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GERM-LINE/SOMATIC DNA COMPARISON IN SPORADIC PATIENTS WITH CEREBRAL CAVERNOUS MALFORMATIONS.

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ABSTRACT

Cerebral cavernous malformations (CCMs) are benign tumours that affect brain capillaries.Although many cases remain asymptomatic,their incidence is steadily increasing. CCMs can arise sporadically or be inherited as autosomal dominant character. Inherited forms result from mutations at three different loci CCM1/KRIT1, CCM2/MGC4607 and CCM3/PDCD10. Etiology of sporadic forms is still unclear. Among the various molecular mechanisms proposed, presence of somatic mutations was sometimes proven. Here we report results obtained by a molecular screening of the three CCMs genes, performed on both germ-line and somatic DNA, isolated from CCM endothelial cells, in eight patients affected by sporadic lesions, who undergone surgery. Comparison of germ-line and somatic sequencing data, for each patient, showed no differences. Our results confirm that presence of somatic mutations is not sufficient to explain CCMs onset in patients affected by sporadic forms and with no CCM genes germ-line mutations. Other possible pathogenic mechanisms are also discussed.

1. Introduction

Cerebral cavernous malformations (CCMs, OMIM#116860) are vascular neoformations that affect cerebral microvascular endothelial cells without involvement ofbrain parenchyma. These lesions lack of pericytes and endothelial cells result defective of both tight and adherens junctions. CCMs incidence ranges around 0,1% - 0,5% worldwide; however, in a high rate of asymptomatic patients are often accidentally discovered. Most common clinical manifestations include headaches, seizures, focal neurological deficits and intracerebral haemorrhages. The most efficient diagnostic method is magnetic resonance imaging (MRI) with gradient-echo sequences. Majority of patients develops sporadic CCMs that usually arise with single lesions, among third and fifth decade of life. Also familial forms (cavernomatosis) were reported and they are inherited with an autosomal dominant pattern; neuroradiological and clinical incomplete penetrance and variable expressivity are the main features of familial CCMs; anticipation was also observed and multiple lesions are often present at pediatric age. CCMs are associated with loss-of-function mutations in any one of the three CCM genes (CCM1/KRIT1, CCM2/MGC4607 or CCM3/PDCD10[1-6])and occur in both sporadic and familial forms [7]. These genes encode for proteins involved in differentiation and maintaining of integrity of blood brain barrier. However, absence of germ-line mutations was reported in about 11% and 43% of familial and sporadic cases respectively [8]. CCMs development in these CCM-genes negative patients may be justified by several causes. While for familial syndromes the most accepted hypothesis is involvement of still undetected genes[9,10],presence of somatic mutations at the CCM genes[11,12], activity of genetic modifiers and involvement of epigenetic factors might underlie pathogenesis of sporadic cases. Furthermore, it was showed that only the sporadic patients negative for mutations in the three CCM genes and whose relatives were negative to MRI, carried variations in the CCM genes which proved to be the known polymorphisms [13-16]. Here we have focused on patients affected by sporadic forms and, particularly, we considered germ-line/somatic DNA comparison in a cohort of 8 Italian sporadic patients.

2. Methods

Sample collection

This study involves 8 patients affected by sporadic CCMs who undergone surgery. Their clinical histories are summarized in table 1. Germ-line

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DNA was extracted from peripheral blood, while somatic DNA was isolated from CCMs biopsies, resected by surgery. DNA extraction was performed as previously described by others [17] Particularly, endothelial cells were isolated from biopsies and selected by Fluorescence Activated Cell Sorting (FACS Canto II flowcytometer Becton Dickinson, Mountain View, CA),using anti-CD31 antibody (Dynabeads® CD31 Endothelial Cell, Invitrogen). DNA was isolated and purified with QIAamp DNA Mini Kit (QIAGEN®).

Molecular analysis

Coding exons and intron–exon boundaries of CCM1, CCM2 and CCM3 genes were screened using the pairs of primers designed according to the CCM1, CCM2 and CCM3 published nucleotide sequence of GenBank (accession no. NG_012964.1, NG_016295.1 and NG_008158.1, respectively). Primer list and PCR conditions are available upon request. Sanger sequencing was run on a 3500 Genetic Analyzer (Applied Biosystems), using the Big Dye Terminator v3.1 chemistry, following manufacturer's procedures. Eventual presence of large genomic mutations was assessed by Multiplex Ligation-dependent Probe Amplification (MLPA) and two different MLPA kits (SALSA MLPA Kits, P130 and P131 CCM, MRC Holland) were used; for the visual inspection, peak heights were compared between the sample and controls to find any alteration in relative peak heights within the test sample. For the normalized peak-area calculations, each peak area was normalized by dividing the individual peak area by the total peak area of all peaks for that sample; statistical analysis was performed by Coffalyser software.

Patient	Gender	Age	Symptom age	Symptom	Number of lesions	Localization
1	M	68	62	Headaches, asthenia, Short-term memory deficits	1	Right frontal
2	M	32	26	Seizures	1	Left fronto-parietal
3	F	43	27	Seizures, right emiparesis	1	Left frontal
4	M	27	18	Headaches	1 with relapse at 24 years	1th lesion: right frontal 2nd lesion: right parietal
5	F	47	42	Headache, asthenia	1	Right semicircular center
6	F	45	40	Seizures	1	Left temporal
7	M	50	47	Seizures, dizziness	1	Right temporal
8	F	67	66	Visual deficits, right paresis	1	Pons

Table 1 - Clinical features of patients involved in the study.

3. Results and discussion

For all considered samples, no differences were observed by comparison of DNA extracted from limphocytes and endothelial cells. No causative mutations were detected, however several single nucleotide polymorphisms(SNPs) were highly represented and they are reported in table 2.

Molecular causes of development of sporadic CCMs are still poorly known. Hypothesis of CCM genes somatic mutation was firstly formulated in 2003 [18] and no evidence of differences between healthy and pathological tissues were detected. However, this idea has never been completely abandoned and loss of heterozygosity (LOH) was also considered. LOH implies a two-hit mechanism of disease development: the first event is the presence of an inherited germ-line mutation and the second is the acquisition of a somatic mutation due to environmental causes. Although in some cases LOH was proven[19], it is not sufficient to explain etiology of all CCMs cases [20]. Our study confirms involvement of other genetic factors and/or molecular mechanisms in CCMs development. CCMs pathogenesis is a very complex network and the base is an angiogenetic dysfunction: high levels of vascular endothelial growth factor were observed in tissues derived from sporadic CCM lesions [21]. Moreover, alteration of expression of genes involved in regulation of cell proliferation such as cyclin D1 [22]and apoptosis due to epigenetic factors may be play an important role, as demonstrated for the tumor-suppressor PTEN gene[23]. Causes of these epigenetic modifications are still unclear. However, maternal trauma during pregnancy or fetal stress conditions during birth seem to be risk factors. It is now, in fact, widely proven the link between oxidative stress, inflammation and angiogenesis [24-28].Among our cases here considered, a young 24 years-old man who developed the first lesion at the age of 17 and the second one at 22 years-old, was born in hypoxic condition due to a late childbirth. He had neither germ-line nor somatic mutations. Certainly, methylome analysis performed on CCMs endothelial cells could open new perspectives. Moreover, recently, a role in development of malformation syndromes was proposed for all those genes who can contribute to phenotype manifestation when a specific causative-gene mutation lacks. These genes are commonly known as modifiers and they can be involved in variable expressivity of several diseases (genetic modifiers in human development and malformation syndromes, including chaperone proteins []). Modifiers genes were identified also in CCMs developments; among these, there are the*TGF-β* and *HEG*genes[29].

GENE	SNP	1	2	3	4	5	6	7	8
KRIT1	rs17164451 c.485+65G>C	G/G	G/G	G/G	G/G	G/G	**G/C**	G/G	G/G
	rs2027950 c.989+63C>G	**C/G**	**G/G**	**C/G**	**G/G**	**C/G**	**G/G**	**C/G**	**C/G**
CCM2	rs2107732 G>A p.Val53Ile	G/G	**G/A**	G/G	G/G	G/G	G/G	G/G	G/G
	rs2304689c.205-36A>G	**A/G**	A/A	A/A	A/A	A/A	A/A	A/A	A/A
	rs11552377 c.358G>Ap.Val120Ile	G/G	G/G	**G/A**	G/G	G/G	G/G	G/G	G/G
	rs73107990 c.472+127C>T	C/C	C/C	C/C	C/C	C/C	**C/T**	C/C	C/C
	rs2289367 c.915G>Ap.Thr305Thr	G/G	G/G	G/G	G/G	G/G	**G/A**	G/G	G/G
	rs2289369 c.915+119C>T	C/C	C/C	C/C	C/C	C/C	**C/T**	C/C	C/C
PDCD10	rs200180968c.268+53C>T	**C/T**	C/C	C/C	C/C	C/C	C/C	C/C	C/C

Table 2 - CCM genes SNPs detected in our cohort of patients. Bold type indicates the presence of the SNP.

4. Conclusion

For several years, LOH was assessed as the major phenomenon involved in CCMs pathogenesis in patients harboring CCM genes mutations in heterozygous conditions. However, absence of somatic mutation observed in all screened patients underlines that molecular pathogenesis of these lesions isn't so simple and more complex mechanisms can lead to the development of these lesions. A highly supported hypothesis is alteration of genic expression, particularly related to epigenetic mechanisms, of genes involved in endothelial cells proliferation, apoptosis and oxidative stress response

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