Identification of a rare BMP pathway mutation in a non-syndromic human brain arteriovenous malformation via exome sequencing

Brain arteriovenous malformations (AVMs) are abnormal connections between arteries and veins that can result in hemorrhagic stroke. A genetic basis for AVMs is suspected, and we investigated potential mutations in a 14-year-old girl who developed a recurrent brain AVM. Whole-exome sequencing (WES) of AVM lesion tissue and blood was performed accompanied by in silico modeling, protein expression observation in lesion tissue and zebrafish modeling. A stop-gain mutation (c.C739T:p.R247X) in the gene SMAD family member 9 (SMAD9) was discovered. In the human brain tissue, immunofluorescent staining demonstrated a vascular predominance of SMAD9 at the protein level. Vascular SMAD9 was markedly reduced in AVM peri-nidal blood vessels, which was accompanied by a decrease in phosphorylated SMAD4, a downstream effector protein of the bone morphogenetic protein signaling pathway. Zebrafish modeling (Tg kdr:eGFP) of the morpholino splice site and translation-blocking knockdown of SMAD9 resulted in abnormal cerebral artery-to-vein connections with morphologic similarities to human AVMs. Orthogonal trajectories of evidence established a relationship between the candidate mutation discovered in SMAD9 via WES and the clinical phenotype. Replication in similar rare cases of recurrent AVM, or even more broadly sporadic AVM, may be informative in building a more comprehensive understanding of AVM pathogenesis.
Written informed consent to participate in this study was obtained, and the study was approved by the institutional review board at the REDACTED. The clinical course and features of the patient are highlighted in Figure 1. In brief, she suffered a hemorrhagic stroke from a brain AVM at age 12 and underwent surgical AVM resection. Two years later, she was observed to have local regrowth of the AVM that was previously completely removed, and a second surgery was performed. The patient had no other known health problems. DNA from tissue obtained at the time of the second surgery was used for WES. Sequencing was performed on the Illumina HiSeq2000 platform using an Illumina Nextera Rapid Capture Exome Kit (Illumina, San Diego, CA, USA).

Table 1. Capture methods and coverage summary of exome data

| Capture method                      | Blood          | AVM            |
|-------------------------------------|----------------|----------------|
| Mean coverage                       | 91.36          | 89.03          |
| % Covered > 10×                      | 96.2           | 95.5           |
| % Covered > 20×                      | 93.3           | 92             |
| % Covered > 30×                      | 88.5           | 86.6           |
| % Zero coverage targets             | 1.9            | 2              |

Abbreviation: AVM, arteriovenous malformation.
DNA obtained from formalin-fixed, paraffin-embedded tissue and whole blood.

Sequences were aligned to NCBI human reference GRCh37 using the Burrows-Wheeler Aligner. The aligned reads were converted to SAM format (Sequence Alignment/Map) and sorted. PCR duplicates were then removed and alignments were recalibrated. Single-nucleotide variant and insertion/deletion calling was performed using HaplotypeCaller from the Genome Analysis Toolkit, and the output was in the gVCF file format. Variant annotation was performed using the ANNOVAR program with references to GRCh37/hg19, dbSNP version 132, 1000 Genomes project (1KGP; 2012 data release), 69 Complete Genomics (2012 update) and exome variant server with ~ 6,500 exomes (NHLBI-ESP project, 2013 update).

Somatic variants were filtered out from changes observed in matched normal DNA to rule out artifacts and generate a list of true somatic and germline variants. The sequencing quality was determined by Genome Analysis Toolkit’s DepthOfCoverage Walker. Quality control steps excluded variants with a sequencing depth < 10 or with genotype quality < 90, those that were found in a selection of frequently mutated genes, and pseudo-genes, as well as those in repetitive regions of genes.

The rare nonsense mutation in SMAD9 (c.C739T:p.R247X, rs553369182) was then confirmed with Sanger sequencing with the following primer sequences: 5’-GTGGTACAGTCACATTCTACACA-3’ and 5’-ACATCTCAGGTGTGCTAGTGAAAT-3’ (Elim Biopharmaceuticals, Hayward, CA, USA).

Within the Exome Aggregation Consortium database, the minor allele frequency was reported at 0.00002 (n = 3). To estimate the damaging character of the stop mutation, we used MutationTaster and Combined Annotation Dependent Depletion. Evolutionary conservation was estimated using genome conservation scores measured by PhyloP.

Immunofluorescence staining
On the basis of the disease-causing prediction, we investigated the role of the SMAD9 mutation in altering protein expression within the BMP signaling pathway. To determine the effect of the mutation on translation,
immunofluorescence staining of AVM samples removed from the patient was used to qualitatively assess both the localization and expression of protein products. Control samples were obtained from samples of a normal human temporal lobe neocortex that were removed as part of a routine protocol for temporal lobe epilepsy. Once isolated, the frozen brain and AVM samples were embedded in an optimal cutting temperature compound (Tissue-Tek, Torrance, CA, USA). Embedded tissues were cryosectioned at a thickness of \(14 \mu m\) with a microtome and subsequently fixed with immersion in ice-cold acetone on positively charged glass slides in preparation for staining. All tissue sections were stored at \(-20^\circ C\) and rehydrated with phosphate-buffered saline on the day of staining. Sections were blocked in 10% swine serum (Vector Laboratories, Burlingame, CA, USA) and incubated overnight in primary antibody at \(4^\circ C\) with the following antibodies: sheep anti-human SMAD9 antibody (R&D systems, Minneapolis, MN, USA); and rabbit anti-human phospho SMAD4 Thr 277 (Thermo Scientific, Waltham, MA, USA). To detect brain endothelial cells, immunofluorescence staining of AVM samples removed from the patient was used to qualitatively assess both the localization and expression of protein products. Control samples were obtained from samples of a normal human temporal lobe neocortex that were removed as part of a routine protocol for temporal lobe epilepsy. Once isolated, the frozen brain and AVM samples were embedded in an optimal cutting temperature compound (Tissue-Tek, Torrance, CA, USA). Embedded tissues were cryosectioned at a thickness of \(14 \mu m\) with a microtome and subsequently fixed with immersion in ice-cold acetone on positively charged glass slides in preparation for staining. All tissue sections were stored at \(-20^\circ C\) and rehydrated with phosphate-buffered saline on the day of staining. Sections were blocked in 10% swine serum (Vector Laboratories, Burlingame, CA, USA) and incubated overnight in primary antibody at \(4^\circ C\) with the following antibodies: sheep anti-human SMAD9 antibody (R&D systems, Minneapolis, MN, USA); and rabbit anti-human phospho SMAD4 Thr 277 (Thermo Scientific, Waltham, MA, USA). To detect brain endothelial cells,
sections were also incubated overnight at 4°C with biotinylated Ulex Europaeus Agglutinin I lectin (Vector Laboratories). Sections were subsequently washed in phosphate-buffered saline with 0.05% Triton X-100 and incubated for 1 h at room temperature with the appropriate secondary antibody. To visualize SMAD9, phospho-SMAD4 and brain endothelial cells, sections were incubated with a Cy3-conjugated donkey anti-sheep antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), Alexa Fluor 488-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch) and DyLight 649-conjugated streptavidin (Vector Laboratories). Following immunodetection, sections were incubated in 1% Sudan Black B (Sigma Aldrich, St. Louis, MO, USA) to quench autofluorescence and counterstained with Hoechst nuclear counterstaining. Sections were mounted with fluorescent mounting media (Dako, Carpinteria, CA, USA) and coverslipped. All imaging was performed with a Leica TCS SP5X confocal microscope, and representative images were prepared with NIH U.S. National Institutes of Health, Bethesda, MD, USA, ImageJ software.

Figure 3. Effect of the SMAD9 mutation on downstream pathway protein phosphorylated SMAD4. Immunohistochemical staining was performed to determine the localization and expression of p-SMAD4 in both control (a, d, g) and lesion (b, c, e, f) tissue. Similar to SMAD9 staining, there was prominent expression of p-SMAD4 in control tissue (a) that co-localized (arrows) to vascular structures identified by lectin staining of the endothelium (b) and Hoechst nuclear counterstaining (c). Decreased expression of p-SMAD4 was observed in arteriovenous malformation tissue, and its expression appeared to be dissociated from vascular structures.

RESULTS

Identification of a rare SMAD9 mutation

WES was performed on DNA extracted from the patient’s brain AVM tissue and circulating leukocytes from peripheral blood (Table 1). Nine potential loss-of-function variants from nine genes were found in both germline and somatic DNA of the patient, which included four single-nucleotide variants and five insertions/deletions (Table 2). Six of the genes were involved in the immune-response or tumor development, two of which had unknown function. Among these, the most interesting was a rare single-nucleotide variant at chr13:37439827, introducing a stop codon in SMAD9 (NM_005905:exon4:c.C739T:p.R247X; NM_001127217: exon5:c.C850T:p.R284X) (Figure 1). The total coverage of the germline and somatic mutations at this location were 129- and 112-fold with mutation frequencies 0.57 and 0.5, respectively. Both genotype quality scores were 99. This mutation was then confirmed by Sanger sequencing. A battery of in silico testing with MutationTaster predicted it to be a disease-causing mutation with a probability of 1, indicating a high “security” of the prediction.
Evolutionary conservation of the amino-acid sequence was high, as estimated by the PhyloP score, which ranged from 2.128 to 4.188 between different transcripts (Table 3). The score generated by the Combined Annotation Dependent Depletion tool was 40, which suggested a strong deleterious effect. Analysis of somatic variations in this patient (insertion-deletion mutation, missense mutation, nonsense mutation and loss of heterozygosity) did not demonstrate any significant changes in genes associated with vascular development (Supplementary Information 1–3).

Cellular localization and reductions of SMAD9 in the peri-nidal brain AVM vasculature

Immunofluorescent staining in control brain tissue revealed prominent SMAD9 expression in the vasculature, including both endothelial and peri-vascular vascular mural cells (Figure 2).

SMAD9 expression was found far less in surrounding neuronal and glial parenchymal cells (data not shown). In the peri-nidal AVM vessels, SMAD9 protein expression was markedly reduced. To demonstrate whether this may impact downstream signal transduction, we next performed immunofluorescent analysis of phosphorylated-SMAD4. In control tissues, there was a ubiquitous and robust phosphorylated-SMAD4 signal in multiple cell types, including vascular cell types (endothelial cells and perivascular mural cells), glia and neurons (Figure 3). In the brain AVM specimen, however, the vascular phosphorylated SMAD4 was nearly absent, with gross reductions also observed in the surrounding parenchyma.

Collectively, these data suggest that AVM-specific reductions in SMAD9 may influence downstream signal transduction, as seen by reductions in phosphorylated SMAD4.

In vivo SMAD9 knockdown results in aberrant vasculogenesis and brain AVMs

To further investigate whether reductions in SMAD9 contribute to brain AVM formation in vivo, we next utilized morpholino knockdown in transgenic zebrafish expressing green fluorescent protein in endothelial cells (Tg(kdr:GFP)). Following morpholino injection, embryos at 68–72 h.p.f. were systematically analyzed for both gross developmental abnormalities, as well as for vascular abnormalities. Overall, the SMAD9 morphants demonstrated slightly smaller heads, smaller eyes and thin, elongated trunks. There was a prominent amount of cardiac edema. Trunk...
circulation was markedly reduced or absent. Although the cardinal vein was often present (albeit faintly visible with fluorescence), the caudal vein plexus was markedly reduced (Figure 4).

The vascular phenotype of the SMAD9 morphant was typified by both developmental abnormalities in the trunk vessels and cranial circulation. There was abnormal intersegmental vessel formation, with ectopic branches above the myoseptum near the dorsal longitudinal anastomotic vessel. Within cranial circulation, there were abnormal arteriovenous connections between the dorsal longitudinal vein, mesencephalic vein and metencephalic artery (Figure 4). There was also a paucity of central arteries that penetrate the hindbrain and usually connect the basilar artery and posterior hindbrain channel. The lateral dorsal aorta was also significantly dilated and irregular (Figures 5 and 6).

**DISCUSSION**

Sporadic brain AVMs are a rare disease, and lesions that recur following complete removal are exceedingly rare. These unique cases provide an opportunity to evaluate the suspected genetic basis of their pathogenesis, which shares a vascular phenotype with syndromic forms of AVM, such as hereditary hemorrhagic telangiectasia. In this report, WES was used to survey the genetic landscape of sporadic brain AVM in an unbiased manner, and a candidate causative mutation in a sporadic AVM was discovered in SMAD9. The nature of the discovery was further investigated by tissue-level protein expression studies, in silico modeling and in vivo vertebrate modeling.

By drawing on the vast experiences of genetic variation in humans represented in the 1000 Genomes Project and others, we were able to establish the rarity of this mutation, which was only observed in 3 alleles out of 121,314 (minor allele frequency = 2.473e-05) in the Exome Aggregation Consortium database v0.3.1. This establishes that the discovered variant is not generally present in healthy humans. While no genotype-phenotype relationship has been previously established with this variant, the majority of brain AVMs are clinically silent unless they produce symptoms from hemorrhage. It is plausible that the three individuals with this rare variant unknowingly harbored occult brain AVMs. Without neuroimaging, it is not possible to exclude the presence of clinically silent AVM. In addition, it is possible that other low-frequency mutations, other than in SMAD9, existed in

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**Figure 5.** Wild-type control imaging of zebrafish cranial circulation. Reference confocal source dataset images of cranial circulation in a representative uninjected Tg(kdrl:eGFP) zebrafish embryo at 72 h post fertilization.
the studied patient, but were not detected because of limitations of the sequencing depth and cellular heterogeneity of the samples sequenced (resulting in potential false negatives). As other alterations in the BMP signaling pathway have been implicated in genetic AVM syndromes, we hypothesized that altered SMAD9 expression could alter downstream signal transduction, resulting in abnormal brain vasculogenesis and/or vascular remodeling. As SMAD9 is routinely phosphorylated and subsequently associates with SMAD4 to translocate to the nucleus, we sought to identify changes in phosphorylated SMAD4 as an indicator of BMP signaling pathway inhibition, as demonstrated by a potential reduction in downstream target activation. Surgical specimens allowed for a unique, direct qualitative observation of the decrease in downstream protein expression. The profound decrease in protein expression despite only a heterozygous loss is not clear, but may be related to a dominant negative effect of nonsense-mediated mRNA decay escape or a truncated protein.

In addition, we provided evidence of the pathogenic effect of this mutation by creating a vertebrate model (zebrafish). While morpholino knockdown of SMAD9 has been conducted using previously validated oligomer sequences, this report is the first to describe the effects of SMAD9 knockdown on vascular development. Zebrafish have traditionally been used as model organisms because of the ease with which their gene expression can be manipulated combined with their high fecundity and the short time-course in which phenotypes can be established. For vascular disease, in particular, the translucent nature of embryos, along with transgenic endothelial cell reporter lines (such as kdr:eGFP), allows for real-time visualization of vascular development from the single-cell stage onward. As vascular development in zebrafish embryos follows a defined, stereotypic anatomical pattern, aberrations in these steps secondary to gene product knockdown can be readily visualized in vivo. Indeed, genetic manipulation in zebrafish that targets an ortholog...
of a known genetic cause of human AVM (ACVRL1) creates a model of cranial arteriovenous shunting.\textsuperscript{35,36}

Not surprisingly, the SMAD9 morphant in the presented experiments has pleiotropic manifestations on overall organism morphology and extracranial vasculature, which reflects the essential role of the BMP pathway in development.\textsuperscript{37} However, these morphants develop abnormal cranial artery-to-vein connections that are strikingly reminiscent of human AVMs, more so than some other models. It is possible that targeting downstream target genes in the canonical BMP signaling pathway to compensate for deficiencies, thereby displaying a more robust AVM phenotype. From a wider perspective, other mutations in SMAD9 have primarily been associated with a rare heritable form of pulmonary hypertension resulting from a heterozygous deletion (OMIM 191600)\textsuperscript{38,39} although the apparent lack of complete penetrance in some of these cases further complicates the interpretation of the genotype–phenotype relationship. Patients with these mutations have also demonstrated a reduction in expression of downstream target genes in the canonical BMP signaling pathway.\textsuperscript{40} The index patient presented here demonstrated no signs or symptoms of pulmonary dysfunction. The heterogeneity of phenotypes resulting from various truncating mutations in SMAD9 in humans, either associated with brain AVMs or with hyperplasia of pulmonary artery smooth muscle cells, may relate to variable microRNA processing or an inability to form various protein products toimerize with SMAD4.\textsuperscript{40–43}

CONCLUSION

_In silico_ modeling, protein expression in lesion tissue and zebrafish modeling provide orthogonal trajectories of evidence that establish a relationship between the candidate gene mutation discovered in SMAD9 via WES and the clinical phenotype. Replication in similar rare cases of recurrent AVM, or even more broadly sporadic AVM, may be informative in building a more comprehensive understanding of AVM pathogenesis.

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COMPETING INTERESTS

The authors declare no conflict of interest.

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