Somatic Variants in SVIL in Cerebral Aneurysms

Pui Man Rosalind Lai, MD,* Jee-Yeon Ryu, PhD,* Sang-Cheol Park, PhD,* Bradley A. Gross, MD, Lawrence D. Dickinson, MD, Sarajune Dagen, BS, Mohammad Ali Aziz-Sultan, MD, MBA, Alan S. Boulous, MD, Daniel L. Barrow, MD, H. Hunt Batjer, MD, Spiros Blackburn, MD, Edward F. Chang, MD, P. Roc Chen, MD, Geoffrey P. Colby, MD, PhD, Garth Rees Cosgrove, MD, Carlos A. David, MD, Arthur L. Day, MD, Kai U. Frerichs, MD, Mika Niemela, MD, PhD, Steven G. Ojemann, MD, Nirav J. Patel, MD, MA, Xiangen Shi, MD, PhD, Edison P. Valle-Giler, MD, Anthony C. Wang, MD, Babu G. Welch, MD, Edie E. Zusman, MD, Scott T. Weiss, MD, MS, and Rose Du, MD, PhD, on behalf of GENIE Investigators

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Abstract

Background and Objectives
While somatic mutations have been well-studied in cancer, their roles in other complex traits are much less understood. Our goal is to identify somatic variants that may contribute to the formation of saccular cerebral aneurysms.

Methods
We performed whole-exome sequencing on aneurysm tissues and paired peripheral blood. RNA sequencing and the CRISPR/Cas9 system were then used to perform functional validation of our results.

Results
Somatic variants involved in supervillin (SVIL) or its regulation were found in 17% of aneurysm tissues. In the presence of a mutation in the SVIL gene, the expression level of SVIL was downregulated in the aneurysm tissue compared with normal control vessels. Downstream signaling pathways that were induced by knockdown of SVIL via the CRISPR/Cas9 system in vascular smooth muscle cells (vSMCs) were determined by evaluating changes in gene expression and protein kinase phosphorylation. We found that SVIL regulated the phenotypic modulation of vSMCs to the synthetic phenotype via Krüppel-like factor 4 and platelet-derived growth factor and affected cell migration of vSMCs via the RhoA/ROCK pathway.

Discussion
We propose that somatic variants form a novel mechanism for the development of cerebral aneurysms. Specifically, somatic variants in SVIL result in the phenotypic modulation of vSMCs, which increases the susceptibility to aneurysm formation. This finding suggests a new avenue for the therapeutic intervention and prevention of cerebral aneurysms.

*These authors contributed equally to this work.

From the Department of Neurosurgery (P.M.R.L., J.-Y.R., S.-C.P., S.D., M.A.A.-S., G.R.C., K.U.F., N.J.P., R.D.), Brigham and Women's Hospital, Harvard Medical School, Boston, MA; Artificial Intelligence and Robotics Laboratory (S.-C.P.), Myongji Hospital, Goyang, Korea; Department of Neurosurgery (B.A.G.), University of Pittsburgh, PA; Department of Neurosurgery (L.D.D., E.E.Z.), Sutter Health, Danville, CA; Department of Neurosurgery (A.S.B.), Albany Medical Center, NY; Department of Neurosurgery (D.L.B.), Emory University, Atlanta, GA; Department of Neurosurgery (H.H.B., B.G.W.), University of Texas Southwestern, Dallas, TX; Department of Neurosurgery (S.B., P.R.C., A.L.D.), University of Texas Health Science Center, Houston; Department of Neurosurgery (E.F.C.), University of California San Francisco, CA; Department of Neurosurgery (G.P.C., A.C.W.), University of California Los Angeles; Department of Neurosurgery (C.A.D.), Lahey Hospital and Medical Center, Burlington, MA; Department of Neurosurgery (M.N.), Helsinki University and Helsinki University Hospital, Finland; Department of Neurosurgery (S.G.O.), University of Colorado, Denver; Department of Neurosurgery (K.S.), Affiliated Fuxing Hospital, Capital Medical University, Beijing, China; Department of Neurosurgery (E.P.V.-G.), Ochsner Medical Center, New Orleans, LA; and Channing Division of Network Medicine (S.T.W., R.D.), Brigham and Women’s Hospital, Harvard Medical School, Boston, MA.

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GENIE Investigators are listed in the appendix at the end of the article.

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Cerebral aneurysms are present in 1%–3% of the general population and cause significant morbidity and mortality when they rupture. The development of an aneurysm is thought to be multifactorial with interactions between environmental risk factors and predisposing genes. Known risk factors for the development of cerebral aneurysm include hypertension, smoking, increasing age, female sex, and family history. Cerebral aneurysms often carry a familial occurrence, although no inheritance pattern has been identified to date. Genome-wide association studies have identified polymorphisms in several genes, including SOX17 (8q11.23), and CDKN2A-CDKN2BAS (9p21.3-23.1), to be associated with aneurysms. These genes have been implicated in mediating inflammatory pathways, vascular remodeling, and cell adhesion. However, despite the success of the genome-wide association studies in identifying the common polymorphisms, which explain more than half of the disease heritability of 41% based on twin studies, there remains a significant role for nonheritable causes. While somatic mutations are largely studied in the context of neoplasms, a small number of studies have examined somatic mutations in vascular diseases including fusiform aneurysms and arteriovenous malformations. Given the significant proportion of nonheritability and the mostly sporadic nature of saccular cerebral aneurysms, we hypothesized that somatic variants in the intracerebral arteries contribute to the formation of saccular cerebral aneurysms.

Methods

Patient Selection

This study has been conducted in accordance with the ethical standards and has been approved by the Institutional Review Boards at the Brigham and Women’s Hospital (BWH), Albany Medical Center, Beijing Sanbo Brain Hospital, Emory University, Helsinki University Hospital, Lahey Hospital and Medical Center, Ochsner Medical Center, Sutter Health, University of California at Los Angeles, University of California at San Francisco, University of Colorado, University of Texas Health Sciences Center, and University of Texas Southwestern. Informed consent was obtained from all patients. Samples were de-identified and sent to the coordinating center (BWH) where they were processed. Tissues from intracranial aneurysms from 30 patients were obtained during surgical clipping of the aneurysms. Paired blood samples from each patient were also collected. Clinical data including age, sex, race, smoking history, and a history of hypertension were obtained. This study was performed according to the Strengthening the Reporting of Genetic Association Studies guidelines.

Whole-Exome Sequencing

Tissue samples were placed immediately in RNA later (Ambion) and stored at −80°C until DNA extraction. DNA was extracted from tissue samples using the AllPrep DNA/RNA kit (Qiagen) according to the manufacturer’s protocol. DNA was extracted from peripheral blood leukocytes using the AutoGenFlex Star system (Autogen, Holliston, MA) with the AutoGenFlex STAR FlexiGene Reagent kit (Autogen) and the AutoGenFlex Blood DNA Finishing kit (Autogen).

Whole-exome sequencing was performed at the Broad Institute (Cambridge, MA) on paired aneurysm and blood samples. Libraries for whole-exome sequencing were constructed as previously described. Libraries were sequenced on Illumina HiSeq 4000 instrument with 151-bp paired-end reads.

Somatic Variant Calling

Alignment was performed with the Burrows-Wheeler Aligner using the hg19 reference genome. Samples were then processed with the bcbio-nextgen v0.9.7 pipeline following best practices for exome sequencing. The minimum read length for mapping was set to 25. MarkDuplicates was performed on the aligned BAM file. After the mapping, we used ensemble calling for genomic variants with 5 variant callers (GATK v3.5.0/Mutect2, Mutect v1.1.7, Freebayes v1.0.2, VarDict v2016.02.19, Strelka v2.9.10) on the regions in which the minimum depth of coverage is more than 4. Thirty matched aneurysm blood samples were used where blood samples were used as controls. From the results, we selected variants present in at least 2 of the 5 callers with parameters for each program as follows: (1) Mutect v1.1.7: -U ALLOW_N_CIGAR_READS --read_filter NotPrimaryAlignment; (2) Freebayes: --min-repeat-entropy 1 --min-alternate-fraction 0.1 --pooled-discrete --pooled-continuous --report-genotype-likelihood-max --allele-balance-priors-off. Then, the variants were filtered by bcftools with “ALT = ‘<*>’ || QUAL > 5” options; (3) VarDict: -c 1 -S 2 -E 3 -g 4 -Q 10; (4) Mutect2: -ploidy 1 -U LENIENT_VCF_PROCESSING --read_filter BadCigar --read_filter NotPrimaryAlignment; and (5) Strelka: default settings. Each caller was run with parameters set in bcbio to minimize the likelihood that ambiguous variants such as low variant quality scores and abnormal genotypes would remain. If some variant callers have variants with missing data, those variants were excluded unless the variants were detected by more than 2 other callers.
Multiallelic variants were split into multiple single-allele variants when normalizing vcf files for ensemble calling.

The effects of the sequence variants were annotated using the SnpEff v4.3T. The final set of mutations were analyzed and visualized with Maftools R package v2.2.10. Gene set enrichment analysis was performed for the genes containing somatic variants via functional profiles of Gene Ontology in clusterProfiler R package v3.14.3. The p values were adjusted with the Benjamini-Hochberg method, and false discovery rate <0.1 was considered statistically significant.

RNA Sequencing and Analysis
RNA was extracted from available remaining aneurysm tissue in 10 patients and from 5 normal middle cerebral arteries obtained from patients without known aneurysms who underwent epilepsy surgeries. The details of the data processing and statistical analysis are presented in the eMethods (links.lww.com/NXG/A563).

Preparation of the Cas9-sgRNA Plasmid
Two 20-bp sequences of 6 sgRNA were designed for targeting the SVIL gene. The designed oligonucleotides are summarized in eTable 1 (links.lww.com/NXG/A563). The oligonucleotides were annealed to form double-stranded duplexes for ligation, and the annealed sgRNA oligonucleotides were ligated into precut pCas vectors. The Cas9-sgRNA plasmids were then purified.

Vascular Smooth Muscle Cell Culture
Vascular smooth muscle cells (vSMCs) were grown in smooth muscle cell medium (ScienCell) supplemented with 10% fetal bovine serum, 1% smooth muscle cell growth supplement, and 1% penicillin/streptomycin at 37°C under 5% CO2. vSMCs were seeded into 24-well plates and were transfected with Cas9-sgRNA plasmids (1 μg) using lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer’s protocol. Cells were harvested 24 hours after transfection and analyzed with reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) and Western blot (see eMethods, links.lww.com/NXG/A563).

Phospho-Kinase Array
Total protein (500 μg) was incubated overnight with the Human Phospho-Kinase Array (R&D Systems), which was spotted with antibodies for 43 human kinases. Signal detection was performed according to manufacturer’s instructions. The signal intensity of each spot was determined by ImageJ (the NIH) software.

Statistical Analysis
Statistical analysis was performed by using the student t test to determine statistical significance. p values <0.05 were considered statistically significant. Calculations were performed using Prism (Graphpad Software, San Diego, CA).

Standard Protocol Approvals, Registrations, and Patient Consents
Institutional Review Board approval was obtained for this study. Informed consent was obtained from the patients included.

Data Availability
The Mutation Annotation Format file for all 30 patients is given in Supplemental Data, links.lww.com/NXG/A563.

Results

Patient Demographics
Table 1 summarizes the patient demographics. The mean age for the cohort is 54 ± 13 years, with a 70% female predominance. The most common location of the aneurysm is the middle cerebral artery (N = 12), followed by anterior communicating artery (N = 7) and posterior communicating artery (N = 6).

Identification of Somatic Variants
Whole-exome sequencing was performed on tissue samples and on matched blood samples from 30 patients. The average target read coverage was 85x (range, 65–106x) for all samples, 88x (range, 72–106x) for blood samples, and 82x (range, 65–98x) for aneurysm samples. A total of 411 nonsilent mutations within exon regions were found in 374 genes. Figure 1 shows the breakdown of variants and the type of

| Characteristic | N (%) |
|---------------|-------|
| Age - median (range) | 53a (30–84) |
| Female sex—no. (%) | 21 (70) |
| Race—n/N (%) | |
| White | 22 (73) |
| Black or African American | 3 (10) |
| Hispanic | 4 (13) |
| Asian | 1 (4) |
| Smoking | |
| Never | 12 (40) |
| Past or current | 18 (60) |
| Location of aneurysm—no./total no. (%) | |
| Middle cerebral artery | 12 (40) |
| Anterior communicating artery | 7 (23) |
| Posterior communicating artery | 6 (20) |
| Other | 5 (17) |
| Ruptured | 18 (60) |

* Median.
mutations in the patients. The most common somatic mutations were missense mutations, followed by in-frame deletions, frame shift deletions, and nonsense mutations. On average, there were 13.7 mutations per patient (median 10.5, range 3–37). The highest number of somatic variants were found on chromosome 1 (42 variants), followed by chromosome 2 (30 variants) and chromosome 4 (27 variants).

Somatic variants in the top 25 genes accounted for 27 (90%) of the mutation-positive patients (Figure 1). The most common variant was p.Leu1374Arg (rs199726033), a missense somatic mutation found in 4 patients (13%) in the supervillin gene (SVIL) that is predicted by SIFT to be deleterious and by Polyphen to be possibly damaging. The average allele frequency of rs199726033 was 0.1424, and the variant was found only in White female patients. When comparing the allele frequencies of this variant at known allele frequencies in The Genome Aggregation Database (gnomAD v2.1.1), the frequencies were higher in patients with aneurysms than in European population frequencies (allele frequency = 0.0916). One other patient had a synonymous variant, p.Pro2168 = , on SVIL that is predicted by GWAS4D to affect the transcription factor binding site for EP300 and decrease binding with a motif-altering p value of $1.85 \times 10^{-3}$. EP300 is a transcription factor that has been shown to be involved in vascular smooth muscle cell (vSMC) phenotypic switching and has been shown to acetylate Krüppel-like factor 4 (KLF4), which increases its transactivation function. There were therefore 5 patients (17%) with variants related to SVIL. Variants in isthmin 2, lysine methyltransferase 2c, myelin transcription factor 1–like, nuclear receptor corepressor 1, and parathyroid hormone 2 were observed in 3 patients each.

**GO Enrichment Analysis**

To understand the overall significance of the mutations and possible functional module involvement, we performed a gene ontology (GO) enrichment analysis of the genes with variants (Table 2). We found that mutations in categories including the vascular endothelial growth factor (VEGF)–activated receptor activity, VEGF binding, DNA-binding transcription activator

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**Figure 1 Somatic Variants From Whole-Exome Sequencing**

(A) The top 25 genes with somatic variants observed in more than 2 samples from aneurysm tissues in 30 patients. The gene, SVIL, was found to have the highest number of somatic mutations (N = 4 missense mutations, N = 1 synonymous mutation not shown) in the transcription factor binding site for EP300 on SVIL. (B) Variant classification and single-nucleotide variant class in the patients. There were 9.4 mutations per patient sample (median 7, range 1–29) with the most common variant being a missense mutation. (C) Overall distribution of 6 different conversions of all variants. SVIL = supervillin.

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activation, RNA polymerase II–specific, solute:cation antiporter activity, and actin binding were identified as enriched in the molecular function domain (Figure 2A). Figure 2B shows genes belonging to each GO term. Genes belonging to these 5 dysregulated modules were found in 80% of patients. No significant GO term was found in the cellular components and biological processes domains.

**Phenotypic Switching of vSMCs in Saccular Cerebral Aneurysms**

To examine the role of phenotypic modulation of smooth muscle cells in cerebral aneurysms, we used RNA sequencing results to examine differential expression between aneurysms and control arteries of the markers associated with phenotypic modulation (Figure 3). Previous studies have shown that synthetic vSMCs have a decreased expression of the contractile markers associated with the contractile phenotype, whereas MMP2 interacts with newly formed extracellular matrix and contributes to the migration of vSMCs. PDGF-BB, which is a soluble protein, is reported to act as an environmental factor that regulates vSMC phenotype.

We found that there was a significantly decreased expression of α-SMA and SM22α and a significantly increased expression of MMP2 and MMP9 in aneurysm tissue relative to control vessels indicative of a phenotypic switch to the synthetic phenotype, but no significant changes in KLF4 or PDGF-B expression.

**SVIL Induces Phenotypic Switching in Vascular Smooth Muscle Cells**

To detect whether the somatic variants in *SVIL* were associated with gene expression changes in aneurysm tissue, mRNA level of the gene was measured in patients with variants in *SVIL* and in control vessels (Figure 4A). It was observed that mRNA levels were significantly decreased in all aneurysm tissues with SVIL mutations, compared with middle cerebral artery controls. In all patients in the aneurysm group, the expression level was less than 30% of that for the control group.

The CRISPR/Cas9 system was used to test the effects of the SVIL knockdown in vSMCs. To determine gene editing efficiency of various sequences, we designed 6 SVIL sgRNA sequences and cloned SpCas9 vectors (eFigure 1A and eTable 1, links.lww.com/NXG/A563). The sgRNA 5 sequence was used to edit SVIL in this study because SVIL mRNA level decreased to the lowest level in this sequence compared with other sequence candidates (eFigure 1B). The target site was PCR amplified, and the T7E1 cleavage assay was performed (eFigure 1C). Cleavage bands indicated that the SVIL loci were modified in the transfected vSMCs. We also confirmed a significant decrease in SVIL mRNA and protein levels in vSMCs with SVIL knockdown compared with controls with scramble vectors using RT-qPCR and Western blot (Figure 4B).

We next confirmed whether SVIL induces the expression of phenotypic switching marker genes in vSMCs (Figure 4C). The phenotypic switch in vSMCs from a contractile state to a synthetic state is considered to play an important mechanism in the remodeling and maintenance of the arteries. We found that SVIL knockdown in vSMCs significantly elevated the mRNA and protein of KLF4, PDGF, MMP2, MMP9, α-SMA, and SM22α, which were confirmed by RT-qPCR and Western blot (Figure 4C). The elevation in KLF4, PDGF, MMP2, and MMP9 are consistent with the phenotypic switch to the synthetic phenotype, which is associated with an increased migration and proliferation.
sgRNA–treated cells, we used the Human Phospho-Kinase Array to profile specific phosphorylation of 43 human kinases in SVIL sgRNA–treated cells (eFigure 2A, links.lww.com/NXG/A563). It is well-known that phosphorylation of p53 at Ser46 transactivates apoptosis-related genes24 and increases the affinity of p53 for KLF4.25 It has also been shown that although KLF4 typically induces SMC dedifferentiation to the synthetic phenotype, it has the opposite effect of inhibiting SMC proliferation when cooperating with p53.26 We showed that SVIL deficiency leads to decreased phosphorylation of p53 at Ser46 (eFigure 2B), which would result in loss of the inhibitory function of KLF4 on SMC proliferation. This shows that SVIL promotes proliferation via the p53/KLF4 pathway. In addition, phosphorylation of proline-rich Akt substrate of 40 kDa (PRAS40) at Thr246 disinhibits mTORC1, which promotes cell proliferation27 and activates MMP2 and MMP9.28 We showed that SVIL deficiency leads to an increased phosphorylation of PRAS40 at Thr246 (eFigure 2C). This suggests that SVIL activates MMP2 and MMP9 via PRAS40. Finally, heat shock protein 27 (HSP27) regulates the rearrangement of actin polymerization.
SVIL sgRNA–treated cells were observed to increase phosphorylation of HSP27 (eFigure 2D). Our results indicate that an increased PDGF may increase phosphorylation of HSP27 to effect the increased migration of vSMCs by actin polymerization.

Effects of SVIL on Migration of Vascular Smooth Muscle Cells

Two cytoskeleton-associated proteins, cofillin and HSP27, are known to control actin polymerization and enhance migration in smooth muscle cells. The phosphorylation of HSP27 suggested the possibility that SVIL affects the cellular migration of vSMCs via actin polymerization. Because cofillin is downstream of the RhoA signaling pathway, we investigated whether SVIL affected the migration in vSMCs through the RhoA signaling pathway. mRNA levels of RhoA and downstream effectors of RhoA such as Rho-associated, coiled-coil-containing protein kinase (ROCK) were measured in vSMCs (eFigure 3, links.lww.com/NXG/A563). We found that SVIL knockdown did not affect the mRNA level of RhoA, whereas the mRNA level of ROCK was significantly increased in SVIL-suppressed vSMCs compared with those in controls. We next found that SVIL knockdown in vSMCs significantly elevated the proteins for ROCK and LIM kinase (LIMK) which were confirmed by Western blot (Figure 5A). ROCK can directly activate LIMK, which leads to the phosphorylation of cofillin resulting in filament stabilization and increased migration. We therefore investigated the changes in F-actin microfilaments in vSMCs using confocal laser scanning microscopy (Figure 5B). The F-actin in SVIL sgRNA–treated cells was increased compared with scrambled sgRNA-transfected cells, which served as the control. SVIL sgRNA–treated cells also had a larger cell area than the control cells. These results suggest that SVIL has distinct morphological effects on the vSMCs. We also confirmed that migration was significantly increased in SVIL sgRNA–treated cells via the wound healing assay (Figure 5C). We next evaluated whether the effects of ROCK were dependent on SVIL. We treated scrambled sgRNA-treated vSMCs or SVIL sgRNA–treated vSMCs with Y27632, an inhibitor of ROCK. Y27632 leads to decreased protein levels of ROCK and LIMK in SVIL-suppressed vSMCs (Figure 5A), which shows that SVIL is an upstream regulator of ROCK. We confirmed a significant decrease in F-actin filaments in SVIL sgRNA–treated cells with ROCK inhibitor compared with those in controls with only SVIL sgRNA–treated cells. Y27632 induced dramatic changes in cell shape (Figure 5D). The cell migration rate of the cells was also reduced by Y27632 (Figure 5E). Our results show that SVIL deficiency may lead to the migration of vSMCs by the upregulation of ROCK, and downregulation of ROCK inhibits the ability to migrate. In summary, our results indicate that SVIL affects the phenotypic switching of vSMCs to the synthetic phenotype with increased cell proliferation and migration via the p53/KLF4, PRAS40/MMP2, MMP9, PDGF/HSP27, and RhoA/ROCK pathways.
Discussion

While somatic variants have been extensively studied in the context of cancers, few studies have been conducted to examine the role of somatic variants in other disease processes. In this study, we found sporadic mutations in SVIL in 17% of cerebral aneurysm tissue and demonstrated the role of SVIL in the modulation of vSMC phenotype.

SVIL is a gene on chromosome 7 that encodes a 205-kDa actin-binding protein that binds to myosin II and F-actin in cells and plays a role in the regulation of cell-cell adhesion and extracellular matrix adhesion, as well as in the cytoarchitecture of plasma membrane in various cell types. However, its role in vSMCs in general, and in cerebral aneurysms particularly, is unknown. The phenotypic switching of vSMCs has been implicated in a number of vascular lesions including aortic aneurysms, atherosclerosis, and hypertension. We therefore investigated the changes in markers of phenotypic switching in cerebral aneurysms and determined the role of SVIL in the phenotypic switching of vSMCs by using the CRISPR/Cas9 system to knockdown SVIL.

(A) The mRNA levels of SVIL in aneurysm tissues where somatic mutations were found in the SVIL gene compared with control middle cerebral artery by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR). (B) The mRNA and protein levels of SVIL using SVIL sgRNA (sequence S) by RT-qPCR and Western blot. (C) Comparison of mRNA and protein levels of markers related to phenotypic switching after SVIL sgRNA delivery by RT-qPCR and Western blot. Error bars represent means ± SD. (n = 3, *p < 0.05, **p < 0.01, ***p < 0.001). SVIL = supervillin.
Similar to aortic aneurysms and atherosclerosis, we found that MMP2 and MMP9 are elevated in cerebral aneurysms, while α-SMA and SM22α are decreased, indicating that phenotypic switching is critical in the formation of cerebral aneurysms. Although it has been shown that KLF4 has a key role in the phenotypic switching of vSMCs in aortic aneurysms and that PDGF-B is upregulated in dissecting thoracic aneurysms and abdominal aortic aneurysms, we found levels of KLF4 and PDGF-B to be unchanged in cerebral aneurysms compared with those in control intracranial vessels.

With the suppression of SVIL, we found changes in vSMCs that are indicative of phenotypic switching, including increased KLF4, PDGF, MMP2, and MMP9. However, unlike mature cerebral aneurysms and vSMCs in aortic aneurysms and atherosclerosis, α-SMA and SM22α are increased rather than decreased. Environmental factors may play a role in this difference. Shear stress decreases the expression of α-SMA and SM22α, which may explain the decreased expression in aneurysm tissue but not in the vSMCs in vitro. Our results indicate that suppression of SVIL alone is not sufficient to decrease the expression of α-SMA and SM22α but creates a vulnerable genetic profile, which may require additional environmental factors such as shear stress for the disease phenotype to manifest.

The increase in KLF4 and PDGF in SVIL-suppressed vSMCs and the unchanged KLF4 and PDGF in aneurysm tissue suggest that there may be a temporal component to the phenotypic switching of vSMCs. It has been shown that in atherosclerosis, the phenotypic modulation of vSMCs away from the contractile phenotype shifts over time with a continuous change in gene expression rather than an abrupt change from one phenotype to another. A study of young and old mice with aortic aneurysms had demonstrated a time-dependent modulation in phenotype where markers such as Mmp2 were expressed in the early stages of disease while other markers such as Tnfrsf11b do not appear until later in older mice. Given that KLF4 and PDGF are involved in proliferation while MMPs are involved in migration and the degradation of the extracellular matrix and vascular wall, these results suggest that proliferation via the KLF4 and PDGF pathways play a less important role in a mature aneurysm with already differentiated vSMCs.
We found that suppression of **SVIL** leads to an increased expression of ROCK, which regulates F-actin filaments and increases the migration rate of the vSMCs. We also confirmed the recovery of the RhoA/ROCK signal and migration of vSMCs with the suppression of ROCK by Y27632. ROCK is known to change the morphology of cells through the formation of actin filaments.\(^{42}\) However, the effects of the **SVIL** on actin cytoskeleton with RhoA/ROCK signaling in vSMCs remains unknown. Our results indicate that the cooperation between **SVIL** and ROCK is required to regulate cell migration. ROCK inhibitors may therefore be a potential new therapy in the treatment of intracranial aneurysms.

**GO Enrichment Analysis**

Gene set enrichment analysis was performed to determine whether the somatic variants in aneurysm tissue are enriched in biologically relevant functions. We found 5 statistically significant GO sets, which account for variants in 80% of patients (N = 24), the majority of which are consistent with what was previously known regarding aneurysms. VEGF is a family of protein growth factors important in angiogenesis and vasculogenesis and has been implicated to play a role in cerebral aneurysm wall remodeling where its expression level is increased in aneurysm wall and in serum in patients who harbor intracranial aneurysms.\(^ {43}\) VEGF induces collagenases, causing endothelial changes by the breakdown of the tunica media and extracellular matrix, accelerating the degradation of this layer and the progression of aneurysm formation.\(^ {43}\) Polymorphism in the VEGF gene has also been implicated to play a role in the development of intracranial aneurysms.\(^ {44}\) This suggests that somatic variants that involve VEGF binding and activated receptor activity may play a role in the pathogenesis of cerebral aneurysms by downstream signaling changes. Another significant gene set was actin binding, which includes **SVIL**. This family of proteins has been proposed to regulate actin filament growth, stability, and disassembly.\(^ {45}\) Because this family can affect vascular smooth muscle cell plasticity by regulating cytoskeleton structure, our result implies that their dysfunction can lead to blood vessels that are more vulnerable to hemodynamic stress, making them more prone to intracranial aneurysms. The other 2 modules, DNA-binding transcription activator activity RNA polymerase II-specific and solute:cation antiporter activity, are composed of many subfunctions, so it is difficult to determine their direct relationship to aneurysms. Our pathway enrichment results imply that somatic genetic variations altering gene function result in downstream effects associated with vascular remodeling, angiogenesis, cellular adhesion, and actin binding. These variants may drive local progression of aneurysms not observed in other normal vascular tissue and explain variations not previously identified by genome-wide association studies.

**Relationship to Environmental Factors**

The association between aneurysms and environmental factors such as smoking and hypertension has been previously demonstrated.\(^ {46,47}\) Smoking has been shown to be associated with somatic mutations in bronchial epithelial cells, which are thought to be related to the development of lung cancer.\(^ {48}\) Although we did not find an association between smoking or hypertension with the top somatic variants (data not shown), it is possible that the somatic mutations seen are related to smoking and/or hypertension. Nevertheless, this does not change our findings regarding the somatic variants and the formation of aneurysms.

The main limitation to our study is the small number of participants, although to date, this is the largest representative cohort for analysis of whole-exome sequencing comparison of aneurysm tissue and matched blood samples. Because of the small sample size, we could not examine the risk of rupture. Because of the generally minute size of the aneurysm tissue samples, we did not have additional aneurysm tissue available for further confirmation with Sanger sequencing. However, we have used the ensemble approach to variant calling to increase the accuracy of the whole exome sequencing, although more stringent thresholds for filtering low-quality variants could be applied.\(^ {49}\) Moreover, it has been shown that whole exome sequencing has a high concordance rate of >99% with Sanger sequencing.\(^ {50}\) Because somatic variants have not been well-studied in complex traits outside of cancer where they are major determinants of cancer pathogenesis and treatment response, it is possible that these variants are the secondary result of some underlying pathophysiologic process in the arterial wall rather than causal. However, we have shown that suppression of **SVIL** leads to the phenotypic modulation of vSMCs, suggesting that the variants in **SVIL** are likely causal. But, as discussed earlier, **SVIL** would not be the sole factor involved in the pathogenesis of aneurysms but a contributor. Other contributors, including other somatic variants found, would merit further study. Furthermore, exome sequencing would not have included all possible regulatory variations, although it provides a more targeted approach to identifying potential protein coding somatic changes. In addition, using control vessels from patients with epilepsy is not ideal because this patient population may harbor genetic disorders. However, this would only bias our results toward the null. Finally, we have focused our attention on **SVIL** in vSMCs. There may be important contributions from the other 2 major vascular cell types, endothelial cells and fibroblasts, which will require further study.

In conclusion, the comparison of cerebral aneurysm and matched serum samples allow for the identification of potential driver somatic mutations of cerebral aneurysms. The most common somatic variants occurred in the **SVIL** gene, which we have shown to contribute to the pathogenesis of cerebral aneurysms via phenotypic modulation of vascular smooth muscle cells. We have demonstrated functionally relevant somatic variants in a large proportion of saccular cerebral aneurysms. Because somatic variants are typically implicated in the pathogenesis of neoplasms rather than vascular diseases, these findings change the fundamental way
in which the pathogenesis of saccular cerebral aneurysms is viewed. Future studies using animal models will be needed to further confirm these findings and to find other pathways involved in the pathogenesis of aneurysms. The results of these studies may provide candidates for a disease-modifying therapy for the noninvasive treatment and prevention of saccular cerebral aneurysms.

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Appendix (continued)

| Name                  | Location                                                               | Contribution                                                                 |
|-----------------------|------------------------------------------------------------------------|------------------------------------------------------------------------------|
| Lawrence D. Dickinson, MD | Department of Neurosurgery, Sutter Health, Danville, CA               | Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data |
| Sarajune Dagen, BS     | Department of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA | Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data |
| Mohammad Ali Aziz-Sultan, MD, MBA | Department of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA | Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data |
| Alan S. Boulos, MD     | Department of Neurosurgery, Albany Medical Center, Albany, NY           | Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data |
| Daniel L. Barrow, MD   | Department of Neurosurgery, Emory University, Atlanta, GA               | Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data |
| H. Hunt Batjer, MD     | Department of Neurosurgery, University of Texas Southwestern, Dallas, TX | Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data |
| Spiros Blackburn, MD   | Department of Neurosurgery, University of Texas Health Science Center, Houston, TX | Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data |
| Edward F. Chang, MD    | Department of Neurosurgery, University of California San Francisco, San Francisco, CA | Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data |
| P. Roc Chen, MD        | Department of Neurosurgery, University of Texas Health Science Center, Houston, TX | Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data |
| Geoffrey P. Colby, MD, PhD | Department of Neurosurgery, University of California Los Angeles, CA | Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data |
| Garth Rees Cosgrove, MD | Department of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA | Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data |
| Carlos A. David, MD    | Department of Neurosurgery, Lahey Hospital and Medical Center, Burlington, MA, USA | Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data |

Continued
Appendix (continued)

| Name                          | Location                             | Contribution                                                                 |
|-------------------------------|--------------------------------------|------------------------------------------------------------------------------|
| Arthur L. Day, MD             | Department of Neurosurgery, University of Texas Health Science Center, Houston, TX | Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data |
| Kai U. Freichs, MD            | Department of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA | Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data |
| Mika Niemela, MD, PhD         | Department of Neurosurgery, Helsinki University and Helsinki University Hospital, Finland | Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data |
| Steven G. Ojemann, MD         | Department of Neurosurgery, University of Colorado, Denver, CO | Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data |
| Nirav J. Patel, MD, MA        | Department of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA | Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data |
| Xiangen Shi, MD, PhD          | Department of Neurosurgery, Affiliated Fuxing Hospital, Capital Medical University, Beijing, China | Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data |
| Anthony C. Wang, MD           | Department of Neurosurgery, University of California Los Angeles, CA | Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data |
| Babu G. Welch, MD             | Department of Neurosurgery, University of Texas Southwestern, Dallas, TX | Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data |
| Edie E. Zusman, MD            | Department of Neurosurgery, Sutter Health, Danville, CA | Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data |
| Scott T. Weiss, MD, MS        | Department of Neurosurgery, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA | Drafting/revision of the article for content, including medical writing for content; study concept or design |
| Rose Du, MD, PhD              | Department of Neurosurgery, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA | Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data; study concept or design; and analysis or interpretation of data |

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