Absence of mutations at *SERPINI1* gene in a cohort of patients with Cerebral Cavernous Malformations

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Abstract

Cerebral Cavernous Malformations (CCM) are vascular lesions affecting brain microvessels. While molecular bases of the sporadic condition are not yet well elucidated, familial forms arise following mutations at three different *loci KRIT1*, *CCM2* and *PDCD10*. However, no germline mutations are detected in a small percentage of families with hereditary history of CCM. In order to detect other possible candidate genes, we performed molecular analysis of *SERPINI1* gene in a cohort of patients carrying no mutations in the three CCM *loci*, aiming to detect mutations likely associated to lesion development. Therefore, we performed molecular analysis of the *SERPINI1* gene in a cohort of 18 unrelated patients affected by both familial and sporadic CCM showing no germline causative mutations. Mutational analysis resulted negative and only few single nucleotide polymorphisms were detected. However, the rs11284733 SNP was detected in a high percentage of patients affected by familial form of the disease. This SNP occurs within a noncoding exon retained in an alternative spliced *SERPINI1* transcript, suggesting its possible role in gene expression regulation.

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Introduction

Intracranial blood vessel disorders include a very heterogeneous group of pathologies both acquired and genetic. Among these, cerebral cavernous malformation (CCM, OMIM #116860) is the most frequent, reaching its worldwide incidence up to 0.8%.[1] Involved vessels appear enlarged and tangled due to absence of pericytes and impairment of endothelial cell junctions. Also cell adhesion to the extracellular matrix is lost. These features result in blood-brain barrier dysfunction and in its increased permeability, with consequent gain of bleeding risk.[2] Together with intracerebral haemorrhage, also seizures, headache, vertigo and focal neurological deficits can represent the main clinical manifestations of the disease. However, only about 70% of patients manifests symptomatology.[3] CCM can arise sporadically or be inherited as autosomal dominant condition. While molecular bases that lead to the sporadic disease are not yet completely clarified, hereditary familial forms are known to be linked to germline mutations at the three *loci KRIT1/CCM1* (HGNC:1573; 7q11.2-21), *CCM2/MGC4607* (HGNC:21708; 7p13) and *PDCD10/CCM3* (HGNC:8761; 3q26.1). Affected patients develop multiple lesions usually already at childhood. However, they can remain asymptomatic due to variable expressivity of the disease.[4] Moreover, incomplete penetrance can determine the absence of lesions in mutation carriers. Penetrance was estimated for the three *loci* to range around 88%, 70% and 66% for *KRIT1*, *CCM2* and *PDCD10*, respectively.[5] Moreover, mutations at the *PDCD10* locus result in a more aggressive clinical phenotype characterized by more frequent haemorrhages, if compared with *KRIT1* or *CCM2* – linked forms.[6] The three CCM genes encode for proteins that are involved in angiogenic-related pathways, contributing to maintenance of cell-cell junctions and cell-extra cellular matrix adhesion,[7,8] to the regulation of apoptosis/proliferation switch of endothelial cells[9] and to oxidative damage response.[2] As known, impairment of system defence against Reactive Oxygen Species (ROS) predisposes to cerebrovascular malformation

onset[10] and several genetic polymorphisms in the *GLO1* and *PON1* were associated to an increased risk of CCM development.[11] To date, more than 300 causative mutations in the CCM genes were reported and the datasets are continuously growing. Moreover, Single Nucleotide Polymorphisms (SNPs) in *KRIT1* and *CCM2* were linked to different prognosis.[12] Mutation rate is about 60%, 20% and 15% for the three *loci* respectively, while no germline mutations are detected in about 5% of patients with familial CCM.[13] Despite the hypothesis of a fourth CCM gene involved in lesion development is commonly accepted, no other associated *loci* have to date been detected. Linkage studies published by Liquori *et al.* revealed a lower frequency of *PDCD10* mutations than expected.[14] This observation has allowed to hypothesize involvement, in CCM pathogenesis, of another gene mapping in the same chromosomic region of *PDCD10*. Further analyses showed that *SERPINI1* gene (HGNC:8943; 3q26.1) is highly close to *PDCD10* and the two genes share a common bidirectional promoter.[15] This structural model is peculiar of homologous gene-pairs. However, no functional correlations are reported for the *PDCD10-SERPINI1* gene-pair and their expression profiles are not comparable. *SERPINI1* encodes for neuroserpin, a serine protease inhibitor that regulates tissue-type plasminogen activator (t-PA).[16] It is organized in cluster with its homologous *SERPINI2* (HGNC:8945). The peculiarity of this chromosomal region is that *PDCD10* is located within this cluster, together with an another gene, *WDR49* (HGNC:26587; Figure 1). Interestingly, extracellular matrix remodelling due to unconventional protease activity of coagulation factors resulted enhanced in CCM-derived endothelial cells.[17] Based on these observations, we chose to study the *SERPINI1* coding sequence in our patients in order to can consider it in CCM pathogenesis.

Materials and Methods

Cohort selection

SERPINI1 gene consists of 10 exons and its molecular analysis was conducted on a cohort of 18 Caucasian non-consanguineous CCM patients. Of these, 9 belonged to families in which CCM segregated, while the other 9 had not affected relatives and they were classified as sporadic. Diagnosis of CCM was based on anamnesis information and magnetic resonance imaging. The previous mutational analysis performed on CCM genes revealed no germline mutations, as well as Multiplex Ligation-dependent Probe Amplification analysis highlighted lo large genomic rearrangements.

Molecular analysis

DNA was purified from peripheral blood and *SERPINI1* coding, noncoding exons and intron-exons boundaries were amplified by polymerase chain reaction and sequenced on 3500 Genetic Analyzer (Thermo Fisher Scientific) by the BigDye Terminator v3.1 chemistry (Applied Biosystems), following manufacturer's guidelines. Primer sequences and reaction conditions are available upon request. The effects of detected variants were *in-silico* predicted by the SIFT dbSNP,[18] MutationTaster[19] and PolyPhen-2[20] tools.

The study was approved by the local Ethical Committee (A.O.U. "G. Martino") and informed consent was obtained for all the subjects involved in the study.

Results

Both coding and noncoding exons of *SERPINI1* gene were sequenced but no mutations in the patients were detected. However, four different SNPs were identified and they are listed in Table 1. Their distribution is not equal between sporadic and famil-ial patients. In detail, three SNPs were detected in the sporadic cohort and these are the rs33917740 and the rs34582040, carried by the 3 same patients and the rs2229697, identified in only anoth-er sample. In contrast, the rs11284733 was detected in 6 patients affected by familial CCM. About their functional consequences, the rs33917740 c.21C>G (Figure 2a) results in the p.Phe7Leu amino acid substitution in the neuroserpin protein. However, SIFT dbSNP, MutationTaster and PolyPhen-2 tools predicted that it is well tolerated and not-disrupting for protein structure. The rs34582040 c.51A>G and the rs2229697 c.576G>C are both syn-onymous substitutions, p.Thr17= and p.Ser192=, respectively. Finally, the rs11284733 c.980-22delAA (Figure 2b) is a dinu-cleotide deletion occurring in a non-coding exon.

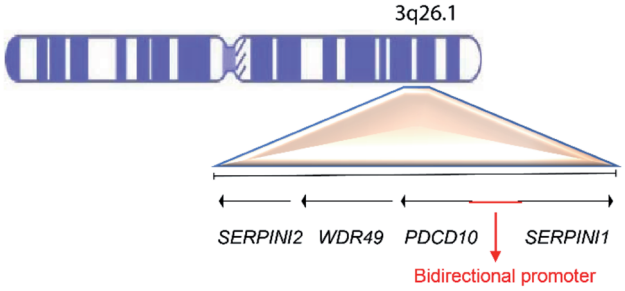

Figure 1. *SERPINI1-SERPINI2* gene cluster. The genomic organization of the 3q26 region shows as *PDCD10* and *WDR49* genes are included within the SERPINI cluster. The arrows indicate the direction of gene transcription (*SERPINI2*, *WDR49* and *PDCD10*: reverse strand; *SERPINI1*: forward strand). The 851 bp *SERPINI1-PDCD10* bidirectional promoter is highlighted in red.

Table 1. SNP report. Single nucleotide polymorphisms (SNPs) detected in both familial and sporadic CCM patients are listed. For each SNP, the nucleotide and amino acid substitution, frequency in patient cohort and Minor Allele Frequency (MAF) according to the GnomAD Database (https://gnomad.broadinstitute.org/) are reported.

Variant	Variant effect	SNP frequency (%) in familial cases	SNP frequency (%) in sporadic cases	MAF (GnomAD database)
rs33917740	c.21C>G; p.Phe7Leu	0	33	0.1262
rs34582040	c.51A>G; p.Thr17=	0	33	0.1030
rs2229697	c.576G>C; p.Ser192=	0	11	0.1263
rs11284733	c.980-22delAA	67	0	0.00007422

Discussion

The study aimed to evaluate *SERPINI1* gene mutations as possible cause of CCM development. *SERPINI1* encodes for the neuroserpin, a serin-protease that acts by inhibiting t-PA. It is highly expressed in fetal brain where guides axonal growth and synaptic plasticity. In adults, its expression is limited to few cerebral areas, including hippocampus, amygdala and hypothalamus. If mutated, it causes a neurodegenerative disease known as FENIB (Familial Encephalopathy with Neuroserpin Inclusion Bodies).[21] According to the observation that *SERPINI1* shares its promoter with *PDCD10*, the third CCM causative gene, we wanted to investigate about its possible role in CCM onset and development. Molecular analysis of *SERPINI1* gene in a cohort of patients affected by both familial and sporadic CCM and with no CCM genes mutations allowed us to detect two highly represented SNPs. The rs33917740 is a nucleotide substitution c.21C>G that leads to the p.Phe7Leu amino acid change. Its frequency is homogeneous among the different ethnic groups and ranges between 0.05 and 0.2 worldwide (https://gnomad.broadinstitute.org/variant/3-167789149-C-G?dataset=gnomad_r3). The rs11284733 (c.980-22delAA) is a deletion that occurs in a non-coding exon. This exon results retained in an alternative spliced *SERPINI1* transcript (Ensembl transcript ID: ENST00000494666). The rs11284733 (c.980-22delAA) allele was detected only in patients affected by familial forms with a frequency equal to 0.335. However, its worldwide frequency is estimated to be 0.00007422, as reported in GnomAD database (https://gnomad.broadinstitute.org/variant/3-167822953-GAA-G?dataset=gnomad_r3). Therefore we think that this difference is deserving of further investigations. In particular, the role of the *SERPINI1* ENST00000494666 transcript could be considered in regulation of CCM gene expression, being it reported in the ANGIOGENES database (http://angiogenes.uni-frankfurt.de/transcript/ENST00000494666), a database collecting both coding and noncoding genes involved in angiogenesis. So, a possible regulatory mechanism for the three CCM genes is not to be excluded.

Moreover, association data do not report any linkage between the rs11284733 and the FENIB phenotype. FENIB is a rare dominant condition linked to *SERPINI1* mutations, characterized by dementia, seizure and progressive myoclonic epilepsy, as consequence of precipitation in neurons of mutated neuroserpin.[22] In order to describe the possible involvement of *SERPINI1* also in CCM development, we previously characterized two different SNPs affecting *PDCD10*/*SERPINI1* bidirectional promoter and a reduced expression level was observed for *PDCD10*. However, they seem not to affect *SERPINI1* expression.[23,24] Neuroserpin acts by inactivating t-PA. In CNS, tPA cleaves both Matrix Metallopeptidase 2 (MMP-2) and MMP-9 enhancing their activity and extra-cellular matrix remodelling rate.[25] Role of serine proteases and their inhibitors is becoming clearer and it was shown that they are required for brain vasculature development in mice.[26] Likewise, serine proteases enhance pericyte coverage on the endothelial tubes under pathological conditions.[27]

This study represents the first investigation about role of the t-PA inhibitor neuroserpin in CCM development and it was driven by the observation that *SERPINI1* gene shares its promoter with *PDCD10*, the third CCM causative gene. Although no mutations

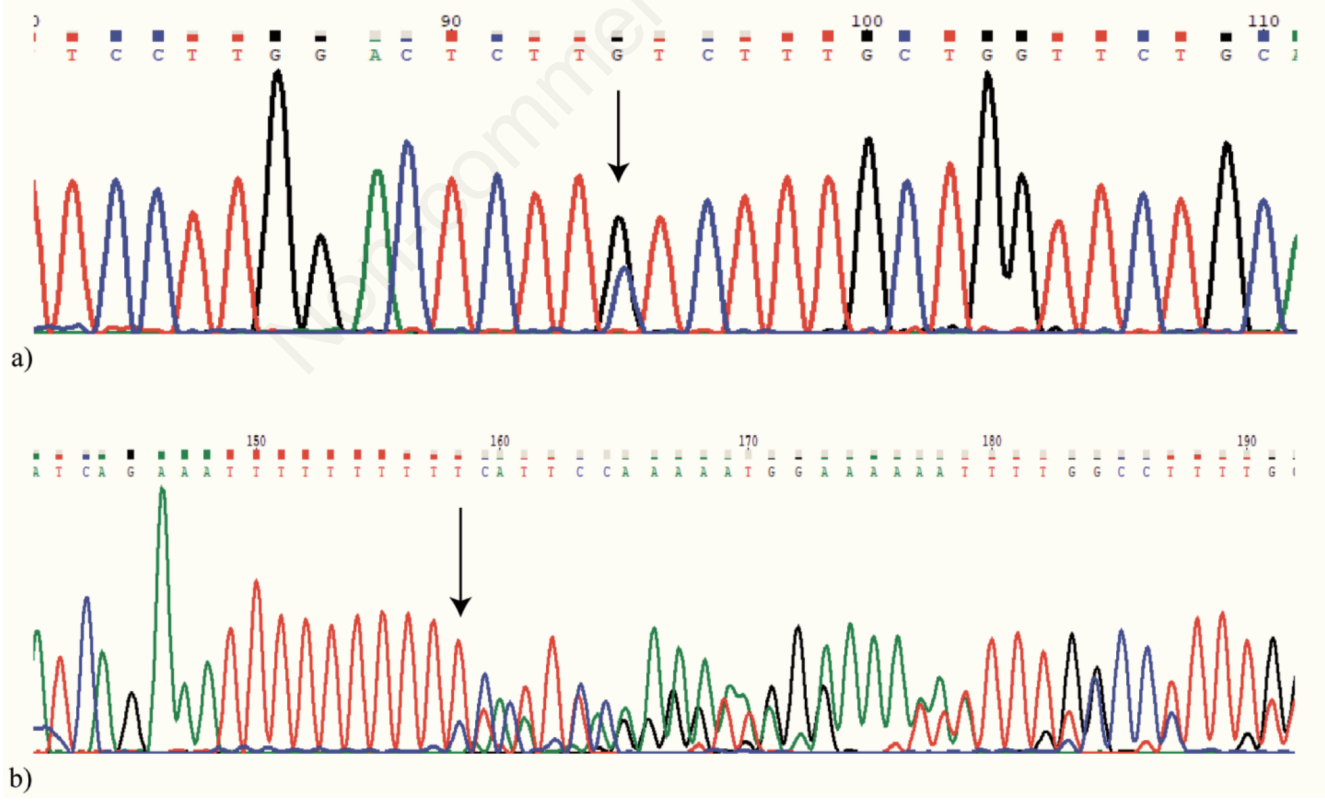

Figure 2. SERPINI1 variants. The electropherograms show the two *SERPINI1* SNPs main represented in our cohort of CCM patients. a) The rs33917740 c.21C>G leading to the missense p.Phe7Leu variant. b) The rs11284733 resulting in the c.980-22del intronic deletion. The arrows indicate the mutated nucleotide.

were found in our cohort of patients, the high frequency of the rs11284733 in patients affected by the familial form of the disease encourages deepening on the research. Clearly, the reduced sample size makes results provisional requiring a larger scale screening and functional validation of collected data.

Conclusions

This study describes results obtained by sequencing analysis of *SERPINI1* gene performed on a cohort of CCM patients and no mutations were detected. However, we identified the rs11284733 in a high percentage of patients affected by familial CCM. By comparison of allele frequency, this SNP resulted more widely represented in our cohort than in general population. Therefore, although involvement of *SERPINI1* in CCM pathogenesis is to date not confirmed, we think that the regulatory role of the noncoding *SERPINI1* ENST00000494666 transcript in angiogenesis and CCM development requires further investigations.

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