

ORIGINAL ARTICLE

RASA1 mosaic mutations in patients with capillary malformation-arteriovenous malformation

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ABSTRACT

Background Capillary malformation-arteriovenous malformation is an autosomal dominant disorder, characterised by capillary malformations and increased risk of fast-flow vascular malformations, caused by loss-of-function mutations in the *RASA1* or *EPHB4* genes. Around 25% of the patients do not seem to carry a germline mutation in either one of these two genes. Even if other genes could be involved, some individuals may have mutations in the known genes that escaped detection by less sensitive techniques. We tested the hypothesis that mosaic mutations could explain some of previously negative cases.

Methods DNA was extracted from peripheral blood lymphocytes, saliva or vascular malformation tissues from four patients. *RASA1* and *EPHB4* coding regions and exon/intron boundaries were analysed by targeted custom gene panel sequencing. A second panel and/or Sanger sequencing were used to confirm the identified mutations.

Results Four distinct mosaic *RASA1* mutations, with an allele frequency ranging from 3% to 25%, were identified in four index patients with classical capillary malformation-arteriovenous malformation phenotype. Three mutations were known, one was novel. In one patient, a somatic second hit was also identified. One index case had three affected children, illustrating that the mosaicism was also present in the germline.

Conclusion This study shows that *RASA1* mosaic mutations can cause capillary malformation-arteriovenous malformation. Thus, highly sensitive sequencing techniques should be considered as diagnostic tools, especially for patients with no family history. Even low-level mosaicism can cause the classical phenotype and increased risk for offspring. In addition, our study further supports the second-hit pathophysiological mechanism to explain the multifocality of vascular lesions in this disorder.

even between members of the same family. The fast-flow vascular malformations are located in the central nervous system, the skin, subcutis, muscles and bones, in the face and neck, or the extremities. They are associated with an increased morbidity and mortality. Two genes have been identified until now: *RASA1*, involved in CM-AVM type 1, and *EPHB4*, involved in CM-AVM type 2.^{1,2} These two genes explain around 50% and 25% of patients, respectively.² The remaining patients could be explained by genomic *RASA1* or *EPHB4* rearrangements, mutations in the promoter or regulatory regions, deep intronic mutations, epigenetic down-regulation, or mosaicism. Other genes could also be involved. Recently, genomic rearrangements involving *RASA1* have been identified in 8.6% of patients with CM-AVM1.⁵ We here report mosaic *RASA1* mutations in four unrelated patients with the classical CM-AVM phenotype.

MATERIALS AND METHODS**Patient recruitment and samples**

Samples and clinical information from patients with CM-AVM included in this retrospective study were sent to our diagnostic or research laboratories. Clinical information was collected in a standardised manner through a questionnaire. Informed consent was obtained from all patients and/or their parents.

DNA extraction**Blood samples**

DNA was extracted from blood (EDTA tubes) using DNA purification kit (Gentra) or Wizard genomic DNA purification kit (Promega), or by the referring laboratories of the hospitals of origin.

Tissue and saliva samples

DNA was extracted using Wizard genomic DNA purification kit (Promega), with protocols according to tissue type.

RNA extraction and cDNA synthesis

Snap-frozen tissue from the AVM of patient 2 was powdered under liquid nitrogen and RNA was extracted using TriPure (Roche). It was retrotranscribed using RevertAid H-Minus First Strand cDNA Synthesis Kit (Fermentas), with random hexamers.

INTRODUCTION

Capillary malformation-arteriovenous malformation (CM-AVM, MIM 608354) is an autosomal dominant disorder first recognised in 2003.¹ The incidence is unknown, but could be as high as 1/10,000.² The phenotype is characterised by CMs, usually small and multifocal, with or without a perilesional pale halo and an increased risk of fast-flow vascular malformations: AVMs, arteriovenous fistulas (AVF) or Parkes Weber syndrome.¹⁻⁴ The penetrance is high and the phenotype is variable



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Table 1 Clinical and genetic data

	Tissue	<i>RASA1</i> variant	Allele frequency (read count) with AmpliSeq panel	Allele frequency (read count) with Sophia Genetics panel	Sanger sequencing	CMs	Fast-flow vascular malformations
Patient 1	Blood	<i>c.1879A>T</i> ; p.(Lys627*)	35.7% (10/28)	25.3% (164/649)	+	3 CMs	Parieto-occipital AVF/spinal AVM from T1 to T8
Patient 2	Blood	<i>c.2035C>T</i> ; p.(Arg679*)	2.7% (3/111)	3.1% (63/2011)	NP	4 CMs, Bier spots on hands and telangiectatic lesions on upper thorax, lower lip and tongue	Facial AVM
	AVM	<i>c.(2035C>T); c.1507C>T</i> ; p.(Arg679*); Gln503*)	13.6% (465/3407)+8% (171/2126)	NP	NP		
Patient 3	Blood	<i>c.1192C>T</i> ; p.(Lys398*)	NP	8.5% (101/1189)	+	More than 20 CMs	Soft tissue and fatty hypertrophy with multiple AV micro fistulas in the right foot
Patient 4	Blood	<i>c.2707C>T</i> ; p.(Arg903*)	NP	6.1% (59/964)	NP	More than 10 CMs	–
	Saliva		NP	4.6% (36/783)	NP		
	CM		6.9% (21/305)	NP	NP		

The symbol – denotes absent and + denotes mutation seen by Sanger sequencing.

AV, arteriovenous; AVF, arteriovenous fistula; AVM, arteriovenous malformation; CM, capillary malformation; NP, not performed.

Targeted next-generation sequencing

Genomic DNA extracted from peripheral blood (patients 1 and 2) and frozen tissue (patient 2—AVM and patient 4—CM) were screened with a custom AmpliSeq gene panel (www.ampliseq.com) and Ion Torrent technology to cover the coding exons and 5 bp of flanking introns of the *RASA1* and *EPHB4* genes (table 1). DNA libraries were prepared with 20 ng of DNA using Ion AmpliSeq Library Kit and according to the manufacturer protocol (Life Technologies). A Personal Genome Machine (PGM, Life Technologies) was used for sequencing with chips 316.

Genomic DNA extracted from peripheral blood (patients 1–4) and saliva (patient 4) were screened with a custom Sophia Genetics panel using IDT (Integrated DNA Technologies) capture probes covering the coding exons and 25 bp of flanking introns of the *RASA1* and *EPHB4* genes (table 1). DNA libraries were prepared with 200 ng of DNA using KAPA HyperPlus Kit (KK8514) according to the manufacturer's protocol (Sophia Genetics). A MiSeq machine (Illumina) was used for sequencing, with a flow cell v3.

Variant analysis

Reads produced by Ion Torrent technology were aligned to the human genome (hg19) to generate .bam files using the Ion Torrent Suite Server V.4 or V.5 (Life Technologies). These files were imported in Highlander software (<http://sites.uclouvain.be/highlander/>) and calling of variants was performed with the embarked Torrent Variant Caller V.5.2 (Life Technologies). PGM_GERMLINE_LOW_STRINGENCY settings were used for caller parameters. We applied the following filters to select variants of interest: frequency ≤ 0.01 in the Exome Aggregation Consortium database (ExAC; <http://exac.broadinstitute.org/>) and the coverage of $50\times$ minimum. The positions of the changes in *RASA1* reported in table 1 were interrogated using Bamcheck tool (implemented in Highlander). No significant noise was detected among 1530 samples screened for this gene using the AmpliSeq panel, except a heterozygous carrier of *c.2035C>T*.

For the samples sequenced with Illumina technology, .fastq files were imported in Sophia DDM software (Sophia Genetics). Alignments to the human genome (hg19) in .bam files and the calling of variants were generated with an algorithm from Sophia Genetics. All variants with an allele frequency $<15\%$ were considered as low confidence and only variants detected

in less than 5% of all analysed patients (n=300) were interpreted. Additional filters applied to keep the variants included: frequency ≤ 0.01 in ExAC and Genome Aggregation Database (gnomAD, <http://gnomad.broadinstitute.org>) and coverage of $50\times$ minimum.

The variants were named according to the guidelines established by the Human Genome Variation Society (<https://www.hgvs.org/mutnomen/>).

Second-hit testing

A 791 bp fragment of *RASA1* cDNA, encompassing both mosaic stop mutations identified in the AVM tissue from patient 2, was amplified with primers 5'-TCAATGACACAGTGGATGGC-3' and 5'-AGAGTATCGTGCTCGAACAC-3' using Qiagen Taq polymerase and annealing temperature at 62°C. The amplified fragment was cloned using the TOPO-cloning kit (Invitrogen). Forty-nine distinct colonies were obtained.

Sanger sequencing

Bidirectional Sanger sequencing was used to confirm the mosaic *RASA1* mutations identified in patients 1 and 3 and to check for the presence/absence of the mutation in family members. Sanger sequencing was also used to sequence the colonies containing the cDNA fragment in order to study the allelic distribution of mutations identified in the AVM from patient 2.

RESULTS

Patients' phenotype

Patient 1 is a young adult with parieto-occipital AVF, diagnosed by MRI, performed in a context of migraine. Clinical examination revealed three CMs. Laser treatment was ineffective. Extended workup showed an asymptomatic spinal AVM. The parents are unaffected. The patient has three siblings, two of which have one CM.

Patient 2 is a middle-aged adult. There is no family history of vascular malformation. At birth, there was a red stain on left ala of the nose and nasolabial fold. This was considered a CM. The patient had several laser sessions, which were ineffective. During teenagehood, the patient had surgical procedure for the facial vascular malformation, which turned out to be an AVM. The postsurgery was complicated by bleeding, delayed healing and increase in volume of the malformation. The clinical

examination revealed four CMs between 0.5 and 2 cm in diameter, numerous telangiectatic lesions on the upper thorax and less than 10 on the lips and tongue and Bier spots on hands. The patient had a second surgery for the AVM with no recurrence over a period of 3 years.

Patient 3 is a teenager with no family history of vascular malformation. At birth, less than five CMs were observed. During childhood, the number of the CMs increased. At the last examination, the patient had more than 20 flat, round-ovoid CMs, mostly with regular borders, some with a pale halo. Clinical examination also revealed some hypertrophy of the right foot with a large flat pink CM on the sole and increased warmth. MR angiography investigation showed increased soft tissue and muscle hypertrophy and arteriovenous microfistulas.

Patient 4 is a middle-aged adult with more than 10 CMs. This patient had three children, all with multifocal CMs.

Molecular analysis

Before this study, *RASA1* testing had been performed by Sanger sequencing and MLPA (Multiplex Ligation-dependent Probe Amplification) in all four index patients and one of the affected children of patient 4. No mutation was identified in the four index patients. A heterozygous *RASA1* mutation was identified in the child of patient 4, as detailed below. Subsequently, *RASA1* and *EPHB4* were analysed using two different custom-designed gene panels. Mosaic *RASA1* mutations, with variable allele frequency, were identified in the four index patients. No mutation and no large deletion/duplication were identified in the *EPHB4* gene.

Patient 1 harbours a c.1879A>T; p.(Lys627*) *RASA1* mutation with an allele frequency estimated at 25.3% with Sophia Genetics panel (table 1). The mutation was subsequently confirmed with the AmpliSeq panel (allele frequency estimated at 35.7%), even if the depth was inferior (28X) to the established threshold (50X). The mutation was also confirmed by Sanger sequencing using new primers. To our knowledge, this mutation has not been reported in the literature or databases, including gnomAD. Nevertheless, as it causes a premature stop codon, it was considered as probably pathogenic. The *RASA1* mutation identified was absent in the DNA from parents and siblings.

Patient 2 harbours a c.2035C>T; p.(Arg679*) *RASA1* mutation with an allele frequency estimated at 2.7% with AmpliSeq panel and 3.1% with Sophia Genetics panel. This mutation has already been published as a pathogenic mutation.³ The mutation was absent in the unaffected mother. Paternal DNA was not available for analysis. Tissue from the AVM was also tested using the AmpliSeq panel. The mutation c.2035C>T; p.(Arg679*) was present with an allele frequency of 13.6%. In addition, a second mutation was identified: c.1507C>T; p.(Gln503*) with an allele frequency of 8% (table 1). The sequencing of the cloned cDNA fragment covering the two mosaic mutations showed three colonies out of 49 with the c.2035C>T change alone and one with the c.1907C>T change alone. The other 45 colonies were wild type.

Patient 3 harbours a c.1192C>T; p.(Lys398*) *RASA1* mutation with an allele frequency estimated at 8.5% with Sophia Genetics panel (table 1). The mutation can be observed as a very weak peak using Sanger sequencing. This mutation has already been published as a pathogenic mutation in several patients.^{3,4} The *RASA1* mutation identified was absent in the DNA from parents.

Patient 4. *RASA1* analysis was initially performed in the index patient and an affected child by Sanger sequencing and

MLPA. No mutation was found in the index patient, but a heterozygous mutation was identified in the child: c.2707C>T; p.(Arg903*). The absence of the mutation in the affected parent was confirmed on a second, independently drawn blood sample. Subsequently, the mutation was identified in two other affected children. Somatic mosaicism affecting the germline was suspected in the affected parent, but was not possible to investigate at the time of diagnosis. Using the AmpliSeq gene panel, the *RASA1* c.2707C>T; p.(Arg903*) mutation was finally identified in the affected parent with an allele frequency estimated at 6.9% in DNA extracted from a CM. The blood DNA and the DNA extracted from saliva were analysed with the Sophia Genetics gene panel. The *RASA1* mutation c.2707C>T; p.(Arg903*) was identified with an allele frequency of 6.1% and 4.6%, respectively (table 1). This mutation has already been published as a pathogenic mutation.^{3,5,6} No second hit was identified in the CM tissue.

DISCUSSION

We report herein distinct mosaic *RASA1* mutations in four unrelated patients with characteristic CM-AVM phenotype. All the patients had multifocal CMs and three of them had in addition fast-flow vascular malformations: intracranial AVF and intraspinal AVM (patient 1), facial AVM (patient 2), or multiple AV microfistulas in a foot (patient 3). Patient 2 had a capillary blush on top of the facial AVM. This had initially been misinterpreted as a CM. Interestingly, patient 2 had, in addition to the multifocal CMs, telangiectatic lesions on the upper thorax, lower lip and tongue. Telangiectatic lesions are frequently seen in patients with hereditary haemorrhagic telangiectasia, and in a minority of patients with CM-AVM type 2, caused by *EPHB4* mutations. To our knowledge, telangiectatic lesions have not been reported before in patients with *RASA1* mutation.

The mutations were absent in the parents of patients 1 and 3, and the mother of patient 2. DNA samples from the father of patient 2 and the parents of patient 4 were not available for testing, but they were all unaffected. Thus, the most plausible explanation for mosaicism in our patients is de novo, postzygotic mutations and not reversion from an inherited mutation to the wild-type allele. Interestingly, the mutation was not present in two siblings of patient 1 with one CM, which thus represent phenocopies. It is not unexpected to identify sporadic CMs, commonly called 'port-wine stain', in relatives of patients with CM-AVM, considering the prevalence of 0.3%.⁷

Three of the mutations have been previously published, one was novel.³⁻⁶ The mutations were identified by targeted next-generation sequencing and confirmed by a different technology and/or in a different tissue. The level of mosaicism varied from 3% to 25% using Sophia Genetics panel (high depth) and was consistent between the two panels (Sophia Genetics and AmpliSeq).

The phenotype does not seem to depend on the level of mosaicism identified in blood. For instance, patient 2 has a severe phenotype, whereas the allele frequency was estimated at only 3%. Interestingly, the allele frequency of the mutation in the AVM tissue was higher (13.6%), suggesting that the level of the mutation in blood does not necessarily allow an estimation of the level of the mosaicism in the critical tissue, underscoring tissular/cellular heterogeneity of the mosaicism. In contrast, in patient 4, the level of the mosaicism in blood and the CM was rather similar: 6.1% and 6.9%, respectively.

In most patients with CM-AVM, the only material available for genetic testing is blood. Thus, careful interpretation and

sensitive enough techniques are necessary to identify low-level mosaicism in patients with no heterozygous *RASA1* or *EPHB4* mutation. However, analysing thousands of different positions for possible low-level mosaicism is a difficult task. High read depth (at least 500X) and filters that enable to identify low-level mosaicism are necessary. On the other hand, the parents of patients with apparently de novo heterozygous mutation could carry the mutation in a mosaic state. This is technically much easier to investigate, once the precise nucleotide concerned is known. One should consider mosaicism in families with an apparently de novo appearance of CM-AVM in an individual with unaffected parents and an affected child. We would recommend performing the initial genetic screening in the child, as the mutation could be present in a mosaic state in the affected parent, as in patient 4.

Patients with somatic mosaicism affecting the germline are at risk of passing the mutation to the offspring. Nevertheless, different levels of mosaicism can be present in different tissues, and the presence and the level of germline mosaicism cannot be predicted from the level of the somatic mosaicism, making genetic counselling difficult. Patient 4 in the current study has similar low-level mosaicism in blood, saliva and the CM (from 4.6% to 6.9%), but had three affected children, suggesting that the level of mosaicism in the germline could be higher.

Many of the *RASA1* mutations reported until now were identified by denaturing HPLC.^{3,4} The sensitivity of this screening method is not high enough to detect low-level mosaicism. A significant proportion of patients with CM-AVM do not carry identifiable *RASA1* or *EPHB4* mutations. Even if other genes are possibly involved, some mutations could have been missed due to the sensitivity of the screening methods, or the location in uninvestigated regions (regulatory regions or deep in introns). Another possibility is large genomic rearrangements. Indeed, *RASA1* genomic rearrangements were reported in 8.3% of patients with *RASA1*-related CM-AVM.⁵ No *EPHB4* genomic rearrangements have been reported as yet, but this is probably only a question of time. As de novo mutation rate is estimated to be as high as 25%,^{3,4} it was logical to also identify mosaic mutations, like in other autosomal dominant disorders with high de novo mutation rate, such as in tuberous sclerosis.⁸ While our manuscript was under review, another group published *RASA1* mosaic mutations in two patients with CM-AVM.⁹ Our study reinforces these results.

Somatic second hit was proposed as the pathophysiological mechanism to explain the multifocality of the vascular malformations in patients with CM-AVM, as previously reported in other vascular malformations.^{10–15} Proving this hypothesis is rendered difficult by the limited availability of tissues. Indeed, surgery is a rare option in patients with CM-AVM. Until now, *RASA1* somatic mutations have been identified in four patients only: in three, the allelic distribution was not studied; in the fourth one, the somatic mutation was in trans with the germline mutation in a CM lesion in support of a second-hit mechanism of disease pathogenesis.^{3,16–18} In this study, patient 2 harbours two mosaic *RASA1* mutations in the AVM lesion: one present in the blood and the malformation (c.2035C>T), and the second one present only in the malformation (c.1507C>T). cDNA analysis showed that the two changes were on different alleles. Thus, this observation strongly supports the second-hit hypothesis in CM-AVM. Interestingly, the level of mosaicism of the first hit (c.2035C>T) was higher in the AVM than in blood.

One could argue that the mosaic mutations identified in this study are not the cause of the phenotype and that another constitutional mutation could have been missed. Although this is a

possibility, the identification of a low-level mosaic mutation in an affected parent (patient 4) of three affected children, with a heterozygous mutation, underscores that the phenotype is explained by the mutation identified.

We have previously reported 118 CM-AVM families with heterozygous *RASA1* mutations.^{1,3,4} We report here four patients with mosaic mutations, which thus represent at least 3% of the patients with *RASA1*-related CM-AVM in our cohort. The percentage of patients with mosaicism could be even higher, if we consider that among the 118 index patients, about 25% have de novo mutations. As those mutations were identified by Sanger sequencing, which is not a quantitative technique, some of them could be present in a high-level mosaicism.

CONCLUSION

We report four index patients with CM-AVM caused by distinct *RASA1* mosaic mutations. In at least one of them, the somatic mosaicism was also present in the germline and this has important consequences for diagnosis and genetic counselling. It is important to keep in mind that even low-level mosaicism can be associated with a severe phenotype. Next-generation sequencing technologies should be used in diagnostic laboratories to increase mutation detection rate in patients with CM-AVM. Finally, two mosaic mutations were identified in an AVM lesion, further supporting the second-hit pathophysiological mechanism in CM-AVM.

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