

## Somatic *MAP2K1* Mutations Are Associated with Extracranial Arteriovenous Malformation

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Arteriovenous malformation (AVM) is a fast-flow, congenital vascular anomaly that may arise anywhere in the body. AVMs typically progress, causing destruction of surrounding tissue and, sometimes, cardiac overload. AVMs are difficult to control; they often re-expand after embolization or resection, and pharmacologic therapy is unavailable. We studied extracranial AVMs in order to identify their biological basis. We performed whole-exome sequencing (WES) and whole-genome sequencing (WGS) on AVM tissue from affected individuals. Endothelial cells were separated from non-endothelial cells by immune-affinity purification. We used droplet digital PCR (ddPCR) to confirm mutations found by WES and WGS, to determine whether mutant alleles were enriched in endothelial or non-endothelial cells, and to screen additional AVM specimens. In seven of ten specimens, WES and WGS detected and ddPCR confirmed somatic mutations in mitogen activated protein kinase kinase 1 (*MAP2K1*), the gene that encodes MAP-extracellular signal-regulated kinase 1 (MEK1). Mutant alleles were enriched in endothelial cells and were not present in blood or saliva. 9 of 15 additional AVM specimens contained mutant *MAP2K1* alleles. Mutations were missense or small in-frame deletions that affect amino acid residues within or adjacent to the protein's negative regulatory domain. Several of these mutations have been found in cancers and shown to increase MEK1 activity. In summary, somatic mutations in *MAP2K1* are a common cause of extracranial AVM. The likely mechanism is endothelial cell dysfunction due to increased MEK1 activity. MEK1 inhibitors, which are approved to treat several forms of cancer, are potential therapeutic agents for individuals with extracranial AVM.

Arteriovenous malformation (AVM) is a congenital vascular anomaly, comprised of abnormal connections between arteries and veins that are missing normal high-resistance capillary beds (Figure 1).<sup>1</sup> Sporadic extracranial AVMs are solitary and may be localized or regional. Rapid blood flow is demonstrable by Doppler ultrasonography. Magnetic resonance imaging reveals signal voids consistent with fast-flow, while angiography shows the early filling of draining veins (Figure 1). With time, arterial to venous shunting causes tissue ischemia that leads to pain, ulceration, bleeding, and destruction of adjacent tissues. Treatment for AVM has been discouraging. Embolization and/or resection are often followed by expansion; there are no drug treatments.<sup>2</sup> The purpose of this study was to identify the genetic basis for sporadic, extracranial AVM in an effort to devise a new therapeutic strategy.

The Committee on Clinical Investigation at Boston Children's Hospital approved this study and informed consent was obtained from study participants. Ten AVM specimens that had been collected during a clinically indicated procedure, including matched unaffected tissue specimens from three of the study participants, had DNA extracted using the DNeasy Blood & Tissue Kit (QIAGEN); saliva DNA was extracted using the prepIT-L2P extraction kit (DNA Genotek). Five affected tissue samples underwent deep WES and another five had WES and WGS on Illumina

platforms at Macrogen. WES and WGS used 101 basepair (bp) or 150 paired-end reads, respectively. Average on-target WES depths were between 224-fold and 281-fold for the AVM samples and between 135-fold and 176-fold for the unaffected tissues. Coverage  $\geq 30$ -fold and  $\geq 390$ -fold were obtained for 90% to 93% and for 81% to 92% of the exomes, respectively (Figure 2). The five AVM specimens and the three matched unaffected tissue samples subjected to WGS yielded average depths between 108-fold and 145-fold and between 37-fold and 50-fold, respectively.

Raw sequencing reads were aligned to the GRCh37 human reference genome using the Burrows-Wheeler Aligner.<sup>3</sup> The reads were processed by Picard and the Genome Analysis Tool Kit<sup>4</sup> for the removal of duplicated and error-prone reads, indel realignment, and base-quality recalibration. Candidate somatic mutations were identified using the single-sample (when only AVM DNA was available) and paired-sample (when AVM and blood/saliva DNA were available) modes of MosaicHunter.<sup>5</sup> Somatic mutations with at least 2% mutant allele fraction and at least five reads supporting the variant allele were considered in subsequent analyses. We excluded common variants that were annotated in the Single Nucleotide Polymorphism,<sup>6</sup> 1000 Genomes Project,<sup>7</sup> Exome Sequencing Project,<sup>8</sup> or Exome Aggregation Consortium<sup>9</sup> databases. Among the protein-altering mutations, only those

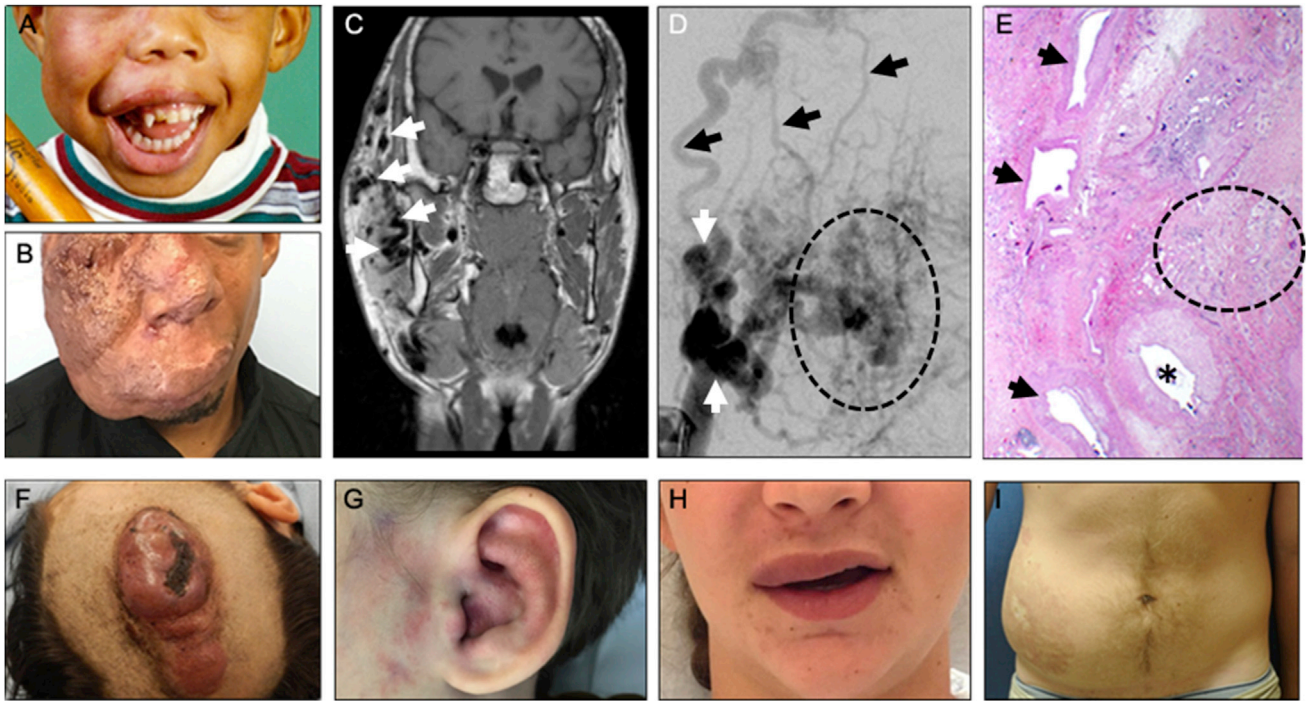
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### Figure 1. Solitary Extracranial AVMs

(A and B) Photograph of participant 23 during (A) childhood (stage I) and (B) adulthood (stage III). Note progressive growth of the facial AVM.

(C) Coronal magnetic resonance image illustrates the extent of the lesion with multiple signal voids consistent with fast-flow (white arrows).

(D) Angiogram showing tortuous arteries (white arrows) that feed the AVM, the “nidus” (dotted oval) where there are direct communications between numerous small arteries and veins, and early filling of draining veins (black arrows).

(E) Hematoxylin and eosin-stained section of participant 23’s affected tissue, obtained after initial resection. Note the large feeder artery (asterisk), hyper-muscularized veins (arrows), and an area where arteries and veins connect in the absence of a normal capillary bed (dotted oval).

(F) Participant 2, scalp AVM (stage III).

(G) Participant 6, ear AVM (stage I).

(H) Participant 12, upper lip AVM (stage I).

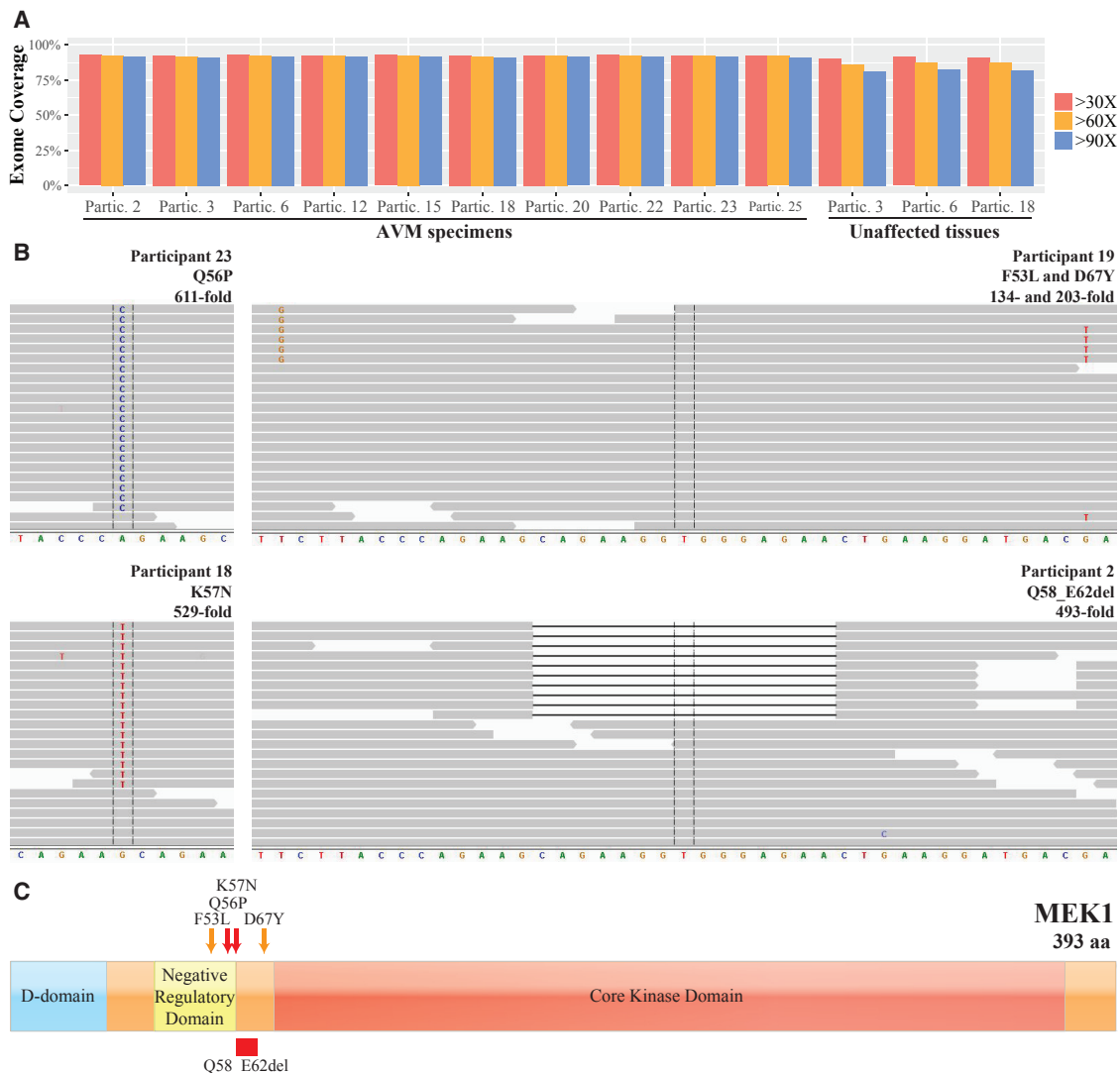
(I) Participant 19, abdominal AVM (stage II).

predicted to be deleterious by Polymorphism Phenotyping (PolyPhen-2) or Sorting Intolerant From Tolerant (SIFT) algorithms<sup>10,11</sup> were analyzed further.

AVMs had between zero and four genes with putative protein-altering somatic mutations, but only *MAP2K1* (GenBank: NM\_002755; MIM: 176872), encoding the dual specificity mitogen-activated protein kinase MEK1 (GenBank: NP\_002746.1), contained somatic mutations (c.171G>T [p.Lys57Asn] or c.167A>C [p.Gln56Pro]) in multiple samples (Table 1). Each AVM had  $\geq 105$ -fold coverage over the entire *MAP2K1* coding sequence. Because several specimens contained somatic *MAP2K1* missense mutations, we reanalyzed samples in which we failed to detect a mutation at reduced stringency by lowering the detection threshold to  $\geq 1\%$  mutant allele fraction and  $\geq 3$  reads supporting the variant allele, and we also looked for short indels. This led to the identification of mutations in three additional deep WES samples (p.Lys57Asn [n = 2], c.173\_187del [p.Gln58\_Glu62del] [n = 1]) (Table 1) and in two of five AVM samples from other participants that had standard<sup>12</sup> WES (Table 2).

The specimen from participant 19 harbored two somatic *MAP2K1* mutations (c.[159T>G;199G>T]; p.[Phe53-Leu;Asp67Tyr]) in *cis* (Table 2).

We developed ddPCR assays<sup>13</sup> for each of the missense mutations that were identified by WES and WGS (Table S1). We used ddPCR to confirm the *MAP2K1* mutations found in deep WES (n = 7) and standard WES (n = 2). We also used ddPCR to screen for somatic mutations in 16 additional individuals: 3 without mutations detected by deep WES, 3 without mutations detected by standard WES, and 10 whom we had not previously examined. Out of these 16 individuals, 7 had *MAP2K1* mutations detected by ddPCR (Table 2). One person had a ddPCR pseudocluster that upon subcloning and sequencing was found to represent the same 15-bp deletion (c.173\_187del [p.Gln58\_Glu62del]) previously detected by WES in another individual. In 13 participants who had affected tissue with *MAP2K1* mutations and a paired unaffected tissue sample, none of the unaffected tissue samples contained mutant *MAP2K1* alleles (Table 2). We also did not identify mutant *MAP2K1* alleles in affected



**Figure 2. Somatic Mutation Detection in AVMs after Whole-Exome Sequencing**

(A) Graph depicts the depth-of-coverage across the exome for ten affected and three unaffected tissue samples. Note  $\geq 90$ -fold coverage for 90% of the exome obtained for each AVM sample.

(B) Integrative Genomic Viewer screenshots showing reads containing variant and reference alleles for four AVM samples. Total read depth at the site of the somatic mutation is indicated as “-fold” coverage. Note 4 reads in participant 19 indicate that the (c.159T>G) p.Phe53Leu and c.199G>T (p.Asp67Tyr) somatic mutations are in *cis*.

(C) Schematic diagram of MEK1 with approximate locations of the D, negative regulatory, and core kinase domains indicated. Note AVM somatic mutations cluster near the negative regulatory domain. The orange arrows indicate that p.Phe53Leu and p.Asp67Tyr variant were found in *cis* in a single individual. All other variants were found in two or more study participants.

tissue from individuals with other types of vascular anomalies, including infantile hemangioma, congenital hemangioma, capillary malformation, lymphatic malformation, venous malformation, and verrucous venous malformation (data not shown).

We re-examined, at reduced stringency, the WES and WGS data for the AVM specimens which did not have detectable *MAP2K1* mutations for mutations in other RAS/MAPK signaling pathway components (*HRAS* [MIM: 190020], *KRAS* [MIM: 190070], *NRAS* [MIM: 164790], *ARAF* [MIM: 311010], *BRAF* [MIM: 164757], *RAF1* [MIM: 164760], *MAP2K2* [MIM: 601263], *MAPK1* [MIM: 176948], and *MAPK3* [MIM: 601795]) as well as for previ-

ously reported AVM-associated genes (*RASA* [MIM: 139150], *PTEN* [MIM: 601728], *ENG* [MIM: 131195], *ACVRL1* [MIM: 601284], *SMAD4* [MIM: 600993], and *GDF2* [MIM: 605120]). No suspicious somatic mutations were found.

We next examined whether somatic *MAP2K1* mutations in AVMs were enriched in a specific cell type by separating endothelial cells from other cell types<sup>14</sup> in three AVM specimens that harbored *MAP2K1* mutations. In brief, specimens were digested with collagenase A (Roche) and dispase (BD Biosciences), filtered through a 70- $\mu$ m strainer, incubated with anti-cluster of differentiation 31 protein (anti-CD31) antibody conjugated to magnetic Dynabeads

**Table 1. MAP2K1 Variant Identification and Filtering for Ten AVM Samples Subjected to Deep Whole-Exome Sequencing**

Participant	Participant									
	2	3	6	12	15	18	20	22	23	25
Somatic mosaic sites identified by MosaicHunter with $\geq 2\%$ variant allele fraction and $\geq 5$ variant reads	34	32	24	14	21	15	22	33	38	63
Considering only variants not present in the general population	7	6	9	3	3	3	2	8	2	43
Considering only non-synonymous mutations	2	0	1	1	0	1	0	1	2	7
Considering only mutations predicted to be deleterious	2	0	1	1	0	1	0	0	2	4
Genes harboring variants found with the above criteria	PALM2-AKAP2, HAUS7	ND	MAP2K1	MAP2K1	ND	MAP2K1	ND	ND	MAP2K1, EPPK1	ZBTB41, OR888, FREM2, ACADVL
MAP2K1 mutation containing samples found using initial or less stringent criteria (variant/total WES reads)	c.173_187del (p.Gln58_Glu62del) (10/493)	ND	c.171G>T (p.Lys57Asn) (13/646)	c.167A>C (p.Gln56Pro) 45/625	ND	c.171G>T (p.Lys57Asn) (17/529)	c.171G>T (p.Lys57Asn) (9/584)	c.171G>T (p.Lys57Asn) (6/583)	c.167A>C (p.Gln56Pro) (21/611)	ND

Abbreviation: ND indicates no mutant alleles were detected.

(Invitrogen), and cultured. CD31<sup>+</sup> cells were grown in endothelial cell growth medium and CD31<sup>-</sup> cells were cultured in mesenchymal stem cell growth medium (both from Lonza). We observed mutant *MAP2K1* alleles only in the DNA we extracted from the endothelial cells (Figure 3).

The most common type of AVM occurs sporadically and is solitary. The prevalence of AVMs is unknown. The Vascular Anomalies Center at Boston Children's Hospital annually evaluates ~50 new individuals with extracranial AVM.<sup>15</sup> Solitary, extracranial AVM is not heritable, has similar incidence in males and females, and exhibits variable severity. These features are typical of disorders caused by a somatic mutation<sup>16</sup> and are consistent with the finding of somatic *MAP2K1* mutations in 16 of 25 tissue specimens. Solitary AVMs progress through four clinical stages: stage I lesions are typically small, warm to the touch, and exhibit arteriovenous shunting by Doppler ultrasonography; stage II lesions enlarge significantly, become pulsatile, and develop venous dilatation; stage III AVMs exhibit pain, ulceration, and/or bleeding; and stage IV lesions are characterized by cardiac failure.<sup>2,17</sup> We cannot exclude the possibility that somatic *MAP2K1* mutations arise as a consequence of AVM expansion, rather than being the cause of AVM. Arguing against the former interpretation is the presence of mutations in stage I lesions (Figure 1 and Table 2) and the absence of mutations in other enlarging vascular malformations. Finding *MAP2K1* mutations solely within the endothelial cell compartment of an AVM strongly suggests that mutant endothelial cells are responsible for the malformation process, perhaps by preventing capillary networks from forming between developing arteries and veins.

We found *MAP2K1* mutations in 64% of the specimens; the remaining samples may contain mutations at levels below our detection limit, in regions of the gene not interrogated by ddPCR, or in other genes. AVMs also occur in individuals segregating three Mendelian genetic diseases; however, these AVMs are clinically, radiologically, and/or histologically distinct from AVMs with *MAP2K1* mutations. AVMs associated with hereditary hemorrhagic telangiectasia (HHT [MIM: 187300, 600376, 175050, 615506]), caused by mutations in *ENG*, *ACVRL1*, *SMAD4*, or *GDF2*, are multifocal, small, and affect lungs, gastrointestinal system, and brain (see McDonald and Pyeritz, GeneReviews, in Web Resources). Families with *RASA1* mutations have capillary malformation-arteriovenous malformation (CM-AVM [MIM: 608354]). This syndrome is characterized by multiple, small, cutaneous fast-flow lesions; some affected individuals also have intracranial or extracranial AVMs (see Beyrak-Toydemir and Stevenson, GeneReviews, in Web Resources). Multiple, intramuscular AVMs occur in individuals with phosphatase and tensin homolog (PTEN) hamartoma-tumor syndrome (MIM: 601728) (see Eng, GeneReviews, in Web Resources).

The *MAP2K1* protein product MEK1 plays an important role in the RAS/MAPK signaling pathway that controls

**Table 2. MAP2K1 Variant Detection in 25 Participants with AVM**

Participant	Age	Sex	Location, Stage	DNA Source <sup>a</sup>	Variant <sup>b</sup>	WES <sup>c</sup>	WGS <sup>c</sup>	ddPCR <sup>d,e</sup>
1	5 y	M	lip, stage II	frozen	p.Gln56Pro	7% (3/45)	–	11% (231/2,628)
				frozen	p.Gln56Pro	–	–	2% (49/2,764)
	6 y		saliva	ND	–	–	0% (0/931)	
2	7 y	M	scalp, stage III	frozen	p.Gln58_Glu62del	2% (10/493)	3% (2/58)	6% <sup>f</sup> (954/15,592)
				saliva	ND	–	–	0% (0/1,745)
3	9 y	F	thigh, stage III	frozen	ND	0% (0/633)	0% (0/68)	0% (0/4,828)
				blood	ND	0% (1/348) <sup>g</sup>	0% (0/31)	0% (0/5,006)
4	9 y	M	forehead, stage II	frozen	p.Lys57Asn	–	–	6% (138/2,654)
				frozen	p.Lys57Asn	–	–	8% (105/1,379)
				CD31 <sup>+</sup> cells, <sup>a</sup> p3	p.Lys57Asn	–	–	31% (187/437)
				CD31 <sup>–</sup> cells, <sup>a</sup> p3	ND	–	–	0% (0/624)
				blood	ND	–	–	0% (0/2,062)
5	11 y	F	cheek, stage II	frozen	p.Lys57Asn	–	–	5% (124/3,445)
				saliva	ND	–	–	0% (0/1,274)
6	11 y	M	ear, stage I	frozen	p.Lys57Asn	2% (13/646)	–	8% (157/2,208)
				blood	ND	0% (0/271)	0% (0/35)	0% (0/6,913)
7	12 y	F	cheek, stage II	frozen	ND	0% (0/37)	–	0% (0/3,207)
				saliva	ND	–	–	0% (0/4,085)
8	12 y	F	ear, stage I	frozen	p.Lys57Asn	4% (2/52)	–	13% (414/3,498)
				saliva	ND	–	–	0% (0/965)
9	13 y	M	foot, stage III	frozen	ND	–	–	0% (0/2,461)
				saliva	ND	–	–	0% (0/1,926)
10	13 y	F	lip, stage II	frozen	p.Lys57Asn	–	–	7% (172/3,117)
				saliva	ND	–	–	0% (0/533)
11	13 y	M	forehead, stage II	frozen	p.Lys57Asn	–	–	4% (68/2,066)
				blood	ND	–	–	0% (0/3,698)
12	14 y	F	lip, stage I	frozen	p.Gln56Pro	7% (45/625)	–	9% (210/2,334)
13	14 y	F	lip, stage II	frozen	p.Gln56Pro	–	–	8% (172/2,980)
				frozen	p.Gln56Pro	–	–	6% (102/1,821)
				frozen	p.Gln56Pro	–	–	10% (209/2,655)
				CD31 <sup>+</sup> cells, <sup>a</sup> p5	p.Gln56Pro	–	–	35% (175/514)
				CD31 <sup>–</sup> cells, <sup>a</sup> p2	ND	–	–	0% (0/563)
				blood	ND	–	–	0% (0/2,396)
14	15 y	M	knee, stage I	frozen	p.Gln58_Glu62del	–	–	10% <sup>f</sup> (1,336/12,887)
15	15 yo	M	cheek, stage III	frozen	ND	0% (0/675)	0% (0/143)	0% (0/3,708)
16	17 y	M	forehead, stage II	frozen	ND	–	–	0% (0/4,025)
				saliva	ND	–	–	0% (0/1,396)
17	18 y	F	lip, stage II	frozen	ND	0% (0/30)	–	0% (0/3,757)
				saliva	ND	–	–	0% (0/2,458)

(Continued on next page)

**Table 2. Continued**

Participant	Age	Sex	Location, Stage	DNA Source <sup>a</sup>	Variant <sup>b</sup>	WES <sup>c</sup>	WGS <sup>c</sup>	ddPCR <sup>d,e</sup>
18	21 y	M	ear, stage II	frozen	p.Lys57Asn	3% (17/529)	4% (3/69)	7% (155/2,559)
				CD31 <sup>+</sup> cells, <sup>a</sup> p4	p.Lys57Asn	–	–	53% (468/946)
				CD31 <sup>–</sup> cells, <sup>a</sup> p4	ND	–	–	0% (0/765)
				blood	ND	0% (0/250)	0% (0/25)	0% (0/3,681)
19	22 y	M	abdomen, stage II	frozen	p.Phe53Leu	5% (6/134)	–	1% (9/1,318)
					p.Asp67Tyr	5% (10/203)	–	1% (7/1,336)
				blood	ND	0% (0/15)	–	–
20	22 y	M	scalp, stage I	frozen	p.Lys57Asn	2% (9/584)	–	4% (63/2,660)
				blood	ND	–	–	0% (0/3,171)
21	23 y	M	face, stage III	frozen	ND	–	–	0% (0/4,061)
22	24 y	M	cheek, stage I	frozen	p.Lys57Asn	1% (6/583)	–	5% (100/2,531)
				saliva	ND	–	–	0% (0/807)
23	32 y	M	cheek, stage III	frozen	p.Gln56Pro	3% (21/611)	4% (2/47)	7% (128/2,103)
				saliva	ND	–	–	0% (0/1,494)
24	39 y	F	scalp, stage III	frozen	ND	–	–	0% (0/3,640)
25	65 y	F	nose, stage II	frozen	ND	0% (0/656)	–	0% (0/2,642)
				saliva	ND	–	–	0% (0/1,325)

Abbreviations are as follows: ND indicates no mutant alleles were detected; dash (–) indicates no study was performed.

<sup>a</sup>For cultured CD31<sup>+</sup> and CD31<sup>–</sup> cells, the passage number (p) at the time DNA was extracted is indicated.

<sup>b</sup>The effect of the mutation at the protein level is indicated.

<sup>c</sup>Mutant allele percentages are provided as whole numbers and are calculated from the number of mutant reads/total reads at that locus.

<sup>d</sup>Mutant allele percentages are provided as whole numbers and are calculated by counting droplets that contain mutant, mutant+wild type, and wild-type amplicons. For simplicity, the ratio of mutant amplicon containing droplets/all amplicon containing droplets is shown.

<sup>e</sup>When no MAP2K1 mutation was detected in affected tissue, the denominator for the ddPCR assay is the sum of wild-type droplets for the four individual assays.

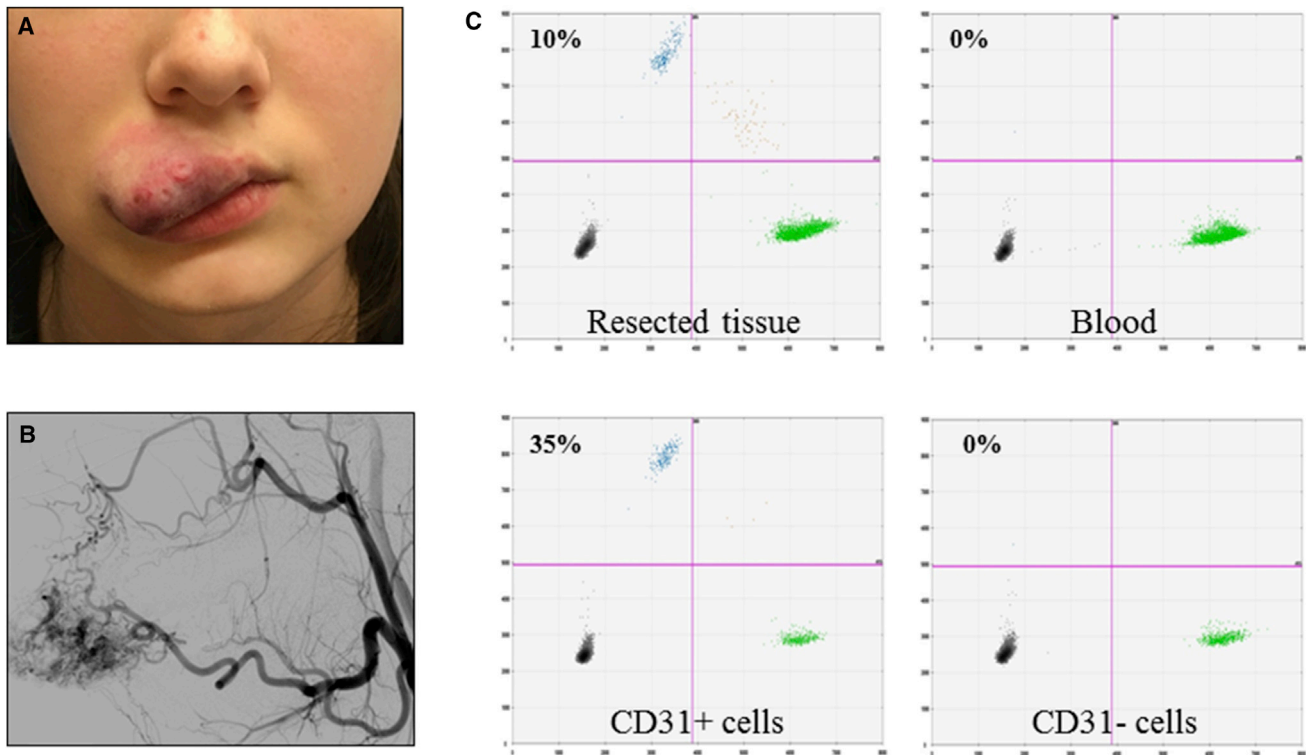
<sup>f</sup>Because no ddPCR assay was developed for this mutation, the mutant allele percentages were determined using pseudocounts, which were observed with each of the other missense mutant ddPCR probes.

<sup>g</sup>We cannot preclude this variant (p.Asp67Tyr) being a true positive somatic mutation; however, this variant was not detected in 3,867 DNA containing ddPCR droplets from affected tissue, nor in ddPCR droplets from the same blood sample. Therefore, we suspect the WES result is a false positive finding.

numerous cellular and developmental processes.<sup>18,19</sup> The somatic mutations we identified cluster within or adjacent to the protein's negative regulatory domain (Figure 2); they have been observed in neoplasms, including melanoma, lung cancer, and hematopoietic malignancies,<sup>20–25</sup> and have been shown to constitutively increase MEK1 activity.<sup>26–28</sup> MEK1 and its paralog MEK2 phosphorylate ERK1 and ERK2. MAPK signaling is activated by receptor tyrosine kinases, integrins, and G protein coupled receptors and is modulated by cross-talk with several other signaling pathways including the AKT-mTOR pathway.<sup>19</sup> Somatic mutations affecting proteins upstream of MEK1 occur in cancer and other types of vascular malformations.<sup>12,19,29–34</sup> The MAP2K1 mutations we detected in AVMs likely alter the function of MEK1 by producing a hypermorphic or neomorphic effect, since these mutations have previously been shown to increase ERK1 and ERK2 phosphorylation in tumors and in cultured cells. Consistent with this hypothesis, mice that are heterozygous for *Mek1* knockout alleles do not exhibit a phenotype, whereas homozygous knockout alleles cause embryonic lethality and placental defects.<sup>35</sup> Germline mutations in MAP2K1 have been

found in persons with Noonan syndrome (NS [MIM: 163950])<sup>36</sup> and cardio-facio-cutaneous syndrome (CFC [MIM: 115150]).<sup>37</sup> Individuals with NS or CFC have cardiac malformations, including pulmonic stenosis and atrial septal defect, but not AVM (see Allanson and Roberts, GeneReviews, and Rauen, GeneReviews, in Web Resources). The MAP2K1 mutations we detected in AVMs differ from those found in NS and CFC.

The AVMs in which MAP2K1 mutations were found do not metastasize, but they enlarge over time.<sup>38</sup> Incompletely resected AVMs often re-expand and attempts to embolize or ligate feeding arteries result in increased flow from collateral vessels.<sup>2</sup> Delineating similarities and differences between MAP2K1 mutation-containing AVMs and cancers will provide insights regarding cell-type-specific functions for MEK1 and broader roles for MEK1 in responding to environmental stress such as tissue injury or hypoxia. MEK1 inhibitors currently are in use against various cancers.<sup>18</sup> Further study is required to determine whether these drugs have efficacy for AVM. Current agents are cytostatic rather than cytotoxic, but might benefit individuals with AVM if they cause mutant



**Figure 3. AVM Endothelial Cells Enriched for the *MAP2K1* Mutation**

(A) Photograph of an upper labial AVM (participant 13) from which CD31<sup>+</sup> and CD31<sup>-</sup> cells were separated.

(B) Angiogram prior to resection shows arteriovenous shunting.

(C) ddPCR assay results performed on DNA extracted from the AVM (resected tissue), endothelial (CD31<sup>+</sup>), and non-endothelial (CD31<sup>-</sup>) cells, and peripheral blood. Droplets containing mutant only, mutant and wild-type, or wild-type only alleles appear in left upper, right upper, and right lower quadrants, respectively (empty droplets are in the left lower quadrant). Percentages of mutant alleles in each sample are indicated (droplet counts are provided in Table 2).

endothelial cells to terminally differentiate into capillary beds or prevent the regrowth of AVMs after resection or embolization.

#### Supplemental Data

Supplemental Data include one table and can be found with this article online at <http://dx.doi.org/10.1016/j.ajhg.2017.01.018>.

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## Supplemental Data

### **Somatic *MAP2K1* Mutations Are Associated with Extracranial Arteriovenous Malformation**

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Table S1. ddPCR Assays Developed for *Q56P*, *K57N*, *F53L*, *D67Y* MAP2K1 Mutations

<b>Product</b>	<b>Assay</b>
Forward Primer	5'-GTATTGACTTGTGCTCCCCAC-3'
Reverse Primer	5'-CCTGTTAATCAAGGCAAACCTCACCT-3'
REF Probe	5'-/5HEX/CCACCTTCTGCTTCTGGGTAAGAAAGGC/3IABkFQ/-3'
F53L Probe	5'-/56-FAM/AGCAAGGCCTCAAGGCGCTT/3IABkFQ/-3'
Q56P Probe	5'-/56-FAM/TCTCCACCTTCTGCTTCGGGGTA/3IABkFQ/-3'
K57N Probe	5'-/56-FAM/CAGTTCTCCACCTTCTGATTCTGGGT/3IABkFQ/-3'
D67Y Probe	5'-/56-FAM/AGTAGTCATCCTTCAGTTCTCCACCT/3IABkFQ/-3'