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Title

Constitutional mosaicism in *RASA1*-related capillary malformation-arteriovenous malformation (CM-AVM)

Author list

Gema Gordo[1], Lara Rodriguez-Laguna[1], Noelia Agra[1], Pilar Mendez[2], Marta Feito[3], Pablo Lapunzina[4-5], Juan C. Lopez-Gutierrez[6], Victor Martinez-Glez[1-4-5]

Affiliations

[1]Vascular Malformations Section, Institute of Medical and Molecular Genetics, INGEMM-IdiPAZ, Hospital Universitario La Paz, Madrid, 28046, Spain.

[2]Unidad de Genética, Servicio de Inmunología y Genética, Complejo Hospitalario Universitario de Badajoz, Badajoz, Spain.

[3]Department of Dermatology, Hospital Universitario La Paz, Madrid, Spain..

[4]Clinical Genetics Section, Institute of Medical and Molecular Genetics, INGEMM-IdiPAZ, Hospital Universitario La Paz, Madrid, 28046, Spain.

[5] CIBERER, Centro de Investigación Biomédica en Red de Enfermedades Raras, ISCIII, Madrid, 28029, Spain.

[6]Vascular Anomalies Center, Plastic Surgery, Hospital Universitario La Paz, Madrid, 28046, Spain.

Correspondence email

Victor Martinez-Glez: vmartineglez@salud.madrid.org. Sección de Malformaciones Vasculares, Instituto de Genética Médica y Molecular INGEMM-IdiPaz-Hospital Universitario La Paz. Paseo La Castellana 261. Edificio Bloque Quirúrgico Planta -1. 28046 Madrid – España. Tel. 912071010 Ext 230

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Abstract

Introduction: Capillary malformation-arteriovenous malformation (CM-AVM; MIM#608354) is caused by germline *RASA1* and *EPHB4* alterations. *RASA1* intralesional second hits have also been reported. Constitutional mosaicism, defined as the presence of a mosaic variant in all cell types of an individual, is detected in clinical practice as mosaic variants in multiple tested samples from one individual or as mosaic variants in blood samples in a disorder affecting another cell/tissue types. Here we report *RASA1* constitutional mosaicism in CM-AVM. **Subjects and methods**: A custom high-throughput sequencing panel was used to search for *RASA1* pathogenic variants in blood samples from two unrelated patients with a clinical diagnosis of CM-AVM. An affected tissue sample from one of the patients was also analyzed. **Results**: Both patients showed different nonsense *RASA1* variants in mosaic in blood samples and in the corresponding affected tissue sample from one of the patients. The mosaicism ranged between 7% and 21,5%. **Conclusions**: We report for the first time the presence of *RASA1* constitutional mosaicism in CM-AVM. Constitutional mosaicism has implications for accurate molecular diagnosis and recurrence risk, and helps to explain the great phenotypic variability in CM-AVM.

Keywords: RASA1; CM-AVM; capillary malformation-arteriovenous malformation; constitutional mosaicism.

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Introduction

Capillary malformation-arteriovenous malformation (CM-AVM; MIM#608354) is characterized by the presence of multiple, small, round-to-oval capillary malformations with a reddish-brown to pink appearance often showing white surrounding halos. One-third of the patients also have fast-flow arteriovenous malformations (AVMs) and arteriovenous fistulas (AVFs) in soft tissue, bone, muscle, spine, and brain[1-3]. When multiple or single large capillary malformations with multiple underlying subcutaneous and intramuscular AVFs are associated with overgrowth of a single affected extremity the condition is known as Parkes Weber syndrome (PKWS; MIM#608355).

Alterations in the *RASA1* gene, an inhibitor of RAS p21 which controls cellular growth, proliferation, survival, and differentiation, have been identified as causing the pathology[3]. Germline inactivating variants located throughout the *RASA1* gene are detected in about 50-85% of the patients, reaching over 90% in familial cases. There are variable inter- and intrafamilial expressivity and penetrance is 90-99%[1,3]. Recently, loss-of-function mutations in *EPHB4*, a tyrosine kinase receptor directly related to *RASA1*, have been described as causing a second type of CM-AVM (CM-AVM2) that mimics *RASA1*-mutated CM-AVM but also hereditary hemorrhagic telangiectasia (HHT) due to the presence of telangiectasias[4].

The intrafamilial variability in CM-AVM together with the presence of multifocal lesions suggested the need for a somatic "second-hit ". Confirming this theory, Revencu et al reported a patient with PKWS in whom a germinal pathogenic variant in *RASA1* and a second somatic mosaic chromosome 5 deletion encompassing *RASA1* were detected[1]. Later, MacMurdo et al reported a patient with CM-AVM showing a germinal variant and a second somatic variant in the *RASA1* gene[5]. Recently, Lapinski et al reported a patient with CM-AVM showing a germline *RASA1* pathogenic variant and in the opposite allele (trans) a somatic *RASA1* pathogenic variant present only within endothelial cells of a CM[6]. Although the presence of a somatic second hit is also a possibility for *EPHB4*, no cases have been described so far for this gene.

The presence of a first germline mutation followed by a somatic second hit is a known pathogenic mechanism associated with multifocal lesions in different vascular malformations such as the cerebral

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cavernous malformation[7], and has also been described for other syndromes as in the Neurofibromatosis type 1 (NF1)[8]. The presence of somatic mutations, without previous germline mutations, has also been widely described in conditions such as the unifocal venous malformations[9], the PIK3CA Related Overgrowth Spectrum (PROS)[10] or in several Rasopathies, including NF1[8,11]. However, the occurrence of a pathogenic, single nucleotide variant (SNV) present both constitutionally and in mosaic seems to be an under recognized and rarely described mechanism[12]. Here we report constitutional mosaic pathogenic variants in the *RASA1* gene in two unrelated patients with CM-AVM.

Materials and methods

The study was performed in the Institute of Medical and Molecular Genetics (INGEMM) at La Paz Hospital, Madrid (Spain). All studies were performed according to the Ethics Committee of the Hospital Universitario La Paz, with written informed consent from the patient and/or the parents. Patient 1 is a 16 months old girl with multiple CMs in glabella, axilla, abdominal region and right thigh, as well as in the left hand and forearm where the CMs are associated with AVM and overgrowth (**Fig 1**). Patient 2 is a 6 years old girl with an intracranial AVM and multiple CMs with white halo all over the body and a large midline CM encompassing the forehead, glabella and nose (**Fig 1**).

DNA extraction from blood and affected tissue was performed by standard procedures. High-throughput sequencing studies were performed using a 20 genes custom panel designed by using NimbleDesign software (Roche NimbleGen, Inc. USA): hg19 NCBI Build 37.1/GRCh37.p13, targeting >98% of all exons (RefSeq) for these genes. For each sample, paired-end (2x150 bp reads) libraries were created according to the standard protocols KAPA HTP Library Preparation Kit for Illumina platforms, SeqCap EZ Library SR (Roche NimbleGen, Inc. USA) and NEXTflex-96 Pre Capture Combo Kit (Bioo Scientific) for indexing. The captured DNA samples were sequenced on a NextSeq™ 500 instrument (Illumina, Inc. USA) using a HIGH v2 300 cycles cartridge, according to the standard operating protocol. Data generated by the NextSeq™ 500 Desktop Sequencer was analyzed using a previously described in-house bioinformatics pipeline for somatic mosaicism detection[13]. To confirm the presence of the mosaic variants a second NGS experiment and/or pyrosequencing with the Pyromark Q96 MD instrument (QIAGEN, USA) were

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performed. Standard PCR and Sanger sequencing were performed using the 96-capillary ABI 3730xl ADN analyzer (Applied Biosystem, Foster, USA) to search for variants in *EPHB4* gene.

Results

Patient 1 showed the variant NM_002890.2 (RASA1):c.1248T>A;p.Tyr416Ter that generates a stop codon in exon 8 of the *RASA1* gene. The variant is detected in DNA obtained from peripheral blood in mosaic of 7% [566/45], as well as in DNA extracted from CM tissue biopsy of the region with overgrowth in a mosaic of 19% [149/36] (**Fig 1**). This variant has already been described as causing the pathology in a family with CM-AVM[5]. Patient 2 showed the variant NM_002890.2 (RASA1):c.2131C>T;p.Arg711Ter that generates a stop codon in exon 16 in mosaic of 21,5% [215/59] in DNA obtained from peripheral blood (**Fig 1**). This variant has already been described in two unrelated patients with multiple CMs[14]. No tissue sample was available from patient 2. None of the 2 variants are present in the Genome Aggregation Database (gnomAD)[15] and according to the ACMG/AMP clinical variant interpretation guidelines[16] both variants are classified as Pathogenic. No variants were found in *EPHB4* gene by Sanger sequencing.

Discussion

In the present report we describe for the first time constitutional mosaic *RASA1* pathogenic variants causing CM-AVM. Two different *RASA1* variants were detected in mosaic in blood samples from two unrelated patients and in the affected tissue sample in one of the patients. The range of mosaicism was between 7% and 21,5%. Both variants were nonsense, thus causing a premature stop codon. The clinical manifestations of the two patients are undistinguishable from the broad phenotypic spectrum described in patients with germline *RASA1* mutations causing CM-AVM.

We cannot discard the presence of a second hit in these patients either as a low mosaic in *RASA1* or in another related gene producing a sum of disadvantages that end in the typical lesions. Additionally, it has not been proven experimentally if these nonsense mutations produce a nonsense-mediated mRNA decay (NMD) phenomenon or if they cause a dominant negative effect, as observed in PTEN[17]. The second case would theoretically be more likely in patient 1 since the stop codon is located near the end of exon 8 and

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does not comply with the precept of being located more than 50 nucleotides upstream of the exon-exon junction to trigger the NMD[18]. On the other hand, the dominant negative interaction between mutant and wild-type *RASA1* alleles could be an explanation for the detection of a single mosaic mutation causing CM-AVM, a pathology in which so far only germline and second hit variants has been described.

There is another circumstance to consider. The temporal window in which a somatic mutation appears during embryonic development can be determinant more than the percentage of affected cells in an individual. In this way, mutations occurring earlier do not necessarily have to produce larger pathogenic consequences. Normal growth and patterning of tissues in embryos are guided by temporal windows involving long-range and dose-dependent responses, short-range signaling activation and dynamics, and transcriptional feedback, thus translating temporal information into spatial information[19]. Therefore, localized and time-specific *RASA1* alterations could be causing deregulations that would not occur the same way in the context of a ubiquitous germinal alteration or even in somatic alterations arising before or after the temporal window.

Somatic mosaicism is defined as the presence of more than one clone of cells with different genotypes, all which are derived from a single cell. Therefore, a specific variant may be specific to tissues or cell types. Conversely, constitutional mosaicism can be theoretically defined as the presence of a mosaic variant in all cell types of an individual. This in clinical practice would mean the presence of a mosaic variant in multiple tested samples from one individual or the presence of a mosaic variant in blood sample in a disorder whose phenotypic consequences are known to be caused by mutations in other cell/tissue types. The percentage of mosaicism present among the cell types may be different. Constitutional mosaicism could be the consequence of a spontaneous and partial reversion of a pathogenic germline variant resulting from a reverse point mutation, mitotic recombination or a gene conversion restoring the aminoacid sequence, a mechanism known as revertant mosaicism[20]. Constitutional mosaicism could also be conditioned by a selective advantage of wild type cells in an embryo with an early somatic mosaicism. The presence of mosaicism in different cell types with different embryological origins has already been described in other pathologies such as Macrocephaly-capillary malformation (MCAP), caused by somatic

activating mutations in the *PIK3CA* gene. In MCAP it is not uncommon to detect different percentages of the pathogenic variant in blood lymphocytes (endoderm), skin with overgrowth (epidermis is ectodermal and cultured skin biopsy fibroblasts are mesodermal) or vascular malformations (endothelium is mesodermal), among others. Although, the percentages of the alternative allele in blood samples are usually lower than in the rest of tissues, probably because the opposite would be incompatible with life in the specific case of the *PIK3CA* gene.

In terms of molecular diagnosis, the possibility of constitutional mosaicism has the same implications as those already known for MCAP. In patients with CM-AVM it would be advisable to initially use high-throughput sequencing studies with high reading depths (> 500x) in DNA samples obtained from peripheral blood to detect a possible constitutional mosaicism with a low frequency of the pathogenic variant. The study should preferably include all genes within the differential diagnosis of the patient. Only if the result is negative could be considered the possibility of obtaining biopsies of affected tissue to search for somatic alterations.

In conclusion, this report documents for the first time the presence of constitutional mosaicism in *RASA1* as cause of CM-AVM. The phenomenon of constitutional mosaicism is under recognized, although it has implications for accurate molecular diagnosis and recurrence risk, and helps to explain the great phenotypic variability in CM-AVM. As knowledge and technology advances, constitutional mosaicism could help us to better understand both normal embryological development and the pathogenic mechanism underlying several diseases.

References

1. Revencu, N. *et al.* RASA1 mutations and associated phenotypes in 68 families with capillary malformation-arteriovenous malformation. *Hum Mutat* **34**, 1632-41 (2013).
2. Boon, L.M., Mulliken, J.B. & Vikkula, M. RASA1: variable phenotype with capillary and arteriovenous malformations. *Curr Opin Genet Dev* **15**, 265-9 (2005).
3. Eerola, I. *et al.* Capillary malformation-arteriovenous malformation, a new clinical and genetic disorder caused by RASA1 mutations. *Am J Hum Genet* **73**, 1240-9 (2003).

4. Amyere, M. *et al.* Germline Loss-of-Function Mutations in EPHB4 Cause a Second Form of Capillary Malformation-Arteriovenous Malformation (CM-AVM2) Deregulating RAS-MAPK Signaling. *Circulation* **136**, 1037-1048 (2017).
5. Macmurdo, C.F. *et al.* RASA1 somatic mutation and variable expressivity in capillary malformation/arteriovenous malformation (CM/AVM) syndrome. *Am J Med Genet A* **170**, 1450-4 (2016).
6. Lapinski, P.E. *et al.* Somatic second hit mutation of RASA1 in vascular endothelial cells in capillary malformation-arteriovenous malformation. *Eur J Med Genet* **61**, 11-16 (2018).
7. Gault, J., Shenkar, R., Recksiek, P. & Awad, I.A. Biallelic somatic and germ line CCM1 truncating mutations in a cerebral cavernous malformation lesion. *Stroke* **36**, 872-4 (2005).
8. John, A.M., Ruggieri, M., Ferner, R. & Upadhyaya, M. A search for evidence of somatic mutations in the NF1 gene. *J Med Genet* **37**, 44-9 (2000).
9. Soblet, J., Limaye, N., Uebelhoer, M., Boon, L.M. & Vikkula, M. Variable Somatic TIE2 Mutations in Half of Sporadic Venous Malformations. *Mol Syndromol* **4**, 179-83 (2013).
10. Keppler-Noreuil, K.M. *et al.* PIK3CA-related overgrowth spectrum (PROS): diagnostic and testing eligibility criteria, differential diagnosis, and evaluation. *Am J Med Genet A* **167A**, 287-95 (2015).
11. Hafner, C. & Groesser, L. Mosaic RASopathies. *Cell Cycle* **12**, 43-50 (2013).
12. Gordo, G. *et al.* mTOR mutations in Smith-Kingsmore syndrome: Four additional patients and a review. *Clin Genet* **93**, 762-775 (2018).
13. Rodriguez-Laguna, L. *et al.* CLAPO syndrome: identification of somatic activating PIK3CA mutations and delineation of the natural history and phenotype. *Genet Med* (2018).
14. Wooderchak-Donahue, W.L. *et al.* Expanding the clinical and molecular findings in RASA1 capillary malformation-arteriovenous malformation. *Eur J Hum Genet* (2018).
15. Lek, M. *et al.* Analysis of protein-coding genetic variation in 60,706 humans. *Nature* **536**, 285-91 (2016).
16. Richards, S. *et al.* Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* **17**, 405-24 (2015).
17. Papa, A. *et al.* Cancer-associated PTEN mutants act in a dominant-negative manner to suppress PTEN protein function. *Cell* **157**, 595-610 (2014).
18. Gehring, N.H., Lamprinaki, S., Hentze, M.W. & Kulozik, A.E. The hierarchy of exon-junction complex assembly by the spliceosome explains key features of mammalian nonsense-mediated mRNA decay. *PLoS Biol* **7**, e1000120 (2009).
19. van Boxtel, A.L. *et al.* A Temporal Window for Signal Activation Dictates the Dimensions of a Nodal Signaling Domain. *Dev Cell* **35**, 175-85 (2015).
20. Kiritsi, D. *et al.* Revertant mosaicism in a human skin fragility disorder results from slipped mispairing and mitotic recombination. *J Clin Invest* **122**, 1742-6 (2012).

Figure legends

Fig 1. Patient 1 shows capillary malformations (CM) an overgrowth (A and B) and Patient 2 shows large and small CMs (D and E). IGV visualization of mosaic *RASA1* pathogenic variants detected by high-

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throughput sequencing in blood (7%) and affected tissue (19%) in patient 1 (C) and in blood sample (21,5%) in patient 2 (F).