Arteriovenous Malformation

MAP2K1 Mutation Causes Local Cartilage Overgrowth by a Cell-Non Autonomous Mechanism

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Extracranial arteriovenous malformation (AVM) is most commonly caused by MAP2K1 mutations in the endothelial cell. The purpose of this study was to determine if local tissue overgrowth associated with AVM is caused by direct or indirect effects of the MAP2K1 mutation (i.e., cell-autonomous or cell-non autonomous). Because cartilage does not have blood vessels, we studied ear AVMs to determine if overgrown cartilage contained AVM-causing mutations. Cartilage was separated from its surrounding tissue and isolated by laser capture microdissection. Droplet digital PCR (ddPCR) was used to identify MAP2K1 mutations. MAP2K1 (p.K57N) variants were present in the tissue adjacent to the cartilage (mutant allele frequency (MAF) 6–8%), and were enriched in endothelial cells (MAF 51%) compared to non-endothelial cells (MAF 0%). MAP2K1 mutations were not identified in the overgrown cartilage, and thus local cartilage overgrowth likely results from the effects of adjacent mutant blood vessels (i.e., cell-non autonomous).

Arteriovenous malformation (AVM) is a fast-flow, congenital vascular anomaly that is locally destructive and characterized by abnormal connections between arteries and veins. Most lesions are sporadic and enlarge to cause significant morbidity. AVMs often involve multiple tissue planes and cause overgrowth of skin, bone, muscle, and cartilage1,2. Treatment includes embolization or resection; drugs for AVM do not exist. We previously reported that extracranial AVMs contain somatic mutations in MAP2K1 that are isolated to endothelial cells (ECs)3. The mechanism by which AVMs cause overgrowth of involved tissues is unknown. The purpose of this study was to determine if tissue overgrowth associated with AVM is caused by direct or indirect effects of a MAP2K1 mutation (i.e., cell-autonomous or cell-non autonomous). Understanding the mechanism by which AVMs enlarge may lead to the development of pharmacotherapy for patients.

Results

Three patients had an auricular AVM causing enlargement of all structures of the ear: Patient 1 (11 year-old male), Patient 2 (18 year-old female), Patient 3 (21 year-old male) (Fig. 1). MAP2K1 (p.K57N) mutations were found in the tissue adjacent to the cartilage (i.e., skin and subcutaneous adipose) in all patients; the mutant allele frequency (MAF) was 6–8% (Table 1, Fig. 2). The mutation was enriched in ECs (MAF 51%) compared to non-ECs (0%). A MAP2K1 mutation was not identified in 1 cartilage specimen, and the other 2 cartilage specimens had a MAF of 0.2% and 0.1% that we considered background noise4. Overgrown AVM ear cartilage in all 3 patients appeared similar to normal ear cartilage histologically. No difference was found between proteoglycan, elastin, type 6 collagen, or type 2 collagen; the cartilage also contained the same chondrocyte and extracellular matrix density and relationship (Fig. 3).
Discussion

Somatic mutations for many types of vascular anomalies recently have been described. However, the mechanism by which these mutations cause vascular anomalies and contribute to their enlargement remains unknown. Extracranial AVM progresses over time and causes overgrowth of tissues, including skin, subcutis, muscle, cartilage, and bone. We previously have shown that extracranial AVMs contain somatic MAP2K1 mutations that are only contained in endothelial cells. Because AVMs involving the ear are associated with significant cartilage overgrowth and cartilage does not contain vasculature, we studied this clinical scenario to gain insight into the pathophysiology of AVMs.

Our data shows that only the vascularized tissue adjacent to cartilage of auricular AVM contains somatic MAP2K1 mutations; the underlying overgrown cartilage does not. Consequently, the enlargement of cartilage does not result directly from a mutation in the cartilage (cell-autonomous). Instead, cartilage hypertrophy occurs secondarily to its surrounding soft tissue containing a vasculature with mutant MAP2K1 endothelial cells (cell-non autonomous).

The histological appearance of the AVM cartilage was no different than normal cartilage. This finding may occur because AVM and cartilage enlargement occurs slowly over many years. Since the overgrowth is gradual, the chondrocyte and extracellular matrix density and protein expression may have time to equilibrate and appear similar to normal cartilage. One potential hypothesis to explain AVM-associated cartilage overgrowth could be paracrine effects from neighboring mutant cells (e.g., secretion of growth factors). Another possibility might be that increased pressure of adjacent tissues on the cartilage causes enlargement. The local vascular environment also may influence the overgrowth of cartilage. Increased blood flow and/or local ischemia causing reactive neo-vascularization may promote enlargement. However, capillary malformation, venous malformation, and lymphatic malformations, which do not exhibit increased arterial flow or shunting/ischemia, also cause underlying cartilage and bony hypertrophy.

In contrast to AVM, which is present at birth and slowly enlarges over time, overgrowth conditions that are fully penetrant at birth and do not exhibit significant postnatal enlargement contain somatic mutations in all tissues. For example, facial infiltrating lipomatosis and CLOVES syndrome (congenital lipomatous overgrowth with vascular, epidermal, and skeletal anomalies) are caused by somatic PIK3CA mutations and exhibit overgrowth of every tissue (i.e., skin, subcutis, muscle, bone, cartilage). These conditions contain PIK3CA mutations in multiple cell types and in all overgrown tissues. Consequently, these disorders likely result from a mutation in a multipotent progenitor cell (cell-autonomous) that contributes to mesodermal derivatives (e.g. stroma, adipose, muscle, bone) as well as to ectodermal structures (e.g., nerves).
We predict that other major vascular malformations (capillary, lymphatic, venous) cause overgrowth of tissues by a similar cell-non-autonomous mechanism as AVM. Causative GNAQ mutations in capillary malformations, like AVM, are enriched in endothelial cells and capillary malformation also results in overgrowth of soft-tissue, cartilage, and bone over time. A dynamic interaction between mutant endothelial cells and other cell types is likely a more favorable paradigm for the development of pharmacotherapy compared to overgrowth conditions such as facial infiltrating lipomatosis which are static and contain mutations in all cell types. Drugs might be able to prevent the progression of AVM and other vascular malformations by blocking overgrowth of tissues (e.g., cartilage) that do not contain a somatic mutation.

Methods
This study was approved by the Committee on Clinical Investigation at Boston Children's Hospital, and the procedures followed were in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 2008. Written informed consent was obtained from participants prior to inclusion in the study. Because auricular AVMs cause overgrowth of the cartilage, and ear cartilage does not have blood vessels containing endothelial
cells that can possess a somatic MAP2K1 mutation, we chose this unique anatomical site to study. We obtained cartilage and its overlying skin and subcutis during a clinically-induced otoplasty procedure to improve the appearance of 3 patients with an AVM causing a prominent ear deformity. The cartilage was separated from its overlying tissue (i.e., skin, subcutis) and in one patient ECs were separated from non-ECs. Because MAP2K1 mutations are known to be enriched in ECs, cartilage was subjected to laser capture microdissection to minimize inclusion of adjacent microscopic tissue that might contain blood vessels with a MAP2K1 mutation. Genomic DNA was extracted from cartilage, skin/subcutaneous tissue, and isolated cells and tested for mutant MAP2K1 alleles using droplet digital PCR (ddPCR).

Cell isolation was performed as previously described. Ear tissue was washed in PBS to remove blood cell contaminants, digested with collagenase A (2.5 mg/mL) (Roche) for 1 hour at 37°C, then filtered through a 100 µm strainer to produce a single cell suspension. Cells were placed on fibronectin-coated (1 µg/cm²) tissue culture plates (Olympus Plastics) in endothelial growth medium-2 (EGM-2, Lonza) 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 (Dynabead, Life Technologies). DNA was extracted from each cell population using the DNaseasy Blood & Tissue kit following the manufacturer’s instructions (Qiagen).

Cartilage from resected AVM tissue was frozen in embedding medium (Sakura), cut into 7 µm sections, and placed on membrane glass slides (ThermoFisher). To distinguish cartilage from non-cartilage tissue microscopically, slides were fixed in 10% formalin and stained with Alcin Blue for the cartilage specific proteoglycan aggrecan and Nuclear Fast Red (Sigma). Using a Zeiss PALM Combi Laser Capture Microscope cartilage was isolated from adjacent non-cartilage tissue. DNA extraction was performed using an Agencourt FormaPure DNA kit (Beckman Coulter) without deparaffinization. PCR primers, fluorescent probes, and methods for detecting wild-type and mutant MAP2K1 alleles have been previously described. For each ddPCR reaction we used 45 nanograms of template DNA, corresponding to ~6000 cells. ddPCR was performed using a QX200 Droplet Generator, QX200 Droplet Reader, and QuantaSoft Software (Bio-Rad).

Additional AVM cartilage sections were digested with chondroitinase ABC (Sigma Aldrich) for one hour at 37°C in a humid chamber, blocked for one hour at room temperature, incubated with primary antibody (1:200 dilution in blocking buffer) overnight at 4°C, and incubated with biotinylated secondary antibodies as well as fluorophore conjugated streptavidin at room temperature for 1 hour and 45 minutes respectively. Primary antibodies used were specific for Aggrecan (Sigma Aldrich AB1031), Collagen Type II (Sigma-Aldrich MAB8887), Elastin (Abcam ab21610), and Collagen Type VI (Abcam ab6588). Other sections were stained with hematoxylin and eosin. Histology of AVM ear conchal cartilage was compared to normal ear conchal cartilage from a control patient who had ear cartilage removed as part of a cosmetic otoplasty procedure. Images were obtained at 20X magnification using Nikon Eclipse 80i microscope with SPOT RT3 camera and SPOT 5.2 software.

Data availability
All data generated or analyzed during this study are included in this published article.

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Competing interests
The authors declare no competing interests.

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