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Endothelial *MAP2K1* mutations in arteriovenous malformation activate the RAS/MAPK pathway

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ABSTRACT

Arteriovenous malformation (AVM) is a locally destructive congenital vascular anomaly caused by somatic mutations in *MAP2K1*. The mutation is isolated to endothelial cells (ECs). The purpose of this study was to determine the effects of mutant *MAP2K1* on EC signaling and vascular network formation. Pathway effects were studied using both mutant *MAP2K1* (K57N) human AVM tissue and human umbilical vein endothelial cells (HUVECs) engineered to overexpress the *MAP2K1* (K57N) mutation. Western blot was used to determine cell signaling along the RAS/MAPK pathway. Geltrex tube formation assays were performed to assess EC vascular network formation. Cells were treated with a *MAP2K1* inhibitor (Trametinib) to determine its effect on signaling and vascular tube formation. Human mutant *MAP2K1*-AVM ECs had similar baseline *MEK1* and *ERK1/2* expression with controls; however, mutant *MAP2K1*-AVM ECs produced significantly more phosphorylated *ERK1/2* than wild-type ECs. Mutant *MAP2K1* HUVECs demonstrated significantly more phosphorylated *ERK1/2* than control HUVECs. Trametinib reduced the phosphorylation of *ERK1/2* in mutant cells and prevented the ability of ECs to form vascular networks. AVM *MAP2K1* mutations activate RAS/MAPK signaling in ECs. ERK activation and vascular network formation are reduced with Trametinib. Pharmacotherapy using *MAP2K1* inhibitors may prevent the formation or progression of AVMs.

1. Introduction

Arteriovenous malformation (AVM) is a congenital vascular anomaly consisting of abnormal connections between arteries and veins through either a fistula or nidus instead of a normal capillary bed. Extracranial lesions enlarge and cause deformity, pain, ulceration, bleeding, and occasionally heart failure. We previously showed that most sporadic extracranial AVMs contain a mutation in *MAP2K1* that is isolated to the endothelial cell (EC) [1]. *MAP2K1* mutations in non-ECs may cause neoplasms (melanoma, lung, hematopoietic) and can increase MEK1 activity [2–11]. The effects of *MAP2K1* mutations on EC function and AVM formation, however, are unknown. The purpose of this study was to determine if the most common AVM *MAP2K1* mutation, *MAP2K1*–K57N, influences EC signaling and whether or not a MEK inhibitor affects mutant cells. Understanding how *MAP2K1* mutations alter EC biology will provide insight into mechanisms by which AVMs form and enlarge.

2. Materials and methods

2.1. Specimen collection

The Committee on Clinical Investigation approved this study and informed consent was obtained. AVM tissue was collected during a clinically-indicated procedure and processed to separate ECs from non-ECs as we have previously described [1]. AVM specimen was washed in PBS to remove blood cell contaminants, digested with collagenase A (2.5 mg/mL) (Roche) for 1 h at 37 °C, then filtered through a 100 μm strainer to produce a single cell suspension. Cells were placed on fibronectin-coated (1 μg/cm^2) tissue culture plates (Olympus Plastics) in endothelial growth medium-2 (EGM-2, PromoCell) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies). After 5–7 days of expansion, cells were fractionated into 2 populations (endothelial and non-endothelial) using anti-human CD31 (endothelial cell marker) magnetic beads (DynaBeads™, Life Technologies). DNA was extracted from each cell population using the DNeasy Blood & Tissue kit (Qiagen) and the mutant allele frequency (MAF) was determined using ddPCR as previously described [1]. CD31$^+$ ECs

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and CD31$^-$ non-ECs were grown in endothelial cell growth medium (PromoCell) and mesenchymal stem cell growth medium (Lonza), respectively.

2.2. Plasmids

A pReceiver-M14 plasmid containing the human *MAP2K1* ORF was obtained from Genecopoeia (Cat# EX-A0826-M14). The K57N mutation was introduced into the *MAP2K1* ORF using the Agilent QuikChange II XL site directed mutagenesis kit (Cat# 200521) (FP: 5′-ctttcttacccagaatcagaaggtgggagaac-3' RP: 5′-gttctcccaccttctgattctgggtaagaaag-3′). pLVX-puro-Ires-zsGreen1 lentiviral overexpression plasmids for wild-type and K57N *MAP2K1* were generated using Infusion HD (Takara) in combination with a high fidelity DNA polymerase (Clone Amp, Takara). 3 PCR fragments were generated: a pLVX-Puro backbone (FP: 5′-gtcgacggtaccgcgggcccgggatc-3'; RP: 5′-tgcagaattcgaagcttgagctcg-3'; template pLVX-Puro (Takara), a *MAP2K1* ORF (FP: 5′-gcttcgaattctgcatccaaaatgcccaagaagaagccgacgcccatc-3'; RP: 5′-gagaggggttagacgccagcagcatgggttg-3'; template: pReceiver-M14-MAP2K1 (wild-type or K57N)) and an IRES-zsGreen1 reporter ORF (FP: 5′-cgtctaacccctctccctcccccccccctaac-3'; RP: 5'-cgcggtaccgtcgactcagggcaaggcggagccggag-3'; template: pLVX-IRES-zsGreen1 (Takara)). The pLVX-puro-zsGreen1 empty vector control was generated with Infusion HD merging 2 PCR fragments: a pLVX-Puro backbone (FP: 5′-gaattctgcagtcgacggtaccgcg-3′ RP: 5′-gaattcgaagcttgagctcgagatc-3'; template pLVX-Puro) and an IRES-zsGreen1 reporter ORF (FP: 5′-ctcaagcttcgaattcccccctctccctcccccccccctaac-3'; RP: 5′-gtcgactgcagaattctcagggcaaggcggagccggag-3'; template: pLVX-IRES-zsGreen1 (Takara). Lentiviral stocks were generated by the Massachusetts General Hospital Viral Vector Core.

2.3. Cell lines

Endothelial colony forming cells (ECFCs) were isolated from human white adipose tissue as previously described [12]. ECFCs were cultured in fibronectin-coated flasks or plates maintained in EGM2 complete medium (EGM2 + endothelial growth supplement (PromoCell) + 20% FBS). Human umbilical vein endothelial cells (HUVECs) were obtained from ThermoFisher Scientific (Cat #C01510C) and were cultured in fibronectin-coated flasks or plates and maintained in EGM2 complete medium. The HUVEC-pLVX, HUVEC-pLVX-WT and HUVEC-pLVX-K57N cell lines were generated using lentiviral infection. 300,000 HUVECs were plated in one well of a 6-well plate. The next day the medium was replaced with 3 ml of EGM2 complete medium containing 8 μg/ml Hexadimethine Bromide (Sigma). 2.4 μl of lentiviral preparation [2.0×10^9 IU (Infectious Units)/mL] was added to the wells. 20 h later the lentivirus containing medium was replaced and cells were cultured for another 72 h. Selection of infected cells was then performed using EGM2 complete medium containing 2 μg/mL puromycin (Invitrogen). On reaching confluency, HUVECs were passaged to a 25 cm^2 flask, kept under puromycin selection, and allowed to reach confluency. Cells then were collected using 0.25% Trypsin-EDTA and sorted for zsGreen1 expression (Green fluorescent). Cells expressing zsGreen1 were plated in a 25 cm^2 flask, kept in culture under puromycin selection, and expanded to a 75 cm^2 flask. A fraction of the cells then was used to analyze the overexpression of wild type and K57N mutant *MAP2K1*, while the remainder of the cells were stored in liquid nitrogen. These cells were designated passage 1 and were not used later than passage 5 for our experiments.

2.4. Trametinib treatment

Patient-derived AVM *MAP2K1* ECs (MAF of 39%) and *MAP2K1* engineered HUVECs were seeded on fibronectin coated dishes at 10,000 cells/cm^2 in complete growth medium. After 24 h, cells were incubated for 18 h with DMSO (vehicle, 1:1000 in complete growth medium) or Trametinib (SelleckChem) at concentrations of 0.1 μM, 1 μM, or 10 μM. Cells were lysed in the culture dish on ice using mammalian lysis buffer (Promega) containing protease and phosphatase inhibitors (Roche) for 10 min. Experiments were repeated 3 times.

2.5. Western blot analysis

Protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific). 15 μg of protein was separated on 4–20% gradient SDS-PAGE gels (Biorad; cat#: 456–1093). Because the protein components of the *MAP2K1* signaling pathway (MEK1, ERK, P-ERK) and the loading controls (GAPDH and ACTIN) all have a similar molecular weight, a master loading mix was made for each sample. 20 μl (containing 15 μg protein) of this mastermix was loaded on 4 separate gels. The gels were transferred to a PVDF membrane (Invitrolon 0.45 μm pore; Life Technologies) and each membrane was detected using an antibody against one specific protein. Membranes were blocked in TBST containing 5% non-fat dry milk for 30 min at room temperature. Primary antibodies were diluted in blocking buffer and incubated with the membranes for 1 h at room temperature. Membranes were washed 3 times for 5 min each with TBST buffer. Alkaline phosphate-coupled secondary antibodies were diluted in blocking buffer and incubated with the membranes for 30 min at room temperature followed by 3 washes with TBST. Membranes then were rinsed two times with water and incubated for 5 min with Tropix CDP star substrate (Applied Biosystems). Immuno-reactive bands were visualized using Hyblot CL autoradiography film (Denville). In order to determine the *MAP2K1* mutation effects on cell signaling, the downstream target of *MAP2K1* (ERK) was targeted. Activation of *ERK* was determined by its phosphorylation (P-ERK). Primary antibodies used were: anti-p44/42-ERK1/2 (Cell Signaling: #9102; 1/1000); anti-Phospho-p44/42 ERK1/2 (Cell Signaling: #9101; 1/1000); anti-MEK1 (MAP2K1) (Cell Signaling: 9124; 1/1000); anti-GAPDH (Cell signaling: #5174; 1/1000), and anti-beta-ACTIN (Sigma Aldrich: #A1978; 1/15,000). Secondary antibodies included: Goat anti-Rabbit IgG (H + L)-AP conjugated (Invitrogen: #31340; 1/10,000) and Goat anti-Mouse IgG (H + L)-AP conjugated (Invitrogen: #31320; 1/10,000). Western blots were repeated a minimum of 3 times.

2.6. Endothelial cell tube formation assay

Tube formation assays were performed using GelTrex (ThermoFisher Scientific, cat#: A1413202). GelTrex was thawed on ice and wells of a 24-well plate were coated with 300 μl GelTrex matrix and heated to 37 °C for 30 min to allow the GelTrex to solidify. HUVECs then were plated in EGM2 complete medium on the surface of the GelTrex matrix at a density of 9000 cells/well (500 μl of an 18,000 cells/ml stock). Cells were placed in a 37 °C CO_2 incubator and the formation of a cell tube network was analyzed after 16 h using an inverted microscope. GelTrex assays were done in triplicate.

2.7. Statistical analysis

Computation was performed used VassarStats [13]. Mean and standard deviation for pERK densitometry data was calculated.

Groups were compared using the Mann-Whitney *U* test. Statistical significance was defined as a p-value < 0.05.

3. Results

Human AVM ECs were obtained from a patient who underwent resection of an AVM of the hand which had a confirmed *MAP2K1*–K57N mutation in ECs (MAF = 39%, passage 5). We found that *MAP2K1*–K57N mutant ECs do not have a proliferative advantage because continuous cultivation revealed a loss of mutant ECs, and by passage 10 mutant ECs were lost from the cultures. Thus, we only used cells up to passage 5 for our experiments.

Because it is not possible to obtain ECs from a non-affected part of a patient undergoing resection of an AVM, we used human white adipose tissue (HWAT) extracted ECFCs as a control. These cells display a stable endothelial phenotype and have robust in vivo blood vessel-forming capacity [12]. Western blot analysis showed that *MAP2K1*–K57N AVM ECs had similar baseline MEK1 and ERK1/2 expression as compared to control HWAT-ECFCs. However, *MAP2K1*–K57N AVM ECs had a significant increase in the levels of phosphorylated and thus active ERK1/2 protein compared to ECFCs (mean increase 622% ± 244%, $p < 0.05$) (Fig. 1).

To independently verify that the higher P-ERK levels in mutant AVM-ECs were the result of the *MAP2K1*–K57N mutation, we used lentiviral infection to overexpress either wild-type *MAP2K1* or K57N mutant *MAP2K1* in HUVECs. HUVECs infected with the empty lentiviral vector were used as control cells. Western blot analysis showed a moderate increase in MAP2K1 protein levels in cells infected with either wild-type or mutant *MAP2K1* overexpressing vectors. While P-ERK levels in cells overexpressing wild type *MAP2K1* were only slightly upregulated, a strong increase in P-ERK proteins levels was found in HUVECs overexpressing *MAP2K1*–K57N compared to both empty vector (mean increase 426% ± 164%, $p < 0.05$) and overexpressing wild-type (mean increase 345% ± 135%, $p < 0.05$) confirming the results obtained in patient derived AVM-ECs.

The FDA approved MEK1 (MAP2K1) inhibitor Trametinib has been used to treat neoplasms that contain activating *MAP2K1* mutations [14] and has been used off-label to treat AVMs [15]. To investigate whether Trametinib was able to counteract the increased phosphorylated ERK1/2 protein level in mutant cells, we exposed AVM derived ECs to increasing concentrations of Trametinib (Fig. 2). A Trametinib concentration of 0.1 μM was able to reduce the P-ERK level in AVM ECs to the level of the control ECFCs.

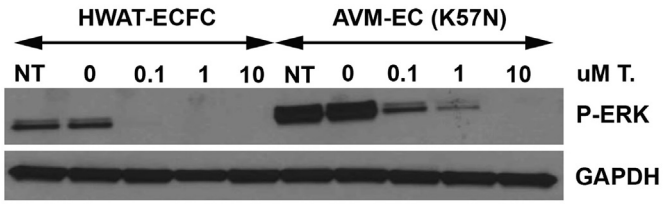

Fig. 2. Western blot shows that a *MAP2K1* inhibitor (Trametinib) reduces P-ERK activation in both ECFCs and human mutant *MAP2K1* AVM ECs (K57N) in a dose-dependent manner. Note that elevated doses are necessary to inhibit the upregulated ERK signaling in mutant cells.

Finally, we tested whether Trametinib could influence vessel formation. Exposure of both AVM-derived ECs and *MAP2K1*–K57N overexpressing HUVECs to Trametinib reduced the ability of mutant ECs to form vascular networks (Fig. 3).

4. Discussion

Our data show that the most common AVM mutation, *MAP2K1*–K57N, over-activates the RAS/MAPK signaling pathway in ECs. This finding is consistent with previous reports that the *MAP2K1* mutation is activating in other cell types that cause cancer [8,9], and when overexpressed in HEK293T embryonic kidney cells [11]. MAPK signaling is enhanced by receptor tyrosine kinases, integrins, and G-protein coupled receptors; MAP2K1 phosphorylates ERK1 and ERK2 [16].

In mammals, this cascade plays a crucial role in development, including fate determination, differentiation, proliferation, survival, migration, growth and apoptosis [17–19]. Interestingly, we have consistently found that prolonged cultivation of AVM derived ECs lowers the MAF in the cultures. This suggests that mutant ECs do not have a proliferative advantage despite their increased RAS/MAP2K1 signaling. We hypothesize that stimulation of RAS/MAPK signaling by mutant ECs might lead to abnormal coordination of artery-capillary-vein formation. The fundamental pathological finding in AVMs is the connection of arteries to veins through a nidus or fistula instead of a normal capillary bed [20]. *MAP2K1* mutant ECs affecting normal vascular development might be the initiating stimulus that causes the pathological connection of arteries to veins. Absence of capillaries in AVMs reduces oxygen delivery to tissues leading to ischemia, ulceration, bleeding, and pain. Reactive neovascularization then contributes to enlargement of the

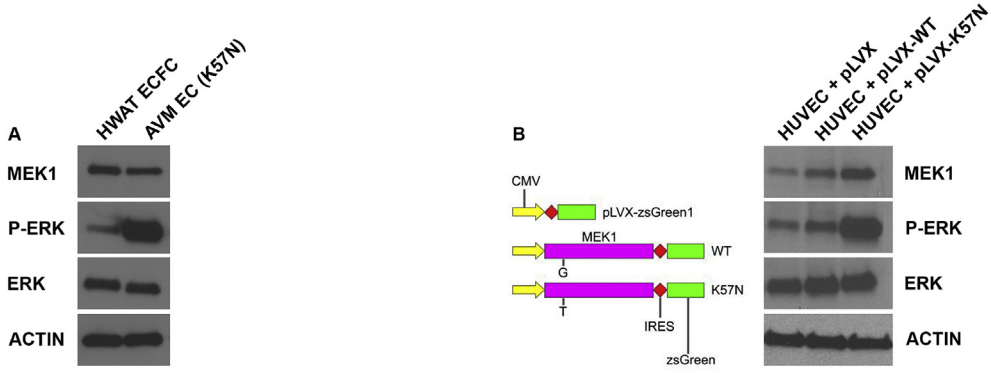

Fig. 1. The *MAP2K1* K57N mutation increases ERK1/2 phosphorylation in endothelial cells (ECs). (**A**) Western blot illustrates MEK, ERK, and P-ERK protein levels in control cells (HWAT ECFCs) and AVM ECs containing a *MAP2K1* K57N mutation. Note the higher levels of phosphorylated ERK1/2 in the mutant ECs. (**B**) (*Left*) Schematic of lentiviral plasmids used to generate engineered HUVECs that overexpress either wild-type or *MAP2K1* K57N mutant protein. (*Right*) Western blot depicting MEK1, ERK, and P-ERK expression in lentiviral infected HUVECs. Higher expression levels of P-ERK protein are detected in HUVECs infected with K57N *MAP2K1* overexpressing lentivirus compared to HUVECs infected with empty (pLVX) lentivirus or wild-type *MAP2K1* overexpressing lentivirus (WT).

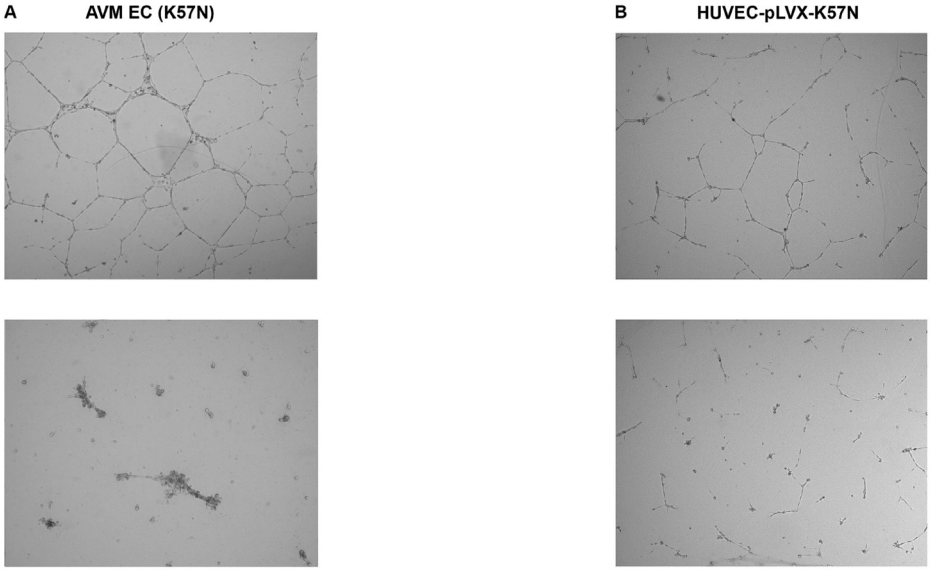

Fig. 3. Trametinib inhibits *MAP2K1* mutant endothelial cell vascular network formation. (**A**) GelTrex assay using mutant human AVM ECs containing the *MAP2K1*−K57N mutation. (*Above*) Vascular network formation without treatment. (*Below*) Failed network development when treated with Trametinib (10 μM). (**B**) GelTrex assay using lentiviral infected HUVECs with the *MAP2K1*−K57N mutation. (*Above*) Vascular network formation in the absence of treatment. (*Below*) Inhibition of network formation in the presence of Trametinib (10 μM).

AVM.

Our finding that Trametinib blocks mutant *MAP2K1* upregulated signaling suggests that it might prove effective for AVMs, similar to its role in treating *MAP2K1* dependent neoplasms (i.e., melanoma, lung adenocarcinoma) [21,22]. Trametinib also stopped vascular network formation. *MAP2K1* inhibition of upregulated EC signaling might prevent the formation and progression of AVMs; regression of lesions also might occur. This hypothesis is supported by a recent case report showing reduction in the size of an AVM treated with Trametinib [15].

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Patent application filed for discovery of MAP2K1 mutation in AVMs (AKG).

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