Novel Oncogenic PDGFRα Mutations in Pediatric High-Grade Gliomas

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Abstract

The outcome for children with high-grade gliomas (HGG) remains dismal, with a two-year survival rate of only 10-30%. Diffuse intrinsic pontine glioma (DIPG) comprise a subset of HGG that arise in brainstem almost exclusively in children. Genome-wide analyses of copy number imbalances previously showed that platelet derived growth factor receptor alpha (PDGFRA) is the most frequent target of focal amplification in pediatric HGGs, including DIPGs. To determine whether PDGFRA is also targeted by more subtle mutations missed by copy number analysis, we sequenced all PDGFRA coding exons from a cohort of pediatric HGGs. Somatic activating mutations were identified in 14.4% (13/90) of non-brainstem pediatric HGGs and 4.7% (2/43) of DIPGs, including missense mutations and in-frame deletions and insertions not previously described. 40% of tumors with mutation showed concurrent amplification, while 60% carried heterozygous mutations. Six different mutations impacting different domains all resulted in ligand-independent receptor activation that was blocked by small molecule inhibitors of PDGFR. Expression of mutants in p53-null primary mouse astrocytes conferred a proliferative advantage \textit{in vitro}, and generated HGGs \textit{in vivo} with complete penetrance when implanted into brain. The gene expression signatures of these murine HGGs reflected the spectrum of human diffuse HGGs. PDGFRA intragenic deletion of exons 8 and 9 were previously shown in adult HGG, but were not detected in 83 non-brainstem pediatric HGG and 57 DIPGs. Thus, a distinct spectrum of mutations confers constitutive receptor activation and oncogenic activity to PDGFRα in childhood HGG.
Introduction

Pediatric high-grade gliomas (HGGs) comprise 15-20% of all childhood tumors of the central nervous system (1). Despite aggressive therapy, prognosis for pediatric HGG remains very poor, with a two-year survival rate of less than 20% (2). A subset of HGGs arise in the brainstem as diffuse intrinsic pontine glioma (DIPG), a disease that occurs almost exclusively in children, and has a two-year survival rate of less than 10% (3). Although the histopathologies of pediatric and adult HGGs can be similar, genome-wide studies have shown significant differences in the frequencies of specific copy number alterations (CNAs) as well as both similarities and differences in the gene expression signatures of HGGs in these two age groups (4-12). Somatic mutations in histone H3 occur in 78% of DIPGs and 36% of non-brainstem pediatric glioblastomas, but occurred only rarely in young adults with glioblastoma, and not in older adult glioblastoma patients (11, 12). Thus, distinct molecular mechanisms drive gliomagenesis at different ages.

Platelet derived growth factor receptor alpha (PDGFRA) is the most frequent target of focal amplification in pediatric HGGs arising within and outside the brainstem (4-7, 10), and somatic mutations of PDGFRA have been recently reported in pediatric HGGs (6, 12). In contrast, epidermal growth factor receptor (EGFR) is the predominant receptor tyrosine kinase targeted by both amplification and mutation in adult glioblastoma (13, 14). Pediatric HGGs with genomic amplification of PDGFRA showed concomitant increases in PDGFRA mRNA by gene expression profiling. Furthermore, PDGFRα overexpression without genomic amplification is commonly found in pediatric HGGs, and amplification of the genes encoding PDGF ligands, or overexpression with and
without **PDGFRA** aberrations were also reported, suggesting both autocrine and paracrine signaling.

PDGF and its receptors are involved in many cellular processes such as migration, survival and proliferation, and they are critical during developmental processes (15). Ligand binding induces receptor dimerization and results in phosphorylation of the receptor at multiple tyrosine residues. Activated PDGFRs transduce signals through multiple downstream pathways, including the PI3K/Akt, RAS/MAP kinase, Src kinase family and PLC/PKC pathways, which have all been implicated in tumorigenesis (15, 16).

Abnormally activated PDGFRα signaling driven by viral expression of PDGFB ligand is sufficient to induce glioma formation *in vivo*, indicating that activation of PDGFR pathways is potentially an early event in tumorigenesis (17-19). Furthermore, simultaneous overexpression of PDGFB and loss of **TP53** induced murine HGG with increased incidence and shorter latency indicating cooperativity between these pathways (20, 21). However, these studies focused on autocrine and paracrine activation of PDGFR signaling pathways by PDGFB ligand overexpression.

Here we report that pediatric HGGs, including DIPGs, carry novel somatic activating mutations of **PDGFRA** that are constitutively active, tumorigenic, and sensitive to small molecule inhibitors.
Materials and Methods

Clinical Samples

Pediatric high-grade glioma samples were obtained from St. Jude Children’s Research Hospital, Memphis, USA, and the Royal Marsden Hospital in the UK (Table S2). Ethical Review Committee approval was obtained from each institution/consortium. Genomic DNA was extracted as previously described from snap-frozen (22) or formalin-fixed paraffin embedded material (10).

Mutation analysis of PDGFRA

All coding exons of PDGFRA were sequenced by direct sequencing of PCR amplified products from genomic DNA in the tumors listed in Table S2, including 90 cases of non-brainstem pediatric HGGs and 43 cases of DIPGs, using primers listed in Table S4, or by exome sequencing for 3 DIPG samples. Additionally, for 51 cases of non-brainstem pediatric HGG, DNA was extracted from formalin-fixed paraffin embedded tissue and amplified and sequenced using primers published previously (9). Identified PDGFRA mutations were validated by independent PCR, and matched normal samples were sequenced when available. Expression of mutated receptor was confirmed by RT-PCR and sequencing using primers listed in Table S4 for available cDNA samples. 83 non-brainstem pediatric HGGs samples were screened by RT-PCR for KDR-PDGFRA gene fusion, and the single case identified was validated by independent PCR and sequencing. cDNA from 83 non-brainstem pediatric HGG and 57 DIPG cases were screened for PDGFRAΔ8,9, the previously reported 243 base pair deletion in exons 8 and 9 as described (23).
**In vitro analyses of overexpression of wild-type and mutant PDGFRA**

Wild type and mutated PDGFRA open reading frames were cloned into the MSCV-IRES-GFP (MIG) retroviral vector and used to generate retrovirus (24). Cortical astrocyte cultures were established from two-day old mice (GFAP-cre;Trp53^{loxP/loxP}) as described previously (25). At passage one, p53-null astrocytes were transduced with retroviruses expressing wild type PDGFRα, PDGFRα mutants or empty vector, and *in vitro* and tumorigenesis experiments were performed before passage six. For proliferation assays, 5.5 x 10^3 cells per well were plated on 96-well plates in triplicate. Cells were grown in DMEM/F-12 supplemented with 10% FBS and 20 ng/mL mouse EGF (Millipore), but without exogenous addition of the PDGF ligand. Proliferation was measured using XTT assay (Roche) at 24 hour intervals over a four day period, without replacing the growth medium. For inhibitor studies, cells were allowed to attach for 4 hours after seeding, then 225 nM (100 ng/mL) Crenolanib (AROG Pharmaceuticals), 50 nM Dasatinib (LC Laboratories) or vehicle (0.1% DMSO) were added to the cells in a single dose, and growth was assayed by XTT as above. Data were normalized to the cell number measured at time zero of the experiment, which was acquired within the first 8 hours from cell seeding (4 hours for cell attachment and 4 hours for development of XTT). For cell cycle analyses, 2 x 10^6 cells were seeded per 10 cm dish and the next day cells were treated with 225 nM (100 ng/mL) crenolanib (AROG Pharmaceuticals), 50 nM dasatinib (LC Laboratories) or vehicle (0.1% DMSO) for 24 hours. Following inhibitor treatment, cells in the supernatant from each dish were collected, pooled with trypsinized cells, and washed with PBS. Cells were fixed in 70% ethanol overnight at
4°C and then stained using Guava Cell Cycle Reagent (Millipore). Data were acquired on the Guava EasyCyte using CytoSoft software (Millipore).

**Tumorigenesis studies**

Mouse experiments were approved by the Institutional Animal Care and Use Committee and are in compliance with national and institutional guidelines. 2 x 10^6 transduced astrocytes were implanted intracranially into athymic nude mice for tumorigenesis studies as previously described (25). On the manifestation of brain tumor symptoms, mice were anesthetized and perfused with PBS. GFP-labeled tumors were dissected using a fluorescent dissecting microscope. For each tumor, a portion was snap-frozen for protein analyses and RNA extraction, and the remainder was fixed in 4% paraformaldehyde in PBS at 4°C overnight, then processed, embedded in paraffin, and cut into 5 μm sections. Hematoxylin and eosin stained sections from all collected tumors were evaluated by a clinical neuropathologist (DWE) and graded according to WHO criteria (26). Immunohistochemistry was performed with microwave antigen retrieval in a citrate solution using the following primary antibodies from Cell Signaling: PDGFRα (#5241), phospho-Akt S473 (#9271) and phospho-4E-BP1 Thr37/46 (#2855). Anti-rabbit biotinylated secondary antibodies were used in conjunction with horseradish peroxidase-conjugated streptavidin (Elite ABC, Vector Labs). Staining was developed with NovaRED substrate (Vector Labs) and the sections were counterstained with hematoxylin (Vector Labs).

**Western blot analysis**
Protein lysates were prepared in ice-cold RIPA buffer (150mM NaCl; 50mM Tris-HCl, pH 8.0; 1% NP-40; 0.5% sodium deoxycholate and 0.1% SDS) supplemented with protease and phosphatase inhibitors (Roche). For in vitro experiments, cells were serum-starved in media containing 0.1% FBS for 16 hours and then treated with or without 50 ng/mL PDGF-AA for 30 minutes. For inhibitor experiments, serum-starved cells were pre-treated with 225 nM (100 ng/mL) crenolanib (AROG Pharmaceuticals) or 50 nM dasatinib (LC Laboratories) for 3 hours and then stimulated with PDGF-AA for 30 minutes. To calculate the IC$_{50}$ and IC$_{90}$ concentrations for crenolanib serum-starved cells were pre-treated for 3 hours with the following doses: 0.023 μM (10 ng/mL), 0.045 μM (20 ng/mL), 0.113 μM (50 ng/mL), 0.225 μM (100 ng/mL) and 1.125 μM (500 ng/mL) and then stimulated with PDGF-AA for 30 minutes. Following treatment, cells were washed twice with ice-cold PBS then scraped and lysed in RIPA buffer. Snap-frozen tissues from brain tumor bearing and control animals were lysed using a microcentrifuge tube pestle (Fisher Scientific). 20 μg of protein extract was separated by electrophoresis on NuPAGE 4-12% Bis-Tris gels run in NuPAGE MES SDS Running Buffer (Invitrogen). Antibodies for phospho-PDGFRα Tyr572/574 (ab5443), phospho-PDGFRα Tyr-742 (ab5452) and phospho-PDGFRα Tyr720 (ab65258) were from Abcam. Antibodies for phospho-PDGFRα Tyr754 (sc-12911), PDGFR-α (sc-338) and α-Tubulin (sc-23948) were from Santa Cruz. Antibodies for phospho-Akt S473 (#9271), phospho-Akt T308 (#9275), pan Akt (#4691), phospho-p44/42 MAPK Thr202/Tyr204 (#9101), p44/42 MAPK (#9102), phospho-MEK1/2 Ser217/221 (#9154), MEK1/2 (#9122), phospho-NDRG1 Thr346 (#3217), NDRG1 (#9408), phospho-PRAS40 Thr246 (#2997), PRAS40 (#2610), phospho-S6 ribosomal protein Ser235/236 (#2211), S6 ribosomal protein
(#2217), phospho-SHP-2 Tyr542 (#3751), SHP-2 (#3752), phospho-Src family Tyr416 (#2101), phospho-Src family Tyr527 (#2105), Src family (#2109), phospho-Stat3 Tyr705 (#9145), Stat3 (#9139), phospho-4E-BP1 Thr37/46 (#9459) and 4E-BP1 (#9452) were from Cell Signaling. Antibody for β-Actin (A5441) was from Sigma. Horseradish peroxidase-conjugated secondary antibodies (NA934, NXA931) were from GE Healthcare. Chemiluminescent signal was detected with ECL reagents (GE Healthcare) or SuperSignal West Dura (Thermo Scientific).

**Gene expression microarray analysis**

Total RNA was extracted from frozen tumor tissue dissected from mice with wild-type PDGFRα, PDGFRα mutants and from parental p53-null mouse primary astrocytes. In addition, RNA was isolated from high-grade astrocytomas generated by implantation of EGFRvIII expressing p53-null PMAs as previously described (25). Gene expression profiles were generated using the Affymetrix Mouse Genome 430 Perfect Match Peg Arrays. Gene expression profiles were generated using the Affymetrix Mouse Genome 430 Perfect Match Peg Arrays in two batches. Three common samples run in both sets were used for batch correction with the program ComBat (27). Unsupervised hierarchical clustering analysis was carried out with the merged data using the 1000 most variable probe sets selected based on median absolute deviation (MAD) score. Single-sample gene set enrichment analysis (GSEA) was performed as described previously (28, 29). For single-sample GSEA, signature gene sets defining human HGG expression subgroups (PN-proneural, Pro-proliferative, Mes-mesenchymal) and murine cell type specific signatures (OPCs- oligodendrocyte progenitor cells, Oligo-oligodendrocyte, N- neurons, A- astrocytes and CA- cultured astroglia) were taken from.

**Statistical Analysis**

There was no apparent association between histological tumor type and the presence of **PDGFRA** mutation in pediatric HGG as assessed by exact chi-square test for independence (p=0.2562). A Kruskal-Wallis test showed that the presence of **PDGFRA** mutations was not significantly associated with age at diagnosis (p=0.068). Statistical significance of the associations of murine tumors driven by different **PDGFRA** mutations with histology, gene expression clusters, human HGGs gene expression signatures and neural cell type specific subgroups was assessed by exact chi-square test for independence. Student’s two-tailed t-test p-values were calculated for cell cycle analysis to assess response to crenolanib and dasatinib versus untreated controls.
Results

**PDGFRA is frequently mutated in pediatric HGGs**

Sequencing of all coding exons of PDGFRA in genomic DNA from 90 pediatric HGGs outside the brainstem, and 43 DIPGs identified 15 cases with mutated PDGFRA (Fig. 1 and Table S1). All mutations were confirmed by independent PCR reactions and were validated as somatic, found only in the tumor and not matching germline DNA when normal sample was available (Table S1). Mutations were identified in the extracellular, transmembrane and intracellular kinase domains and included missense mutations, in-frame insertions or deletions (Fig.1). Analysis of cDNA showed that one of the somatic point mutations, N468S, generated a new splice site that removed 13 amino acids (E10del2) (Fig.S1A and Table S1). 60% (9/15) of the mutations were present as heterozygous alleles, and 40% (6/15) of mutations were also amplified. SNP array analyses previously showed copy number imbalances for the majority of the samples, demonstrating sufficient tumor purity to detect clonal alterations (4, 5). Importantly, all mutations reported here were readily detected by Sanger sequencing, indicating clonal expansion of the population containing the mutation, particularly in the cases in which PDGFRA was not amplified. There was no association between the presence of PDGFRA mutation and histopathological features (p=0.26). Three mutations were found in anaplastic astrocytomas (3/24), one in an anaplastic oligodendroglioma (1/6), and the remaining mutations in grade IV glioblastomas, including two DIPGs (11/102) (Table S1 and S2). Both mutations in the kinase domain, N659K and D842V were previously reported in gastrointestinal stromal tumors but not in gliomas (32, 33). The remaining mutations are all novel, including four different missense mutations (E229K, C235R,
Y288C and C290R), three in-frame insertions (C450ins, A491ins and V544ins) and three in-frame deletions (E7del, E10del and E10del2) (Table S1) not found in previous studies of a large collection of pediatric HGGs or adult HGGs (6, 12, 23, 34). Three of the identified mutations (E7del, E10del2 and N659K) were recurrent, each of them found in two different HGG cases.

We also evaluated whether activating PDGFRA mutations previously shown in adult HGG occur frequently in pediatric HGG. 40% of adult glioblastomas with PDGFRA amplification harbor an in-frame deletion of 243 base pairs in exons 8 and 9 of the extracellular portion of the receptor (PDGFRA<sup>Δ8</sup>,<sup>9</sup>) that renders the receptor constitutively active (23, 35). A gene fusion between PDGFRA and KDR (VEGFR2), which rendered the receptor constitutively active and tumorigenic in vivo, has been reported in one out of 215 cases of adult glioblastoma. We used RT-PCR to screen for these alterations that would have been missed by exonic sequencing. We found one case of KDR-PDGFRA in 83 non-brainstem pediatric HGGs analyzed (Fig. S1B). There were no examples of the PDGFRA<sup>Δ8</sup>,<sup>9</sup> deletion in 83 non-brainstem pediatric HGG, and 57 DIPG screened by RT-PCR (Fig. S1C). Thus, a different spectrum of intragenic mutations target PDGFRA in HGGs from different age groups.

**PDGFRA mutants are constitutively active**

To better understand the consequence of PDGFRA mutation in pediatric gliomagenesis, retroviral constructs expressing wild-type PDGFRA or six selected PDGFRA mutants that affect different regions of the receptor were generated for functional studies. p53-
null primary mouse astrocyte (PMA) cultures were chosen as a relevant cellular background to assess PDGFRα function, because 70% of pediatric HGGs outside of the brainstem and 46% of DIPGs with amplified and/or mutated PDGFRα also have inactivating mutations of TP53 (Table S2). All six mutants were constitutively active, inducing strong phosphorylation of PDGFRα at Tyr-572/574, Tyr-742, Tyr-720, and Tyr-754 when expressed in p53-null PMAs in the absence of serum. These phosphotyrosine sites have been shown to associate with activation of different downstream signaling pathways. Src family members bind to phosphorylated PDGFRα at Tyr-572/574, phospho-Tyr-742 promotes interaction with p85, the regulatory subunit of PI3K, and leads to PI3K pathway activation while phosphorylated Tyr-720 and Tyr-754 recruit and activate SHP-2 (Src homology-2 domain-containing phosphatase) which regulates activation of Src family kinases and the MAPK pathway (16, 36-38). Different levels of receptor phosphorylation were observed depending on the specific mutation, with the E10del2 exhibiting the lowest receptor activation. All mutants were expressed to similar levels as shown by total PDGFRα (Fig. 2A, left). In contrast, overexpression of wild-type PDGFRα did not lead to receptor activation in the ligand-free condition. However, treatment of these cells with PDGF-AA, triggered wild-type PDGFRα phosphorylation to levels similar to or greater than the mutant receptors (Fig.2A, right). Furthermore, phosphorylation of known downstream signaling targets of PDGFRα confirmed the constitutive activity of PDGFRα mutants. In the absence of ligand, elevated levels of phospho-Akt (Ser473 and Thr308) and higher phosphorylation levels of S6 ribosomal protein and PRAS40 were observed in comparison to wild-type PDGFRα expressing cells, indicating ligand-independent activation of the PI3K pathway.
by the mutated receptor. Activation of MAPK signaling, detected by phosphorylation of p44/42 MAPK was not increased by mutant PDGFRα in the absence of ligand. Ligand stimulation further increased phosphorylation levels of PI3K pathway components but had only modest effect on the MAPK pathway activation (Fig.2A and Fig.S2).

Expression of PDGFRα mutants as well as wild-type receptor added a significant proliferative advantage in comparison with empty vector control cells as determined by XTT assays (Fig.2B).

**PDGFRα signaling is abrogated by inhibitors**

To analyze whether wild-type and PDGFRα mutants have differential responses to small molecule inhibitors, two distinct compounds were selected. Dasatinib (BMS-354825) is a potent, multi-tyrosine kinase inhibitor that exerts broad antiproliferative activity by targeting PDGFRα, PDGFRβ, ABL, SRC family kinases, KIT and several other tyrosine kinases (39, 40). In contrast, crenolanib (CP-868,596) is a relatively specific inhibitor of PDGFRα and PDGFRβ, that is more than 100 fold more selective for PDGFRs versus a variety of other kinases (e.g. KIT, VEGFR-2, TIE-2, FGFR-2, EGFR, ERBB2, and SRC) (41, 42). Both drugs have been shown to inhibit PDGFR kinase activity by competing with ATP. To compare wild-type and mutant PDGFRα signaling alterations in response to these inhibitors, cells were serum starved overnight, then treated with crenolanib or dasatinib for 3 hours before adding PDGF-AA ligand. As previously reported, the D842V mutant was resistant to dasatinib treatment, however it was effectively inhibited by crenolanib (42). Both inhibitors significantly blocked activation of wild type PDGFRα and all other mutants analyzed as shown by decreased
phosphorylation of the receptor at Tyr-572/574, Tyr-742, Tyr-720, and Tyr-754 (Fig. 3A). Furthermore, downstream signaling activation was significantly diminished, including decreased phosphorylation of PI3K and MAPK pathway effectors (Fig. 3A and Fig. S3A). D842V and V544ins were the only two mutants that induced a substantial increase in SHP2 phosphorylation (Fig S2), and the only two mutants for which crenolanib induced a substantial decrease in phospho-SHP2 (Fig S3A).

The effect of each inhibitor on cell proliferation was examined by XTT assay (Fig. 3B and Fig. S3B), using a concentration greater than the IC90 determined to block receptor phosphorylation (Fig. S4). Crenolanib selectively inhibited proliferation of PDGFRα expressing cells, both wild-type and all analyzed mutants, while it did not affect proliferation of the empty vector control cells (Fig. 3B and Fig. S3B). In contrast, dasatinib exerted broad anti-proliferative effects and significantly inhibited empty vector control cells in addition to PDGFRα expressing cells. Furthermore, concordant with the inability of dasatinib to block phosphorylation of the D842V mutant, cells expressing this mutant showed a diminished growth inhibitory response compared to other mutants (Fig. 3B). Crenolanib and dasatinib both exert cytostatic effects on PDGFRα-expressing p53-null PMAs, arresting cells in G0/G1 (Fig. S5). However, neither inhibitor induced significant cell death, as assessed by annexin V staining and TUNEL assays (data not shown).

**PDGFRα mutations are oncogenic in vivo and drive development of HGGs**
To determine if expression of wild-type PDGFRα or PDGFRα mutants renders p53-null PMAs tumorigenic, two million cells were implanted intracranially into the parietal lobe of two month old athymic nude mice and monitored daily for signs of morbidity. Mice were euthanized and brain tumor tissue was collected when mice became symptomatic. Brain tumors formed in 100% of mice implanted with cells expressing any of six different mutant forms of PDGFRα. Only one of 19 mice implanted with cells overexpressing wild-type PDGFRα developed a brain tumor, and none of the empty vector control cells developed into brain tumors in vivo. The tumor-induced morbidity was detected between 23 and 72 days for all mutants except E7del expressing tumors, which occurred at 103-119 days and the single tumor from wild-type PDGFRα, which occurred at 120 days post-implantation (Fig.4A). Standard histopathologic preparations from all tumors were evaluated (DWE) and classified according to WHO criteria (Fig 4B and Table S3). Overall, 63% (44/70) of tumors analyzed were grade III anaplastic astrocytoma, 26% (18/70) were grade III anaplastic oligoastrocytoma, 10% (7/70) were grade IV glioblastoma, and the single tumor that developed from cells expressing wild-type PDGFRα was the only low-grade tumor, a grade II oligoastrocytoma. Tumors expressing the D842V or V544ins mutation were significantly associated with anaplastic astrocytoma histology (p<0.0001 and p=0.0012 respectively) and tumors with E10del were significantly associated with anaplastic oligoastrocytoma histology (p=0.002). The rest of the evaluated tumors expressing C540ins, E10del2 and E7del mutations were not significantly associated with a specific morphology (p>0.2).

Immunohistochemical analysis for PDGFRα showed strong overexpression of the receptor throughout all tumors. PDGFRα-driven murine gliomas showed growth patterns
similar to human high-grade gliomas including an easily visualized focal mass as well as diffuse infiltration into the normal brain parenchyma (Fig. 4C and Fig. S6A). These tumors were immunopositive for p-4E-BP1 (Thr37/46) and p-Akt (Ser473) suggesting activated PDGFRα signaling (Fig. 4C). Furthermore, activation of PDGFRα in tumor tissues was confirmed by western blot analysis, which demonstrated high levels of phosphorylated receptor on Tyr-572/574, Tyr-742, Tyr-720, and Tyr-754 in all tumors examined but not in normal brain (Fig. 4D). Moreover, phosphorylation of PI3K pathway components, SHP2 and Src family kinases were strongly elevated compared to normal cortex. In contrast, levels of total STAT3 and phosphorylated STAT3 were similarly increased in all analyzed tumors compared to normal brain tissue, which may indicate cell-type specific differential expression of STAT3, not necessarily selective activation of STAT3 signaling in tumor. There was no significant difference in levels of MAPK pathway activation observed between tumor tissues and normal brain (Fig. 4D and Fig. S6B).

**Gene expression signatures of PDGFRα-driven murine HGGs resemble human HGGs**

To evaluate the similarity of the molecular signatures of PDGFR-driven murine HGGs to human disease, gene expression profiles were analyzed for representative mutant and wild-type PDGFRα generated brain tumors. Additionally, gene expression profiles of EGFRvIII-driven murine HGGs were assayed for comparison (25). Unsupervised hierarchical clustering (UHC) segregated the tumors into two distinct subgroups (Fig. S7). Interestingly, PDGFRα and EGFRvIII expressing tumors were distributed among
both subgroups, and there was no significant association of specific PDGFRα mutations or tumor histopathology with either of the identified subgroups. Furthermore, using single sample gene set enrichment analysis (GSEA) the gene expression profile of each murine tumor was compared to published signature gene sets for human HGG subgroups (proneural, proliferative and mesenchymal) and murine cell type-specific signatures (oligodendrocyte progenitor cells, oligodendrocytes, neurons, astrocytes and cultured astroglia) (30, 31). We also compared the gene expression signatures of seven independent untransduced p53-null PMA cultures, which showed a significant similarity to the published expression signatures of astrocytes and cultured astroglia (31).

Interestingly, transformation of p53-null PMAs by PDGFRα mutants resulted in gliomas with a range of expression signatures representing the three major expression subgroups observed in human HGGs (Fig. 5) (30). 37.8% (14/37) of PDGFRα tumors showed proneural, 32.4% (12/37) proliferative and 16.2% (6/37) mesenchymal subgroup expression signatures. There was no significant association of specific PDGFRA mutations with a particular expression subgroup described in pediatric and adult HGGs (4, 5, 30). Interestingly, tumors driven by E10del2 mutation were significantly associated with the oligodendrocyte progenitor cell gene expression signature (p=0.03), however there was no significant association between specific mutation and expression signature of cell types.
Discussion

Aberrations of PDGFRα signaling via amplification and/or mutation of PDGFRα are frequent in pediatric HGGs. Here, targeted sequencing identified novel activating somatic mutations of PDGFRα in pediatric HGGs. Two recent studies reported PDGFRα mutations in pediatric HGGs, but the functional consequence on PDGFRα activation was not explored, and none of the described mutations overlapped with our findings (6, 12). Interestingly, mutations were not represented by a single hotspot as observed in gastrointestinal stromal tumors. One of the identified mutations in this study, V544ins, a small duplication resulting in a 14 amino acid in-frame insertion, was found in the transmembrane domain, potentially facilitating oligomerization of the receptor via the transmembrane domain or disrupting the inhibitory conformation of the juxtamembrane region and consequently leading to ligand independent receptor dimerization and activation as observed for PDGFRβ, c-Kit and other RTKs (43-45).

In adult glioblastomas, the frequency of PDGFRα amplification and mutation is lower than in pediatric tumors and PDGFRα mutations comprise a distinct set of alterations compared to childhood disease (8, 9, 23, 34). Approximately 40% of amplified PDGFRα in adult glioblastomas is not wild-type, but contains recurrent genomic deletions of exons 8 and 9 resulting in an in-frame deletion in the extracellular domain and constitutive receptor activation. Several in-frame deletions occur in pediatric HGG; however, the recurrent alterations in adult glioblastoma were not found in pediatric tumors. Instead, mutations were not restricted to a single functional domain, but were distributed among different regions of the PDGFRα protein, including the kinase domain, and extracellular regions involved in ligand binding and receptor-receptor
interaction. The six PDGFRα mutants analyzed represented alterations in different functional domains, and all were constitutively active and tumorigenic. Thus, alternative genetic mechanisms are used to generate oncogenic mutations of PDGFRA in childhood and adult HGGs.

Numerous mouse glioma models have been generated by expression of exogenous PDGF, driving paracrine stimulation of cells expressing endogenous PDGFR (17-19, 46-48). In this study, gliomas were driven by the PDGFRA mutations found in pediatric HGGs. Both wild-type and mutant PDGFRα conferred a proliferative advantage to p53-null PMAs in vitro, however, the wild-type showed minimal tumorigenic activity, resulting in only 1 of 19 mice developing a low-grade glioma while all six of the mutants induced high-grade glioma formation with 100% penetrance. In contrast, a previous study showed that wild-type PDGFRα transformed Ink4a/Arf-null PMAs to generate gliomas (49). The difference in the gliomagenic activity of wild-type PDGFRα may be explained in part by a higher propensity for transformation in the recipient cells, as the Ink4a/Arf-null PMAs formed tumors at lower frequency in the absence of PDGFRα, while the early passage p53-null PMAs in the present study did not form any tumors. Additionally Ink4a/Arf and p53 loss may differentially cooperate with PDGFRα overexpression to drive glioma (49). It is also possible that the levels of wild-type receptor expression in our model system were not sufficient to model the gene amplification that drives tumorigenesis in human tumors. However, the wild-type receptor was expressed at levels similar to the E7del, E10del and E10del2 mutations (Fig. 2), which were also amplified in human tumors.
The fact that all PDGFRα mutations showed ligand-independent phosphorylation while wild-type receptor activation required ligand stimulation, suggests lack of sufficient ligand in the brain of implanted adult animals, or different selective advantage conferred by wild-type PDGFRA amplification versus mutation with or without amplification. Amplification of wild-type PDGFRA occurred more frequently in tumors within the brainstem (26%, 11/43 DIPG vs. 11%, 9/84 non-brainstem HGG, p=0.04), while PDGFRA sequence mutations were more common in pediatric HGG arising outside the brainstem, although this was not statistically significant (14%, 13/90 non-brainstem HGG vs. 5%, 2/43 DIPG, p=0.14) (4, 5). Moreover, all six samples with concomitant amplification and mutation of PDGFRA were from non-brainstem tumors. Given the higher frequency of wild-type amplification in DIPGs, it is possible that wild-type PDGFRα requires a more specific or alternative cell of origin to drive transformation compared to the mutants, which may not be adequately recapitulated in our experimental system. These effects can vary among oncogenic mutations. For example EGFRvIII drives tumorigenesis equivalently from Ink4a/Arf-null cortical astrocytes or neural stem cells, while BRAF duplications show a differential growth response in cerebellar neural stem cells compared to astrocytes, and in neural stem cells from the cerebellum compared to those from neocortex (50, 51). Furthermore, comparison of DIPG and non-brainstem pediatric HGG also revealed distinctive gene expression profiles, significant differences in the frequency of histone mutations and specific large-scale genomic imbalances, as well as the absence of focal deletion of CDKN2A in DIPG, which is common in pediatric non-brainstem HGG (4, 5, 11). This suggests that distinct tumor microenvironments within different locations of the brain.
may exert different selective pressures driving pediatric HGG, which could include differential susceptibility to PDGFRA mutation/amplification-driven tumorigenesis.

The tumors that arose from mutant PDGFRα-transformed PMAs showed a range of morphologies. Although the majority of murine HGGs were diagnosed as anaplastic astrocytomas or glioblastomas, a significant number of tumors showed a mixed glial (oligoastrocytic) phenotype. Because tumors were generated with virally-transduced astrocytes, this suggests that in a subset of tumors, signaling activated by mutated PDGFRα triggered astrocyte dedifferentiation as described for tumors induced by PDGFB ligand overexpression in cultured astrocytes (18). Alternatively, rare progenitor cell populations in the starting populations may give rise to some of the tumors. In several different studies, viral introduction of PDGF-B into brain induced gliomas with a predominantly proneural signature, perhaps driven in part by the endogenous cells that expressed PDGFR, and were therefore competent to respond to the exogenous ligand (18, 47, 52). In contrast, the tumors arising from transduced PMAs in this study displayed a range of gene expression signatures similar to the spectrum observed in human HGGs, and with similarities to expression signatures of a range of different types of normal cells, despite the relatively uniform astrocytic gene expression signature in the parental PMAs. This may indicate significant plasticity in the neonatal PMAs, especially in the context of aberrant signaling driven by mutant PDGFRα.

Since PDGFRA signaling alterations are frequent in pediatric HGGs, including amplification and/or activating mutation of the receptor, understanding the therapeutic and phenotypic consequences of PDGFRA mutations is important to guide clinical intervention. The model system reported here showed that all mutants tested were
constitutively active and capable of driving high-grade gliomas with complete penetrance and a range of gene expression signatures relevant to human tumors. This model varies from previous models driven by expression of exogenous ligand in that it recapitulates the autocrine signaling that the mutant receptor drives in tumor cells.

The consequence of oncogenic mutations to therapeutic inhibitors can have significant clinical ramifications. Some oncogenic mutations confer resistance to small molecule inhibitors. For example, PDGFRA D842V is a recurrent hotspot mutation in gastrointestinal stromal tumors that confers resistance to imatinib (32). In contrast, some oncogenic mutations cause enhanced sensitivity to small molecule inhibitors and are associated with significantly prolonged survival, such as BRAF mutations in melanoma, or EGFR mutations in small cell lung cancer (53, 54). This is highly dependent on the specific mutation rather than the therapeutic target, as illustrated by the different spectrum of EGFR mutations in adult glioblastomas that are not sensitive to the same small molecule inhibitors that work well in lung cancer (55). All of the PDGFRα mutants tested here were inhibited by crenolanib, and most were inhibited by dasatinib. While it is promising that these mutations do not confer resistance to available small molecule PDGFR inhibitors, these compounds induced a cytostatic, but not cytotoxic response. This strongly suggests that clinical use of PDGFR inhibitors as a single agent may not be sufficient to cause regression of pediatric HGG, but could be a useful addition to other therapeutic approaches.
Acknowledgments

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References


Figure Legends

**Figure-1** *PDGFRA* somatic mutations identified in pediatric HGGs and DIPGs.

Sanger sequencing of *PDGFRA* from genomic DNA revealed multiple mutations, including missense mutations, in-frame insertions and in-frame deletions. The schematic shows the location of the mutations and affected domains of PDGFRα. A star indicates samples with concomitant mutation and amplification of the *PDGFRA* locus. The signal peptide is designated by an orange oval and the transmembrane domain by a brown rectangle.

**Figure-2** PDGFRα mutants are constitutively active and confer proliferative advantage.

A) Western blot analysis of whole cell lysates from p53 null PMAs transduced with retroviruses expressing wild type PDGFRα (WT), PDGFRα mutants or empty vector. Cells were grown in serum free conditions overnight and then with or without PDGF-AA for 30 minutes. PDGFRα signaling activation of downstream targets was monitored using indicated antibodies in the presence and absence of PDGF-AA.

B) Proliferation of p53 null PMAs transduced with retroviruses expressing wild type, PDGFRα mutants or empty vector was measured by an XTT assay in triplicate. Error bars show standard deviation. Shown is a representative figure of three independent experiments.

**Figure-3** Wild-type and mutant PDGFRα are sensitive to small molecule inhibitors.

A) Western blot analysis of whole cell lysates from p53 null PMAs transduced with retroviruses expressing wild type PDGFRα (WT), PDGFRα mutants or empty vector. Following overnight serum starvation, cells were pre-treated with Crenolanib (C),
Dasatinib (D) or vehicle (-) for 3 hours and then stimulated with PDGF-AA for 30 minutes. Inhibition of PDGFRα signaling was analyzed using indicated antibodies.

B) Growth of p53 null PMAs transduced with retroviruses expressing wild type, PDGFRα mutants or empty vector was measured with XTT assay in triplicate in the presence of crenolanib (left graph), dasatinib (right graph) or vehicle. Single dose of inhibitors (dashed lines) or vehicle (solid lines) was administered and growth was measured. Error bars show standard deviation. Shown are representative figures of three independent experiments.

**Figure-4** PDGFRα mutants are tumorigenic *in vivo*.

A) Kaplan–Meier curves showing time to morbidity of mice following intracranial implantation of p53 null PMAs expressing wild type PDGFRα (WT), PDGFRα mutants or empty vector.

B) Diverse histologic phenotypes were generated in PDGFRα-driven HGGs. Most tumors diffusely infiltrated brain parenchyma, while also producing circumscribed masses. (a) Representative anaplastic astrocytoma, showing moderately pleomorphic tumor cells, some with astrocytic differentiation, diffusely infiltrating cerebral white matter (D842V; H&E x200), (b) Representative anaplastic oligoastrocytoma with admixed oligodendrogial and astrocytic phenotypes (C450ins; H&E x200), (c) Representative glioblastoma with focal giant cell phenotype (V544ins; H&E x200).

C) Immunohistochemical analysis of a representative brain tumor stained for PDGFRα, p-4E-BP1 (T37/46), p-Akt (S473) and counterstained with hematoxylin. Scale bar is 50 μm.
D) Western blot analysis of whole cell lysates from tissues of wild-type and mutant PDGFRα-driven brain tumors. Lysates from normal adult cortex (lanes N #1 and #2) were included as controls. Signaling pathway activation in PDGFRα-driven murine HGGs was assayed using the indicated antibodies.

**Figure-5** Gene expression profiles of murine PDGFRα-driven HGGs resemble human HGGs.

Heat map of single sample gene set enrichment analysis of representative murine brain tumors and untransduced p53-null PMA cultures using gene sets defining human HGG expression subgroups (PN-proneural, Pro-proliferative, Mes-mesenchymal) and murine cell type specific signatures (OPCs- oligodendrocyte progenitor cells, Oligo-oligodendrocyte, N- neurons, A- astrocytes and CA- cultured astroglia). The order of the tumor samples is arranged based on the unsupervised hierarchical clustering analysis using 1000 most variable probe sets, which revealed two major expression subgroups HC1 and HC2 (Figure S7). The histology of each tumor is indicated above the heat map: anaplastic astrocytoma (purple), anaplastic oligoastrocytomas (green), glioblastoma (brown), oligoastrocytoma (yellow) and white mark tumors with no diagnosis available. Untransduced p53-null PMA cultures are marked with black.
**Figure 1**

Diagram illustrating the domain structure and key mutations in a protein, including:

- **Immunoglobulin-like domain**
  - Deletion mutations: C450ins, E10del2, E10del, A491ins
  - Insertion mutations: V544ins

- **Receptor-receptor interaction**
  - Missense mutations: N659K, D842V
  - Amplified: * symbol

- **Tyrosine kinase domain**

Additional annotations for domain interactions and key residues are also indicated on the diagram.
Figure 2

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Relative XTT

- Vector
- WT
- D842V
- V544ins
- C450ins
- E10del
- E10del2
- E7del

p < 0.001
Figure 3

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- p-PDGFRA (Y572/574)
- p-PDGFRA (Y742)
- p-PDGFRA (Y720)
- p-PDGFRA (Y754)
- PDGFRA
- p-AKT (S473)
- p-AKT (T308)
- AKT
- p-S6 (S235/236)
- S6
- p-PRAS40 (T246)
- PRAS40
- α-Tubulin

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