]
Age at onset of SAH (≤ 50 / > 50 years)	0.521	0.681
Hunt and Hess grade (≤ 2 / > 2)	0.295	0.135
Fisher grade (≤ 2 / > 2)	0.882	0.085
GOS (≤ 3 / > 3)	0.721	0.644
IAs side (Anterior / Posterior)	0.918	0.777
IAs number (Single / Multiple)	0.870	0.355
IAs size (≤ 6 / > 6 mm)	0.701	0.446
Smoke (Yes / No)	0.092	0.301
History of hypertension (Yes / No)	0.385	0.397
Ischemia (Yes / No)	0.812	0.096
Hydrocephalus with permanent shunt (Yes / No)	0.497	0.398

[a]Patients with VEGF polymorphisms (18 bp deletion and+936 C>T genotype) were respectively compared with not deleted and C/C patients

presence versus absence of ischemia during the follow-up period and hydrocephalus requiring permanent shunt), no significant difference was found in multiple comparisons (Table 4). In addition, the comparison of the clinical characteristics of the disease, according to the different genotypes of the two analyzed polymorphisms, showed no significant difference.

Discussion

The results of our study in an Italian population do not support the hypothesis of a significant genetic association between VEGF gene and aneurysmal SAH. Both allelic and genotypic frequencies were similarly distributed between cases and controls. In addition, clinical characteristics of the disease were not significantly influenced by different genotypes, suggesting that this gene is not major genetic risk factor for the disease. However, to the best of our knowledge, this is the first association study investigating potential role of VGEF gene in SAH, and additional studies in different populations are warranted.

We acknowledge some potential limitations of this study. First, we did not measure VEGF concentrations in serum of patients with cerebral aneurysms. Second, the sample size was limited to 200 patients and confirmatory studies are needed in order to better elucidated the involvement of VEGF in the disease. Finally, population stratification may have limited the power of our study.

The biological mechanisms supporting a role of VEGF in the development and rupture of cerebral aneurysms are still under investigation. Skirgaudas et al. [26] found increased VEGF expression on aneurysm walls. Experimental in vitro studies suggested that the aneurysm development could be induced by an imbalance of VEGF and its receptors (VEGFR1 and VEGFR2), through a mechanism involving endothelium nitric oxide (NO). Bussolati et al. [6] demonstrated that the release of NO, VEGF stimulated, is inhibited by blocking VEGFR1. VEGFR1 via NO negatively regulates VEGFR2-mediated proliferation; therefore, the inhibition of VEGFR1 induces the formation of aneurysm-like structures. Frosen et al. [12], evaluating VEGF receptor expression in normal artery walls, 24 unruptured and 64 ruptured aneurysms, reported high positivity for VEGFR2 in all aneurysms, and lower VEGFR1. Finally, Maderna et al. supported the hypothesis of aneurysm formation associated with a loss of expression of VEGFR1, moderate expression of VEGFR2 and high concentration of nitrate [20]. Therefore, additional genetic studies with different polymorphism may disclose a role for this gene or its receptors in the pathophysiology of the disease.

Despite the attempts that have been made to better understand the pathophysiology of aneurysms, structural, molecular, biological and genetic features involved in the development of aneurysms are still under investigation. Proangiogenic factors appear to contribute to abdominal aortic aneurysm formation, but their relationship is still unclear for cerebral aneurysms. [23]. Several investigations have suggested that the inflammatory response promotes the formation of aneurysms by the production of reactive oxygen species that enhance apoptosis of smooth muscle cells [21]. In our previous studies, we demonstrated a significant genetic association between proinflammatory cytokines genes and aneurysmal SAH [8–10]. Hence, a multifactorial pathogenesis is the best likely explanation of the disease.

In conclusion, we found no significant association between polymorphisms within the VEGF gene and aneurysmal subarachnoid hemorrhage in an Italian population. Additional studies are warranted to better elucidate potential correlations between the VEGF gene and the disease.

Conflicts of interest None.

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