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Somatic Activating KRAS Mutations in Arteriovenous Malformations of the Brain

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BACKGROUND
Sporadic arteriovenous malformations of the brain, which are morphologically abnormal connections between arteries and veins in the brain vasculature, are a leading cause of hemorrhagic stroke in young adults and children. The genetic cause of this rare focal disorder is unknown.

METHODS
We analyzed tissue and blood samples from patients with arteriovenous malformations of the brain to detect somatic mutations. We performed exome DNA sequencing of tissue samples of arteriovenous malformations of the brain from 26 patients in the main study group and of paired blood samples from 17 of those patients. To confirm our findings, we performed droplet digital polymerase-chain-reaction (PCR) analysis of tissue samples from 39 patients in the main study group (21 with matching blood samples) and from 33 patients in an independent validation group. We interrogated the downstream signaling pathways, changes in gene expression, and cellular phenotype that were induced by activating KRAS mutations, which we had discovered in tissue samples.

RESULTS
We detected somatic activating KRAS mutations in tissue samples from 45 of the 72 patients and in none of the 21 paired blood samples. In endothelial cell–enriched cultures derived from arteriovenous malformations of the brain, we detected KRAS mutations and observed that expression of mutant KRAS (KRAS\textsuperscript{G12V}) in endothelial cells in vitro induced increased ERK (extracellular signal-regulated kinase) activity, increased expression of genes related to angiogenesis and Notch signaling, and enhanced migratory behavior. These processes were reversed by inhibition of MAPK (mitogen-activated protein kinase)–ERK signaling.

CONCLUSIONS
We identified activating KRAS mutations in the majority of tissue samples of arteriovenous malformations of the brain that we analyzed. We propose that these malformations develop as a result of KRAS-induced activation of the MAPK–ERK signaling pathway in brain endothelial cells. (Funded by the Swiss Cancer League and others.)
ARTERIOVENOUS MALFORMATIONS of the brain are high-flow vascular malformations that occur in approximately 15 per 100,000 persons and cause hemorrhagic stroke in children. They are tortuous, morphologically abnormal vascular channels between arteries and veins that lack an intervening capillary network, allowing high-pressure arterial blood from feeding arteries to shunt directly into the venous outflow system. The underlying cause of sporadic arteriovenous malformations of the brain is unknown, but similar lesions are found in rare genetic syndromes, such as hereditary hemorrhagic telangiectasias (a group of disorders caused by inactivating germline mutations in regulators of the transforming growth factor β–SMAD pathway), and in the capillary malformation–arteriovenous malformation syndrome (a RASopathy that is caused by inactivating mutations of RASA1 or EPHB4). This pattern indicates that a genetic cause may underlie the development of arteriovenous malformations of the brain, but most of these malformations occur as sporadic lesions in persons without a family history of the disease. We hypothesized that arteriovenous malformations of the brain arise from somatic mutations in the cranial vasculature.

METHODS

STUDY PATIENTS

Patients were eligible for inclusion in the study if they had sporadic, unifocal arteriovenous malformations of the brain with a defined nidus and evidence of arteriovenous shunting on digital subtraction angiography. In addition, patients had to have no family history of arteriovenous malformations and no documented history of genetic vascular disease. Clinical and surgical information was obtained from patients’ charts by the authorized study team. All diagnoses of arteriovenous malformations of the brain were confirmed by the study pathologists, radiologists, and surgeons (see Supplementary Appendix 1, available with the full text of this article at NEJM.org). All the patients, who were 18 years of age or older, provided written informed consent.

STUDY PROCEDURES, OUTCOMES, AND OVERSIGHT

The main study group comprised patients from Canada. Tissue samples of arteriovenous malformations of the brain and blood samples were obtained from these patients and deidentified, flash-frozen, and stored at Toronto Western Hospital, University Health Network, Toronto. Whole-exome sequencing was performed to detect somatic mutations. The results of DNA sequencing were confirmed with the use of droplet digital polymerase-chain-reaction (PCR) analysis. All the procedures performed with the use of samples obtained from patients were approved by the Institutional Research Ethics Review Board of the University Health Network. Details about the immunohistochemical analyses of the tissue samples are provided in Supplementary Appendix 1.

The independent validation group comprised patients from Finland. DNA samples were obtained from formalin-fixed, paraffin-embedded tissues of arteriovenous malformations of the brain from these patients. The samples were treated with a DNA glycosylase to reduce the risk of false positives on droplet digital PCR analysis, as described by Do and Dobrovic. Droplet digital PCR analysis was performed to detect KRAS mutations. All the procedures performed with the use of samples obtained from patients were approved by the Research Ethics Board at the University of Eastern Finland. Additional information regarding the tissue samples that were obtained from the patients is provided in Supplementary Appendix 1.

CELLULAR AND MOLECULAR STUDIES

Freshly resected tissue samples of arteriovenous malformations of the brain or control samples of normal tissue from temporal lobectomy specimens were obtained for the main study group from Toronto Western Hospital. Endothelial-cell cultures were established according to a modification of methods that had been described previously and were enriched and depleted with the use of anti-CD31 magnetic beads. Cell cultures were isolated from freshly resected tissue. Frozen tissues were used for exome sequencing and digital droplet PCR assays. The cell cultures were used for the digital droplet PCR assays and Western blotting. Details about the cellular and molecular studies to model mutant KRAS expression in cultured human cells are provided in Supplementary Appendix 1.

STATISTICAL ANALYSIS

Pairwise comparisons were made with the use of Student’s t-test. Comparison of three or more
groups was performed with the use of a one-way analysis of variance with the Newman–Keuls post hoc test. A P value of 0.05 or less was considered to indicate statistical significance. The Q value is the adjusted P value that is found by means of an optimized false discovery rate approach, and the r value is the linear correlation coefficient.

RESULTS

PATIENTS

From January 2013 through October 2017, a total of 39 patients were included in the main study group and had samples of arteriovenous malformation stored in the neurosurgical tissue bank. Of those, 21 had matched blood samples and 6 had fresh arteriovenous-malformation tissue that was used to derive cell culture. In addition, fresh vessel samples that had been obtained from 3 patients undergoing temporal-lobe resection for epilepsy were included. Tissues from 2 patients with cavernous malformations and 1 with a dural arteriovenous fistula as well as 2 samples of normal cortical vessels and 1 sample of gliosis tissue were included as controls. In the Finnish validation group, archived paraffin-embedded samples that had been obtained from 33 patients with arteriovenous malformations of the brain were included; 54 samples of arteries from the circle of Willis, 18 samples of cavernous malformations, and 2 samples of dural arteriovenous fistulas were also obtained for the control group.

DETECTION OF SOMATIC ACTIVATING KRAS MUTATIONS

In the main study group, whole-exome sequencing was performed on tissue samples of arteriovenous malformations of the brain that were obtained from 26 patients. Using the default criteria of MuTect2 variant caller, we detected six somatic missense variants in three genes, including two variants of unknown clinical significance (one in PCSK5 and the other in TP53BP1) (see the Supplemental Methods Section in Supplementary Appendix 1). The other four identified variants mapped to the same genomic coordinate in KRAS (chr12:25398284), which corresponded to a c.35G→A (p.Gly12Asp) mutation (in Patients 1, 4, and 10) or a c.35G→T (p.Gly12Val) mutation (in Patient 2). These variants were present in 2.4 to 4.0% of the sequence reads per sample (mean sequence coverage, 339±64×). On analysis of the 17 paired blood samples, the exomes had good coverage for this site (mean sequence coverage, 121±41×); no sequence reads contained a variant (Fig. 1). On further analysis that was performed with the use of relaxed criteria (the presence of variants in >0.5% of sequence reads, with reads on both DNA strands; see the Supplemental Methods Section in Supplementary Appendix 1), KRAS mutations were present in tissue samples obtained from 12 of the 26 patients and in none of the 17 paired blood samples (Fig. 1). KRAS mutations were present in 0.9 to 4.1% of the sequence reads. Activating KRAS mutations were found at codon 12: the c.35G→T (p.Gly12Val) mutation was found in 4 patients and the c.35G→A (p.Gly12Asp) mutation in 8 patients, including 1 patient (Patient 1) in whom the nidus sample and the draining-vein sample contained the c.35G→A (p.Gly12Asp) mutation in 2.0% and 1.3% of the sequence reads, respectively. These mutations are known to confer constitutive activity on KRAS.12 (Details of the analyses are shown in Table S1 in Supplementary Appendix 2 and Tables S2 and S3 and Fig. S1 in Supplementary Appendix 1.)

CONFIRMATION OF KRAS MUTATIONS

To confirm the results of whole-exome sequencing and to detect additional KRAS variants that may have been missed because of low representation in the sample, we performed droplet digital PCR analysis of the tissue samples obtained from the 26 patients (primer sequences are shown in Table S4 in Supplementary Appendix 1). We detected 12 putative KRAS mutations that had been detected previously on whole-exome sequencing and 6 KRAS mutations in previously negative samples (Fig. 1). We also tested 13 additional tissue samples, 4 of which had matching blood samples, with the use of droplet digital PCR analysis only; we detected the c.35G→A (p.Gly12Asp) mutation in 9 of these samples and the c.35G→T (p.Gly12Val) mutation in 2 of them (Fig. 1). The other 2 samples were nonmutated. Altogether, droplet digital PCR analysis revealed KRAS mutations in tissue samples from 29 of the 39 patients: 19 samples had the c.35G→A (p.Gly12Asp) mutation, 9 had the c.35G→T (p.Gly12Val) mutation, and 1 had the c.183A→T (p.Gln61His) mutation. The fractional abundance of variants in the unpurified tissue samples ranged from 0.43 to
Figure 1. Detection of KRAS Mutations in Samples Obtained from Patients.

The top chart shows the allele frequency of KRAS variants, determined by either the percentage of sequence reads that contained variants on whole-exome sequencing or the fractional abundance of variants on digital droplet polymerase-chain-reaction (PCR) analysis, in tissue samples of arteriovenous malformations of the brain. The samples are shown according to patient number in order of highest to lowest frequency, first in the main study group, which included 39 patients from Canada (29 [74%] with KRAS mutations), and then in the independent validation group, which included 33 patients from Finland (16 [48%] with KRAS mutations). The bottom chart shows details about the samples, including the sample type (fresh-frozen or formalin-fixed, paraffin-embedded tissue), sample site (nidus or draining vein), the presence or absence of a paired blood sample, and the specific activating mutation detected. No KRAS mutations were detected in paired blood samples. The CD31+ cells from Patient 32 in the main study group were positive for a c.35G→A KRAS mutation (data not shown in this figure), but there was insufficient tissue remaining for whole tissue analysis. Two patients from the main study group (Patients 1 and 39) had KRAS mutations in the draining-vein sample that matched those observed in the nidus sample.
4.37% (Fig. 1), a finding consistent with the results of whole-exome sequencing \( r=0.72 \). The 21 paired blood samples were negative for KRAS mutations (fractional abundance, 0%; positive droplets, 0). Moreover, droplet digital PCR analysis of 3 samples from patients with vascular lesions of the brain that were not arteriovenous malformations (2 of cavernous malformations of the brain and 1 of a draining vein of a spinal dural arteriovenous fistula), 2 samples of normal cortical vessels, and 1 sample of gliosis tissue surrounding a cavernous malformation were negative for KRAS variants (fractional abundance, 0%; positive droplets, 0). (Details of the analyses are shown in Fig. S2 and in Tables S3 and S6 in Supplementary Appendix 2.)

**DETECTION OF KRAS MUTATIONS IN AN INDEPENDENT VALIDATION GROUP**

The results in the main study group were validated in an independent validation group that involved 33 Finnish patients who had arteriovenous malformations of the brain. Genomic DNA was extracted from formalin-fixed, paraffin-embedded tissue samples of arteriovenous malformations (2 of cavernous malformations of the brain and 1 of a draining vein of a spinal dural arteriovenous fistula), 2 samples of normal cortical vessels, and 1 sample of gliosis tissue surrounding a cavernous malformation were negative for KRAS variants (fractional abundance, 0%; positive droplets, 0). (Details of the analyses are shown in Fig. S2 and in Tables S3 and S6 in Supplementary Appendix 1 and Table S5 in Supplementary Appendix 2.)

**DETECTION OF KRAS MUTATIONS IN ENDOTHELIAL CELLS**

To identify the types of cells that have KRAS mutations in arteriovenous malformations of the brain, we used magnetic-activated cell sorting to enrich and deplete endothelial cells from six fresh cell cultures derived from arteriovenous malformations of the brain (from Patients 29, 30, 31, 32, 38, and 39) and to enrich and deplete endothelial cells from three control cultures of normal vascular cells derived from cortical vessels (from three different patients undergoing temporal lobectomy for epilepsy). The endothelial-cell–enriched fractions of the cultures were, as expected, enriched with CD31+ cells (CD31 is an endothelial marker) (Fig. 2A); however, these fractions retained some cells that expressed alpha smooth-muscle actin (\( \alpha \)-SMA). In contrast, the endothelial-cell–depleted fractions did not have cells that expressed CD31 and were highly enriched with \( \alpha \)-SMA+ cells (Fig. 2A). Droplet digital PCR analysis showed that the CD31+
cultures derived from five of the six samples obtained from patients were positive for the c.35G→A (p.Gly12Asp) or c.35G→T (p.Gly12Val) mutation in KRAS. Those cultures also had a fractional abundance of variants that was 2.8 to 61.8 times as high as the level in whole tissue before fractionation, reaching 14.90% in Patient 30, 45.80% in Patient 32 (for whom whole un-fractionated tissue was not available owing to the small amount of initial material), and 52.15% and 17.54% in the nidus and draining-vein samples, respectively, from Patient 39 (Fig. 2B).

In some samples, the fractional abundance of variants in the CD31+ cultures correlated with the percentage of CD31+ cells seen on immunostaining of low-passage (passage 1 to 2) CD31+ cultures: the nidus sample from Patient 32 (fractional abundance, 45.80% [i.e., >90% mutant KRAS cells]) had 87% CD31+ cells, and the draining-vein sample from Patient 39 (fractional abundance, 17.54% [i.e., >30% mutant KRAS cells]) had 30% CD31+ cells. These findings suggest that most endothelial cells derived from these samples were positive for KRAS mutations. In contrast, the CD31− cultures from the same samples were negative for KRAS mutations (Fig. 2B). Droplet digital PCR analysis of the control cell cultures did not reveal KRAS mutations in CD31+ or CD31− fractions. (Details of the analyses are shown in Figs. S2 and S4 and in Table S9 in Supplementary Appendix 1.)

**ERK1/2 ACTIVATION IN ENDOTHELIAL CELLS WITH KRAS MUTATIONS**

KRAS is an effector molecule that lies downstream of receptor tyrosine kinases, and it activates diverse cellular signaling networks, such as the RAF–MEK (mitogen-activated protein kinase [MAPK] kinase)–ERK (extracellular signal-regulated kinase) (MAPK–ERK) signaling pathway and the PI3K (phosphoinositide 3 kinase)–AKT–mTOR (mechanistic target of rapamycin) pathway. We tested for phosphorylation of ERK1/2, AKT, and a noncanonical KRAS target, p38 MAPK, in endothelial cell–enriched cultures derived from arteriovenous malformations of the brain with KRAS mutations and in human umbilical-vein endothelial cells (HUVECs) that expressed one of two isoforms (proteins encoded by differently spliced messenger RNA) of mutant KRASG12V: KRAS4AG12V or KRAS4BG12V. The results for these cell cultures were compared with the results for

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>CD31+ Cell Culture</th>
<th>CD31+ Cell Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>0.76</td>
<td>4.38</td>
</tr>
<tr>
<td>30</td>
<td>1.60</td>
<td>4.90</td>
</tr>
<tr>
<td>31</td>
<td>0.75</td>
<td>1.23</td>
</tr>
<tr>
<td>32</td>
<td>NA</td>
<td>14.37</td>
</tr>
<tr>
<td>38</td>
<td>0.28</td>
<td>0.28</td>
</tr>
<tr>
<td>39 (Nidus)</td>
<td>2.34</td>
<td>17.54</td>
</tr>
<tr>
<td>39 (Draining Vein)</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

**Enrichment:**

- 5.7×
- 9.3×
- 2.8×
- NA
- 0×
- 22.3×
- 61.8×

**Fractional Abundance of KRAS Variants (%):**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Unfractionated Sample</th>
<th>CD31+ Patient Sample</th>
<th>CD31− Patient Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>0.76</td>
<td>4.38</td>
<td>1.60</td>
</tr>
<tr>
<td>30</td>
<td>1.60</td>
<td>4.90</td>
<td>0.75</td>
</tr>
<tr>
<td>31</td>
<td>0.75</td>
<td>1.23</td>
<td>NA</td>
</tr>
<tr>
<td>32</td>
<td>NA</td>
<td>14.37</td>
<td>2.34</td>
</tr>
<tr>
<td>38</td>
<td>0.28</td>
<td>0.28</td>
<td>0.00</td>
</tr>
<tr>
<td>39 (Nidus)</td>
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<td>17.54</td>
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</tr>
<tr>
<td>39 (Draining Vein)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>
primary endothelial cell–enriched cultures derived from normal brain vessels or HUVECs that received an empty expression construct. In both experiments, we observed increased levels of ERK1/2 phosphorylation in cells expressing mutant KRAS (Fig. 3A, and Fig. S5 in Supplementary Appendix 1) but did not observe increased levels of AKT or p38 phosphorylation. These findings suggest that mutant KRAS specifically activates the MAPK–ERK pathway in endothelial cells. Moreover, immunohistochemical staining of 25 tissue samples of arteriovenous malformations of the brain that were obtained from patients with arteriovenous malformations in the main study group and of 3 control samples of normal brain tissues was performed. There was strong staining for ERK phosphorylation in endothelial cells in the samples obtained from patients, regardless of the presence of a KRAS mutation, and little or no staining for ERK phosphorylation in the control samples (Fig. 3B).

**PHENOTYPE OF ENDOTHELIAL CELLS WITH KRAS MUTATIONS**

We performed RNA sequencing of HUVECs that expressed KRAS4AG12V, KRAS4BG12V, or control plasmid under growth factor–starved and serum-starved conditions. Assessment of categories of gene ontology that were altered in endothelial cells with KRASG12V expression revealed an enrichment of genes involved in the categories of angiogenesis or vascular development, proliferation, and migration. We also observed up-regulation of genes in the Notch pathway (e.g., DLL4, JAG1, JAG2, NOTCH1, HES1, and HEY2), which are involved in angiogenesis and arteriovenous specification and have been implicated in the pathogenesis of arteriovenous malformations. Arterial specification markers (e.g., NRPI and EFNB2) and venous specification markers (e.g., EPHB4 and NR2F2) were unchanged. The expression of genes that are known to have a role in angiogenesis (e.g., VEGFA, VEGFC, DUSP5, and HLF) was also induced, as was the expression of genes that are characteristic of the endothelial-to-mesenchymal transition (e.g., SNAI1, SNAI2, ZEB1, and PCDH1), a process implicated in other vascular malformations. These results imply that active KRAS dysregulates angiogenesis and vascular remodeling in endothelial cells. (For details, see Fig. S6 in Supplementary Appendix 1.)

The angiogenic factors vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) activate intracellular signaling in a RAS-dependent manner. Gene set enrichment analysis to compare the dysregulated genes in HUVECs that express KRASG12V with those in HUVECs that have been stimulated with VEGF showed a robust correlation (normalized enrichment score, 2.01; Q<0.0001 for false discovery rate) (Fig. S6D in Supplementary Appendix 1). This finding suggests that, in the absence of exogenous stimulation, HUVECs that express KRASG12V behave like angiogenic endothelial cells.

HUVECs with KRAS4AG12V expression had a highly elongated, mesenchymal-like phenotype (Fig. S7A in Supplementary Appendix 1). Time-lapse confocal imaging revealed that HUVECs that expressed KRAS4AG12V were more migratory than control cells on a scratch-wound assay performed in the absence of exogenous angiogenic factors and appeared to have more rapid rearrangements of actin during migration, as assessed by live imaging of actin dynamics with the use of LifeAct-GFP (Video 1, available at NEJM.org, and Fig. 4A and Video 2). Cell proliferation and apoptosis were unchanged in HUVECs that expressed KRAS4AG12V (Fig. S7C and S7D in Supplementary Appendix 1). A phenotype with similar morphologic features, migratory patterns, and actin dynamics was seen in human aortic cells that had been transfected with KRAS4AG12V (Video 3, and Fig. 5 in Supplementary Appendix 1).

Since cells that expressed KRAS4AG12V did not form typical cobblestone monolayers (Fig. S7A in Supplementary Appendix 1), we assessed formation of adherens junctions. Expression of KRAS4AG12V led to disassembly of vascular endothelial cadherin junctions (Fig. 4B). The inhibition of MAPK–ERK signaling with the use of a MEK inhibitor suppressed ERK phosphorylation, restored localization of vascular endothelial cadherin to the junctions of endothelial cells, and appeared to reduce lamellipodia formation (Fig. 4B). In addition, inhibition of the MAPK–ERK pathway abrogated the VEGF-like gene expression.
signature that was induced by KRAS4A(G12V), but inhibition of the PI3K pathway did not (Fig. 4C), a finding that reinforces the notion that the phenotype induced in endothelial cells by mutations that constitutively activate KRAS is specifically mediated by the MAPK–ERK pathway.

**Figure 3. Detection of ERK Phosphorylation in Endothelial-Cell–Enriched Cell Cultures and Tissue Samples.**

In Panel A, endothelial-cell–enriched (CD31+) cell cultures derived from tissue samples of arteriovenous malformations of the brain with KRAS mutations (from Patients 29, 30, and 31 in the main study group) show increased phosphorylation of ERK1/2 (extracellular signal-regulated kinase 1 or 2) but not of p38 or AKT, whereas three control CD31+ cell cultures derived from normal brain vasculature do not show increased phosphorylation of ERK1/2. Densitometry is shown on the right, normalized to beta-actin. The asterisk indicates a P value of 0.002. In Panel B, immunohistochemical staining of a tissue sample of an arteriovenous malformation of the brain with a KRAS mutation (from Patient 17 in the main study group) shows strong staining for ERK phosphorylation in endothelial cells lining the vascular lumen (arrows) and in vascular smooth-muscle cells in the vessel wall, whereas normal brain parenchymal vessels show little or no staining for ERK phosphorylation in endothelial cells (arrowheads).
A

Control

KRAS4A G12V

0 Min 80 Min 160 Min

B

Vascular Endothelial Cadherin ERK Phosphorylation F-Actin

Control

KRAS4A G12V

KRAS4A G12V and MEK Inhibition

C

Relative Expression (KRAS{G12V} vs. control)

Vehicle PI3K inhibitor MEK inhibitor
tend to be associated with activating mutations of the brain, “high flow” vascular anomalies, including somatic mutations in genes in the PI3K pathway,21,22 whereas MAPK pathways. “Slow flow” vascular malformations are caused by dysregulation of the MAPK–ERK pathway. This finding is consistent with the fact that other genes in other types of vascular malformations27 and the small fraction of endothelial cells therein and is consistent with the low frequencies of somatic mutations found in other genes in other types of vascular malformations.

Somatic activating KRAS mutations were present in the majority of tissue samples of sporadic, nonfamilial arteriovenous malformations of the brain that we analyzed. They were accompanied by dysregulation of the MAPK–ERK pathway. This finding is consistent with the fact that other types of vascular malformation are caused by somatic mutations in genes in the PI3K or RAS–MAPK pathways. “Slow flow” vascular malformations are often associated with activating mutations in genes in the PI3K pathway,21,22 whereas “high flow” vascular anomalies, including sporadic arteriovenous malformations of the brain, tend to be associated with activating mutations in genes in the RAS–MAPK pathway.23,26

The finding of relatively low allele frequencies of KRAS variants in sporadic arteriovenous malformations of the brain (0.5 to 4%) is consistent with the cellular heterogeneity of arteriovenous malformations27 and the small fraction of endothelial cells therein and is consistent with the low frequencies of somatic mutations found in other genes in other types of vascular malformation (0.8 to 27%).22,23,25,26 The detection of KRAS mutations in endothelial cell–enriched fractions from primary cultures but not in endothelial cell–depleted fractions from the same cultures suggests that KRAS mutations are probably specific to endothelial cells and that dysregulation of the biology of endothelial cells is a key feature of the formation of arteriovenous malformations of the brain. Moreover, the allele frequencies of the KRAS variants correlated with the percentages of endothelial cells in enriched fractions, which suggests that these variants are present in most endothelial cells isolated from arteriovenous malformations of the brain, arise early in the development of the malformations, and are probably primary events in the pathogenesis of the malformations.

How might alterations in RAS signaling in endothelial cells induce arteriovenous malformations? The mutations that we have identified are known to drive strong and constitutive MAPK–ERK signaling28 and are important drivers of tumorigenesis.29 Arteriovenous malformations of the brain are not associated with cancer, which suggests a context-dependent role for KRAS mutations in the endothelium. The finding that somatic activating KRAS mutations in endometriosis do not cause cancer is consistent with this interpretation.30

Our initial in vitro exploration of endothelial-cell phenotypes after KRAS activation showed increased expression of angiogenic genes such as VEGF/A and genes encoding proteins in the Notch signaling pathway. These transcriptional changes are accompanied by a cellular phenotype that includes enhanced migratory behavior and disassembly of adherens junctions. Previous studies have shown that increased VEGF and Notch signaling are relevant to the development and maintenance of arteriovenous malformations of the brain.31–37 Some of the in vitro phenotypes that we observed could be reverted to normal by means of inhibition of MAPK–ERK signaling. Our finding of increased MAPK–ERK signaling in endothelial cells from arteriovenous malformations of the brain without a KRAS variant suggests that activation of the MAPK–ERK pathway may be a defining feature of arteriovenous malformations of the brain. In the absence of available direct pharmacologic inhibitors of KRAS, small-molecule MEK inhibitors, which are used in clinical practice for treating cancers,38 represent candidates for testing in clinical trials to treat arteriovenous malformations of the brain.
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APPENDIX

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