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Somatic *PDGFRB* Activating Variants In Fusiform Cerebral Aneurysms

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SUMMARY

The role of somatic genetic variants in the pathogenesis of intracranial aneurysm formation is unknown. We identified a 23-year-old man with progressive right sided intracranial aneurysms, ipsilateral to an impressive cutaneous phenotype. The index individual underwent a series of genetic evaluations for known connective tissue disorders that were unrevealing. Paired sample exome sequencing between blood and fibroblasts derived from the diseased areas detected a single novel variant within the platelet-derived growth factor receptor β gene (*PDGFRB*) juxtamembrane-coding region predicted to cause a p.(Tyr562Cys) [g.149505130T>C (GRCh37/hg19); c.1685A>G] change. Variant allele fractions ranged from 18·75% to 53·33% within histologically abnormal tissue, suggesting post-zygotic or somatic mosaicism. In an independent cohort of aneurysm specimens, we detected somatic activating *PDGFRB* variants in the juxtamembrane domain or kinase activation loop in 4/6 fusiform aneurysms (and 0/38 saccular aneurysms, Fisher's Exact Test p<0·001). *PDGFRB*-variant, but not wild type, patient cells were found to have overactive auto-phosphorylation with downstream activation of ERK, SRC and AKT. Expression of discovered variants demonstrated non-ligand dependent auto-phosphorylation, responsive to the kinase inhibitor sunitinib. Somatic gain-of-function variants in *PDGFRB* are a novel mechanism in the pathophysiology of fusiform cerebral aneurysms and suggest a potential role for targeted therapy with kinase inhibitors.

Intracranial aneurysms occur in approximately 2% of the population with a rupture risk of 6 per 100,000 individual-years [1,2]. There are two types of aneurysms, the more common saccular (90-95%) and fusiform (4-8%)[1,2]. Saccular aneurysms are abnormal arterial outpouchings at branch points with histological loss of the media and intima. Fusiform aneurysms are circumferential abnormal arterial dilatation with histological medial and intimal hyperplasia. The size, location in the cerebrovascular tree and type of aneurysm all influence the natural history of this disease[1,2]. Abundant evidence supports a genetic component to the etiology of intracranial aneurysms[1-7]. The pathogenesis of cerebral aneurysm formation and rupture is complex and involves both environmental[2] and genetic factors defined by twin, linkage and genome wide association studies[3,4,5]. Several genetic syndromes are associated with intracranial aneurysms, conferring increased risk compared to the general population[1-3]. Studies in mono and dizygotic twins also suggest both genetic and environmental contributions[5]. Established environmental risk factors, which may somatically alter coding regions of the genome, include cigarette smoking and hypertension[1,2]. The role of post-zygotic variants of genes that function in critical intracellular signaling pathways has been established for several types of overgrowth syndromes[8,9] and vascular malformations[10-16], but the role of somatic genetic alterations or mosaicism in intracranial aneurysms remains unknown.

The index individual was first treated for a dissecting fusiform paraclinoid internal carotid artery aneurysm at 9 years of age. All individuals' data and specimen collection were reviewed and approved by the University of Washington Institutional Review Board and Human Subjects Division. The individual was noted to have an impressive ipsilateral cutaneous phenotype. Fourteen years later, he developed a giant dissecting fusiform aneurysm of the right vertebral artery, which was previously normal by angiography (Fig. 1A-C and Fig. S1 in the Supplementary

Appendix). He had apparently normal cognition with no neurological deficits or other birth defects. No other abnormalities were found on brain (including intracranial calcifications), cardiovascular, or peripheral vascular imaging. He later developed both radial and coronary artery aneurysms (Fig. S1 J,K in the Supplementary Appendix), but never aortic aneurysm or dissection, underlying his dermal phenotype. Family history was negative. The left neurovascular tree remained normal (Fig. S1 C,E in the Supplementary Appendix). Detailed phenotype information for this individual is shown in Supplementary Appendix Fig. S1. He underwent a series of operations for treatment of the giant, rapidly growing fusiform vertebral aneurysm. DNA was extracted from multiple vascular and perivascular tissue samples (Fig. 1K) obtained during surgery. Initial variant discovery was carried out using paired sample exome sequencing to an average depth of ~150x between blood and fibroblasts derived from the diseased areas (>99% of the exome was covered for all samples). Exome sequencing was performed on blood and abnormal tissue using a customized exome capture probe set from the UW Medicine Center for Precision Diagnostics that is built upon the xGen Exome Research Panel v1.0 (IDT) backbone. Initial variant discovery was carried out using a comparison between blood and diseased area cultured fibroblast exomes sequenced to an average depth of ~150x on the Illumina HiSeq 2500 platform. Subsequent exome sequencing was performed on other diseased specimens and healthy radial artery (Fig. 1K and L) to at least 40x average depth (>99% of exome). Resulting reads were aligned using BWA-MEM (v0.7.5) following the Broad Institute's GATK best practices. Somatic variants were identified using MuTect (v1.1.7) with default parameters. Our analysis detected a single novel variant within the platelet-derived growth factor receptor β gene (*PDGFRB*) juxtamembrane-coding region (p.Tyr562Cys [g.149505130T>C (GRCh37/hg19); c.1685A>G]). Variant allele fractions ranged from 18·75% to 53·33% within histologically abnormal tissue (Fig. 1D-L). No other somatic

variants were found. The highest allele fractions were found in a specimen from an occipital artery aneurysm (Fig. 1D-I and Fig. S2 in the Supplementary Appendix). This *PDGFRB* variant was not found in DNA isolated from blood or histologically normal, left-sided, radial artery (Fig. 1J-L), confirming post-zygotic or somatic mosaicism.

PDGFRB encodes a conserved transmembrane receptor tyrosine kinase involved in diverse signaling processes during embryonal development[17-21]. PDGFRB is normally expressed in several cell types, including pericytes and vascular smooth muscle cells, and has an essential role in vascular progenitor cell signaling[19-21]. Based on the findings in the individual described above, we performed targeted sequencing of *PDGFRB* in a validation cohort of 50 aneurysm and arterial walls (Fig. S3 in the Supplementary Appendix). The validation cohort was sequenced similarly to the exome sequencing performed on the index individual with the exception that a custom capture probe set (IDT) was used rather than the full exome. Variants were batch-identified across the cohort using the Platypus variant caller (v0.8.1), using a minimum variant allele fraction of 2%, a minimum coverage of 5 reads, and a minimum posterior probability of 0 (no variant reads) allowing more inclusive initial analysis. Germline variants and sequencing artifacts were further filtered out with an in-house script. All somatic variants were analyzed with IGV (v2.3.71) and functionally annotated with Oncotator (v1.9.3.0). Targeted sequencing revealed four variants in three additional sporadic individual cases: a variant within the juxtamembrane domain predicted to result in a four amino acid in-frame deletion (p.Tyr562_Arg565del) in exon 12, and two additional variants in the activation loop of the kinase domain (p.Asp850Tyr and p.Arg849_Lys860delinsHisAlaGlyLeuGluLeuHisLeuGln) in exon 18 (Fig. 2A). The latter variant was comprised of two deletions located in *cis* that together are predicted to result in a complex in-frame insertion-deletion (Fig. S6 in the Supplementary Appendix). Variants were only found in

fusiform aneurysms (3/5, 60%), radiographically and histologically similar to our index individual (Fig. 2B-F and Fig. S4 in the Supplementary Appendix). All saccular aneurysms had wild-type *PDGFRB.*

Exome sequencing was performed on aneurysm walls and control tissues from all three *PDGFRB*-variant sporadic fusiform aneurysms (see the Supplementary Appendix)**.** All aneurysm samples were sequenced to at least 175x (>99% of exome) and control samples to at least 90x (>99% of exome) average coverage with the exception of lymph node DNA from the VAL-44 individual. Additional sequencing was added to the *PDGFRB* variant region of the lymph node DNA (non-aneurysm control DNA) with the custom capture probe set in order to study a germline contribution for VAL-44. The aneurysm exome of VAL-44 was analyzed on its own. For every available control tissue, complete pairs were analyzed with a variant using FreeBayes (v1.0.2), Strelka2 (v2.0.17), VarDict (v1.5.1), and VarScan2 (v2.4.3), and the output was filtered with an in-house script and confirmed with manual inspection on IGV. For the VAL-44 aneurysm without a good-quality control, variant calling was done with FreeBayes, Platypus, and Vardict and the output was filtered with an in-house script and confirmed with manual inspection on IGV. Exome sequencing of aneurysm and normal tissue DNA revealed only recurrent *PDGFRB* variants (Fig. S6 and S7 in the Supplementary Appendix), suggesting a causal role in the formation of sporadic fusiform aneurysms. DNA was available from blood and/or unaffected healthy tissue, to explore the germline contribution of the variant in all cases (Fig. S7 in the Supplementary Appendix).

The skewed *PDGFRB* allele fractions, in sporadic fusiform aneurysms, ranged from 5·6 to 21·4% (Fig. 2A) also consistent with post-zygotic, somatic variants. These results were also confirmed by next generation sequencing after independent primer pair amplification across the variant locations, with similar allele fractions (Fig. S5 and S7 in the Supplementary Appendix).

Two of the *PDGFRB* missense variants observed (p.(Tyr562Cys) and p.(Asp850Val)) have recently been found in sporadic myofibromas[22,23]. None of the variants we detected in intracranial aneurysms were seen in >120,000 normal genomes (dbSNP, 1000Genomes, NHLBI-EVS, gnomAD) which support their pathogenicity and suggest that they may be embryonic lethal. These variants altered conserved regions and were predicted to be protein altering and pathogenic by PolyPhen2, SIFT and MutationTaster (Fig. S9 in the Supplementary Appendix).

In fusiform aneurysms, missense variants and in-frame deletions occurred in either the -ArgTyrGluIleArg- motif of the juxtamembrane region or adjacent -AspPheGly- motif in the activation loop (Fig. 3A-C, Fig. S9 in the Supplementary Appendix) of PDGFRB. Disruption of juxtamembrane region auto-inhibitory sites causes constitutive activation[24,27]. All four variants occur in known homologous *PDGFRA* and *KIT* "hot spots" within the juxtamembrane or activation loop of the kinase domains[24,25]. The conserved residues are found in all tyrosine protein kinases, and the analogous residues (Tyr555 and Tyr552) in the PDGFRA and KIT kinases are somatically altered in cancers[24,25] (Fig. 3B). The aneurysm alterations in *PDGFRB* are predicted to result in p.Tyr562Cys and p.Tyr562_Arg565del. Aligned amino-acid sequences of the activation loops of human KIT, PDGFRA and PDGFRB start and end with residues - AspPheGly - and -AlaProGlu-. The two alterations within the kinase loop known to be important for autoregulation[24,25,28,29] were p.(Asp850Tyr) and an in-frame deletion/insertion spanning this region (Fig. 3C). These data suggest that variants found in cerebral aneurysms act via gain-of-function mechanisms. Deep targeted sequencing of the genes coding for the kinases *KRAS, PDGFRA*, *BRAF*, *TGFBR1* and *TGFBR2* identified no variants in the cohort of 50 aneurysms, consistent with an etiology specific to *PDGFRB.*

Several heterozygous germline or mosaic gain-of-function variants in *PDGFRB* result in

infantile myofibromatosis (IM, MIM #228550) or sporadic myofibromas, while single heterozygous germline variants were found in the rare Kosaki overgrowth (MIM #616592) and Penttinen syndromes (MIM #601812) (Fig. 3 and Fig. S10 in the Supplementary Appendix). Several other heterozygous germline loss-of-function variants cause primary familial brain calcification (PFBC, MIM #615007). None of the above phenotypes were found in our four aneurysm individuals (Fig. S10 in the Supplementary Appendix). Individuals with genome copy number alterations, including chromosome 5 deletions encompassing *PDGFRB*, have been associated with developmental delay but not with aneurysm development[26]. Mice deficient in *Pdgfb* or *Pdgfrb* die from multiple developmental defects including hemorrhages due to a lack of pericytes and vascular smooth muscle cells in blood vessels[17-20]. *Pdgfrb* activating mutations in mice cause vascular smooth muscle cell de-differentiation, hyperplasia and increased extracellular matrix synthesis[20]. The wide range of phenotypes suggests a complexity of PDGFRB function and downstream signaling that is likely due to cell lineage and developmental timing specific expression.

To study the functional status of PDGFRB variants, we performed assays using cells collected from skin punches of the index individual (healthy and affected regions) and site-directed mutagenesis was performed using the QuickChange II Site-Directed Mutagenesis Kit (Catalog #*200518,* Agilent Technologies). For details, see the Supplementary Appendix**.** Ligand binding induces PDGFRB dimerization and activating autophosphorylation *in trans*. Phosphorylation on multiple tyrosine residues creates docking sites for signaling proteins, including phosphatidylinositol-3 kinase (PI3K), AKT, STAT transcription factors, and phospholipase Cγ (PLCγ)[27-31]. Starved mosaic fibroblasts harvested from the index individual, predicted to express the Tyr562Cys variant, had higher basal levels of pPDGFRB, pSRC (Tyr416), pAKT (Ser473)

and pERK 1/2 (Thr202/Tyr204) (Fig. 4A, B). PDGFBB was able to further autophosphorylate PDGFRB and activate downstream signaling (Fig. 4B). To investigate the pathogenic mechanism of all discovered variants, we ectopically expressed wild-type (WT), aneurysm-associated variants, and two controls in HEK cells (Fig. 4C). All four aneurysm-associated variants caused higher levels of autophosphorylation of PDGFRB when compared to WT. We next tested the ability of the multi-targeted receptor tyrosine kinase inhibitor sunitinib (Sutent) to down regulate auto-phosphorylation of PDGFRB variants (Fig. 4D). While all intracranial aneurysm variants exhibited relative resistance to sunitinib compared to WT PDGFRB under these conditions, three could be strongly inhibited. In contrast, the p.(Asp850Tyr) variant exhibited marked resistance to sunitinib under these conditions. Interestingly, kinase inhibitor resistance was also reported for p.(Asp850Val), similar to the p.(Asp842Tyr) PDGFRA and p.(Asp816Val) KIT variants in gastrointestinal stromal tumors[24,25].

Exome sequencing of the index individual allowed study of the role of somatic alterations in aneurysm formation. A single post-zygotic, somatic variant in *PDGFRB*, predicted to cause a gain-of-function protein, was discovered. This observation in the index individual provided the proof in principle that alterations in *PDGFRB* might be found in sporadic, non-mosaic, individuals. Here we describe variants of *PDGFRB* as a cause of single and multiple fusiform aneurysms. The role of somatic mosaicism has been described in overgrowth syndromes (*AKT1*[8], *PIK3CA*[9]), Sturge-Weber Syndrome (*GNAQ*[11]), head and neck[12-15] and cerebral arterial-venous malformations (*KRAS*[16]). Following this theme, *PDGFRB* activating variants of the cerebral vasculature seem to drive aneurysm formation. We describe the first reported genetic cause of any type of sporadic intracranial aneurysm: activating variants in *PDGFRB*. We used exome sequencing and targeted deep sequencing to explore the genetic landscape of cerebral aneurysms. A somatic point variant,

predicted to result in a tyrosine to cysteine (p.(Tyr562Cys)) change, within the conserved, auto-inhibitory, juxtamembrane region of PDGFRB was identified. To explore the possibility of additional driving mutations and genetic similarities we performed exome sequencing in all fusiform aneurysms. This confirmed the following: 1. Exome sequencing of *PDGFRB*-variant fusiform aneurysms revealed no additional detectable recurrent gene alterations consistent with a causal relationship, 2. Two of the fusiform aneurysms carried wild type *PDGFRB* and had no evidence of novel driver variant and 3. Exome sequencing of multiple abnormal tissue specimens, from *PDGFRB*-variant patients, revealed a definitive role for somatic mosaicism in two of four individuals.

Activating *PDGFRB* variants underlie sporadic fusiform aneurysms, suggesting an important role in cerebral artery angiogenesis. Four of six fusiform aneurysms carried *PDGFRB* activating variants, highlighting the importance of this pathway. Two fusiform aneurysms did not harbor *PDGFRB* alterations even on deep sequencing, suggesting another gene or mechanism in formation or that ultra-low variant allele fractions were not detected. While our exome sequencing was sensitive enough (150x coverage) to detect *PDGFRB* variants, it is possible that we were unable to detect ultra, low-level variants in other novel contributing genes. Re-sequencing of *PDGFRB*, with on average 400x coverage, in both wild-type fusiform and saccular aneurysms should detect allele frequencies down to 1%. Although we examined pathologic tissue specimens, we concede our techniques would not allow detection of low frequency fractional (<1%) variants with certainty.

Protein kinase activation by somatic variant or chromosomal alteration is a common mechanism of overgrowth syndromes[8,9], vascular malformations[10-16] as well as tumorigenesis[24,25]. While variants in *PDGFRA* and *PDGFB* may be oncogenic, very few variants of *PDGFRB* have

been described in cancer[24] (Fig. 3D). Chromosome translocations causing gene fusions have been reported in rare cases of myeloid neoplasms with eosinophilia that were sensitive to imatinib[34]. Collectively, our functional studies demonstrate elevated auto-phosphorylation of variant PDGFRB and consequent activation of AKT, SRC and ERK. PDGFRB is essential in the propagation of cerebral pericytes and is a surface marker for these cells, which are thought to give rise to the vascular smooth muscle layer of the arterial media[18-21,27-31]. Since PDGFRB is highly expressed in and plays an essential role in pericyte development[18-21,27-31], our data suggest that aneurysms may originate from cerebral pericytes, vascular smooth muscle cells or their progenitors.

Alterations result in activated alleles of PDGFRB with differential sensitivity to kinase inhibitors, suggesting a potential role for therapeutic intervention. Direct inhibition of activated receptor tyrosine kinases may be a promising approach to aneurysm therapy, and further research is warranted. Where resistance is encountered, for example in the p.(Asp850Tyr) variant described in this study, kinase inhibitors to downstream targets (e.g. AKT, ERK) represent an alternative strategy. The *PDGFRB* variants detected in both fusiform intracranial aneurysms and myofibromas cluster in the same two regions (juxtamembrane and activation loop of the kinase domain), with one variant in common (p.(Tyr562Cys)) and another involving the same codon (p.(Asp850Tyr) in intracranial aneurysms, p.(Asp842Val) in myofibromas). It is striking that none of the variants are reported to occur in the germline, but are tolerated in a mosaic pattern. It is unknown if *PDGFRB* variants cause both cerebral aneurysms and myofibromas in the same individual but there may be overlap. While the individuals described in this manuscript had no evidence of myofibromas, renal and iliac aneurysms have been reported in an individual with IM[35]. More research is necessary to understand the role of age or timing specific intracranial aneurysm

formation. PDGFRB targeted therapy for treatment of severe IM in an individual with a heterozygous germline mutation *PDGFRB* has been reported[36].

Fusiform aneurysms of the neurovascular tree are difficult to treat and can rupture, cause vessel occlusion or mass effect on nearby perforator vessels resulting in neurologic deficits[1,2,32,33]. Securing of the aneurysm, prior to rupture with maintenance of cerebral blood flow are the goals. Fusiform cerebral aneurysms are difficult to treat with surgery or with endovascular techniques, such as stent or coil reconstruction[32,33]. Regardless of treatment option, outcomes for fusiform aneurysm are poor[32,33], highlighting a need for improved therapies for this select group of vascular lesions. The data presented suggests that some fusiform cerebral aneurysms are caused by activating variants in *PDGFRB.* The identification of *PDGFRB* variants in a subset of human fusiform aneurysms and the association between variant and kinase inhibitor efficacy extend the role of overactive kinase activity in vascular pathogenesis. The striking differences in *PDGFRB* variants found in fusiform versus saccular aneurysms raise the possibility of unique genetic landscapes and molecular pathogenesis underlying this heterogeneity. Our cohort is limited, and adequate study will require a much larger cohort from multiple centers. These findings provide a model for understanding post-zygotic genetic alterations causing sporadic aneurysms, which may occur over the lifetime of individuals. The role of somatic *PDGFRB* variants in fusiform aneurysms has shed light on a pathogenic mechanism in intracranial aneurysms. This finding may aid in the identification of additional causative somatic intracranial aneurysm genes. Identification of recurrent *PDGFRB* alterations provide an avenue of study for tyrosine kinase inhibition as a future therapeutic strategy in fusiform cerebral aneurysms.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

Figure 1: Index individual phenotype and *PDGFRB* genotype. A, Body map skin mosaicism (in red) and specimens used for exome sequencing. **B**, Cutaneous appearance. **C**, Angiogram 3D reconstruction of right vertebral injection illustrating a giant vertebral fusiform aneurysm. **D-I,** Abnormal extra cranial soft tissue vasculature associated with the occipital artery (Specimen MOS-WES-3): **D**, Low power (4x) and **E**, medium power (10x) magnification of a hematoxylin and eosin (H&E) stained slide demonstrating a markedly affected vessel with focal evidence of dissection (arrowhead). There is severe intimal hyperplasia and the tunica media (*) becomes markedly attenuated. **F**, Low power (4x) and **G**, medium power (10x) magnification of Gomori trichrome (GT) stained slide with the intima (**) and the tunica media (*) highlighted. **H**, Low power (4x) and **I**, medium power (10x) magnification of Verhoeff-Van Gieson (VVG) stained slide. The internal elastic lamina (*) associated with the relatively better preserved fragment of tunica media, as well several areas with attenuated internal elastic lamina are highlighted (**). **J**, Specimen MOS-WES-6, medium power magnification (10x), H&E stained slide of an unremarkable left arm radial artery. Well-defined intimal layer, tunica media, and tunica adventitia along with an intact internal elastic lamina (*) are visualized. **K**, Specimens used for exome sequencing and coverage of the p.Tyr562Cys variant. **L**, Next generation sequencing reads across the area of the missense variant, with the variant nucleotides (C) in blue. The reference nucleotide and amino acid sequences are at the bottom.

Figure 2: Sporadic fusiform aneurysms harbour *PDGFRB* variants. A, Demographics of sporadic fusiform aneurysm subjects and variants, including age at treatment**. B, C, F,** Angiogram and/or 3D reconstruction from angiogram representative images illustrating the fusiform morphology. **D** and **E**, Representative H&E stained sections of specimen VAL-44 at low (1·25x) and medium (10x) magnification showing a markedly affected vessel with vascular wall attenuation, an intraluminal thrombus with early organization and dissecting haemorrhage. The tunica media (*) is focally present and becomes attenuated (**). **G**, H&E stained sections at low magnification (1·25x) of specimen VAL-61 stained showing a representative portion of the 3 cm aneurysm with a large partially organizing thrombus and a markedly attenuated vascular wall. Abbreviations: AF = Allele frequency, ICA = Internal carotid artery, MCA = Middle cerebral artery, PCA = Posterior cerebral artery, RCCA = Right common carotid artery.

Figure 3: Variants in PDGFRB within the juxtamembrane region and the kinase activation loop found in fusiform aneurysms. A, Schematic representation of the PDGFRB protein, amino acid sequence of the two hotspots, and the location of variants. Germline and somatic PDGFRB variants with known or implied functional consequences in other syndromes and diseases included for comparison. **B**, Homologous juxtamembrane amino acid sequences for KIT, PDGFRA and PDGFRB and location of aneurysm variants. **C**, Homologous kinase domain activation loop amino acid sequences for KIT, PDGFRA and PDGFRB and location of aneurysm mutations. **D**, All somatic variants reported in the COSMIC database with possible activating consequences (missense and in-frame insertion/deletions) in KIT, PDGFRA and PDGFRB. Notice the increased frequency of variants in both juxtamembrane region and kinase activation loop of KIT and PDGFRA. There is a comparative lack of variants reported in PDGFRB.

Figure 4: PDGFRB variants are constitutively phosphorylated, sensitive to sunitinib kinase inhibition and activate downstream signalling pathways. A, Western blot analysis of non-starved normal (wild-type PDGFRB) and mosaic affected Tyr562Cys fibroblast cells from the index individual. **B**, Western blot analysis of starved and PDGF-BB stimulated normal (wild-type PDGFRB) and mosaic affected Tyr562Cys fibroblast cells from the index individual. **C**, Western blot analysis of HEK cells stably expressing the described aneurysm variants and Trp566Arg (IM - gain of function) and Asp844Gly (PFBC - loss of function) control variants showing varying levels of phosphorylation and expression of PDGFRB. **D**, Sensitivity of PDGFRB auto-phosphorylation to sunitinib.