Novel Oncogenic PDGFRA Mutations in Pediatric High-Grade Gliomas

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Introduction

Pediatric high-grade gliomas (HGG) comprise 15% to 20% of all childhood tumors of the central nervous system (1). Despite aggressive therapy, prognosis for pediatric HGG remains very poor, with a 2-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 2-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 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 nonbrainstem pediatric glioblastomas, but occurred only rarely in young adults with glioblastoma and not in older patients with adult glioblastoma (11, 12). Thus, distinct molecular mechanisms drive gliomagenesis at different ages.

Platelet-derived growth factor receptor α (PDGFRA) is the most frequent target of focal amplification in pediatric HGGs (6, 12). In contrast, EGF receptor (EGFR) is the predominant receptor tyrosine kinase (RTK) targeted by both amplification and mutation in adult glioblastoma (13, 14). Pediatric HGGs with genomic amplification of PDGFRA showed concurrent increases in PDGFRA mRNA by gene expression profiling. Furthermore, PDGFRA overexpression without genomic amplification is commonly found in pediatric HGGs, and amplification of gene 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, BAS/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 PDGFβ 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 PDGFβ 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 PDGFβ 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 HGG samples were obtained from St. Jude Children’s Research Hospital (Memphis, TN) and the Royal Marsden Hospital (London, United Kingdom; Supplementary 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-embedded 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 the Supplementary Table S2, including 39 cases of nonbrainstem pediatric HGGs and 43 cases of DIPGs, using primers listed in the Supplementary Table S4, or by exome sequencing for three DIPG samples. For an additional 51 cases of nonbrainstem 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 reverse transcription (RT)-PCR and sequencing using primers listed in the Supplementary Table S4 for available cDNA samples. Eighty-three nonbrainstem pediatric HGGs samples were screened by RT-PCR for KDR–PDGFRα gene fusion (23) and the single case identified was validated by independent PCR and sequencing. cDNA from 83 nonbrainstem pediatric HGG and 57 DIPG cases were screened for PDGFRA<sup>exons 8,9</sup> by 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 2-day-old mice (GFAP<sup>-creTrp53<sup>fl<sup></sub>/loxP<sub></sub></sup></sup>/<sup></sup>/loxP<sub></sub></sup>) as described previously (25). At passage one, p53-null astrocytes were transduced with retrovirus expressing wild-type PDGFRA, PDGFRA mutants or empty vector, and in vitro and tumorigenesis experiments were carried out before passage six. For proliferation assays, 5.5 × 10<sup>5</sup> cells per well were plated on 96-well plates in triplicate. Cells were grown in Dulbecco’s Modified Eagle Medium (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 (2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt) assay (Roche) at 24-hour intervals over a 4-day period, without replacing the growth medium. For inhibitor studies, cells were allowed to attach for 4 hours after seeding, then 225 nmol/L (100 ng/mL) crenolanib (AROG Pharmaceuticals), 50 nmol/L dasatinib (LC Laboratories), or vehicle (0.1% dimethyl sulfoxide, 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 × 10<sup>5</sup> cells were seeded per 10 cm dish and the next day cells were treated with 225 nmol/L (100 ng/mL) crenolanib (AROG Pharmaceuticals), 50 nmol/L 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. A total of 2 × 10<sup>5</sup> 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 fluorescence 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 (H&E)–stained sections from all collected tumors were examined by a clinical neuropathologist (D.W. Ellison) and graded according to World Health Organization (WHO) criteria (26). Immunohistochemistry was conducted with microwave antigen retrieval in a citrate solution using the following primary antibodies from Cell Signaling Technology: PDGFRα (#2521), 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 radioimmunoprecipitation assay (RIPA) buffer (150 mmol/L NaCl; 50 mmol/L Tris–HCl, pHi 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% PBS for 16 hours and then treated with or without 50 ng/mL PDGF-AA for 30 minutes. For inhibitor experiments, serum-starved cells were pretreated with 225 mmol/L (100 ng/mL) crenolanib (AROG Pharmaceuticals) or 50 mmol/L 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 controls or 50 nmol/L dasatinib (LC Laboratories) for 3 hours and with 225 nmol/L (100 ng/mL) crenolanib (AROG Pharmaceuticals) or 50 mmol/L dasatinib (LC Laboratories) for 3 hours 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). Twenty microgram of protein extract was separated by electrophoresis on NuPAGE 4% to 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 Tyr-720 (ab65258) were from Abcam. Antibodies for phospho-PDGFRα Tyr-754 (sc-12911), PDGFR-α (sc-338), and α-tubulin (sc-23948) were from Santa Cruz Biotechnology. Antibodies for phospho-Akt S473 (#9271), phospho-Akt T308 (#9275), pan Akt (#4691), phospho-p44/42 mitogen-activated protein kinase (MAPK) Thr202/Tyr204 (#9101), p44/p42 MAPK (#9102), phospho-MEK1/2 Ser217/211 (#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-4E-BP1 Thr37/46 (#9459), and 4E-BP1 (#9452) were from Cell Signaling Technology. 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). Gene expression microarray analysis

Total RNA was extracted from frozen tumor tissue dissected from mice with wild-type PDGFRA, PDGFRA 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 primary mouse astrocyte (PMA) 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 (UHC) analysis was carried out with the merged data using the 1,000 most variable probe sets selected on the basis of median absolute deviation score. Single-sample gene set enrichment analysis (GSEA) was conducted as described previously (28, 29). For single-sample GSEA, signature gene sets defining human HGG expression subgroups (PN, pronuclear; Pro, proliferative; and 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 previously published work (30, 31). Data are deposited at www.ncbi.nlm.nih.gov/geo/, accession GSE46190.

Statistical analysis

There was no apparent association between histologic tumor-type and the presence of PDGFRA mutation in pediatric HGG as assessed by exact χ² 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 χ² test for independence. Student 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 Supplementary 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 (Supplementary 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, N6468S, generated a new splice site that removed 13 amino acids (E10del2; Supplementary Fig. S1A and Table S1). Sixty percent (9 of 15) of the mutations were present as heterozygous alleles and 40% (6 of 15) of mutations were also amplified. Single-nucleotide polymorphism array analyses previously showed copy number imbalances for the majority of the samples, showing 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 histopathologic features (P = 0.26). Three mutations were found in anaplastic astrocytomas (3 of 24), one in an anaplastic oligodendroglia (1 of 6), and the remaining mutations in grade 4 glioblastomas, including two DIPGs (11 of 102; Supplementary Table S1 and S2). Both
that would have been missed by exonic sequencing. We found alterations in glioblastoma. We used RT-PCR to screen for these alterations previously shown in adult HGG occur frequently in pediatric different regions of the receptor were generated for functional studies. p53-null 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 (Supplementary 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 PMA 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, whereas 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 with 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 pMAPK pathway components but had only modest effect on the MAPK pathway activation (Fig. 2A and Supplementary 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

Figure 1. **PDGFRα somatic mutations identified in pediatric HGGs and DIPGs.** Sanger sequencing of **PDGFRα** 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 **PDGFRα** locus. The signal peptide is designated by an orange oval and the transmembrane domain by a brown rectangle.

**PDGFRα mutants are constitutively active**
To better understand the consequence of PDGFRα mutation in pediatric gliomagenesis, retroviral constructs expressing wild-type PDGFRα or six selected PDGFRα mutants that affect different regions of the receptor were generated for functional studies. p53-null 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 (Supplementary 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 PMA 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, whereas 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 with 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 pMAPK pathway components but had only modest effect on the MAPK pathway activation (Fig. 2A and Supplementary 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).
of three independent experiments is shown.

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 Supplementary Fig. S3A). D842V and V544ins were the only two mutants that induced a substantial increase in SHP2 phosphorylation (Supplementary Fig. S2) and the only two mutants for which crenolanib induced a substantial decrease in phospho-SHP2 (Supplementary Fig. S3A).

The effect of each inhibitor on cell proliferation was examined by XTT assay (Fig. 3B and Supplementary Fig. S3B), using a concentration greater than the IC_{90} determined to block receptor phosphorylation (Supplementary Fig. S4). Crenolanib selectively inhibited proliferation of PDGFRα-expressing cells, both wild-type and all analyzed mutants, whereas it did not affect proliferation of the empty vector control cells (Fig. 3B and Supplementary Fig. S3B). In contrast, dasatinib exerted broad antiproliferative 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 with other mutants (Fig. 3B). Crenolanib and dasatinib both exert cytostatic effects on PDGFRα-expressing p53-null PMAs, arresting cells in G0 (Supplementary Fig. S5).

PDGFRα mutations are oncogenic in vivo and drive development of HGGs

To determine whether expression of wild-type PDGFRα or PDGFRα mutants renders p53-null PMAs tumorigenic, 2 million cells were implanted intracranially into the parietal lobe of 2-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 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 to 119 days and the single tumor from wild-type PDGFRα, which occurred at 120 days after implantation (Fig. 4A). Standard histopathologic preparations from all tumors were evaluated (by D.W. Ellison) and classified according to WHO criteria (Fig. 4B and Supplementary Table S3). Overall, 63% (44 of 70) of tumors analyzed were grade 3 anaplastic astrocytoma, 26%...
(18 of 70) were grade 3 anaplastic oligoastrocytoma, 10% (7 of 70) were grade 4 glioblastoma, and the single tumor that developed from cells expressing wild-type PDGFRα was the only low-grade tumor, a grade 2 oligoastrocytoma. Tumors expressing the D842V or V544ins mutation were significantly associated with anaplastic astrocytoma histology \( (P < 0.0001 \text{ and } P = 0.0012, \text{ respectively}) \) 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 HGGs including an easily visualized focal mass as well as diffuse infiltration into the normal brain parenchyma (Fig. 4C and Supplementary 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 showed 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 with normal cortex. In contrast, levels of total STAT3 and phosphorylated STAT3 were similarly increased in all analyzed tumors compared with 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 Supplementary 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. In addition, gene expression profiles of EGFRvIII-driven murine HGGs were assayed for comparison (25). UHC segregated the tumors into two distinct subgroups (Supplementary Fig. S7). Interestingly, PDGFRα and EGFRvIII-expressing tumors were distributed between 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 GSEA the gene expression profile of each murine tumor was compared with 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; refs. 30, 31). We also compared the gene expression signatures of seven independent untransduced p53-null PMAs, 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; ref. 30). Of note, 37.8% (14 of 37) of PDGFRα tumors showed proneural, 32.4% (12 of 37) proliferative, and 16.2% (6 of 37) mesenchymal subgroup expression signatures. There was no significant association of specific PDGFRα 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 PDGFRA are frequent in pediatric HGGs. Here, targeted sequencing identified novel activating somatic mutations of PDGFRA in pediatric HGGs. Two recent studies
used to generate oncogenic mutations of different functional domains, and all were constitutively active mutants analyzed represented alterations in different regions of the PDGFR extracellular domain and constitutive receptor activation. 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 PDGFR 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 PDGFRα 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, whereas all six of the mutants induced HGG 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, whereas the early passage p53-null PMAs in the present study did not form any tumors. In addition, 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, whereas 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 PDGFR amplification versus mutation with or without amplification. Amplification of wild-type PDGFR occurred more frequently in tumors within the brainstem (26%, 11 of 43 DIPG vs. 11%, 9 of 84 nonbrainstem HGG, P = 0.04), whereas PDGFRα sequence

Figure 5. Gene expression profiles of murine PDGFRα-driven HGGs resemble human HGGs. Heat map of single sample GSEA 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 (OPC, oligodendrocyte progenitor cells; Oligo, oligodendrocyte; N, neurons; A, astrocytes; and CA, cultured astroglia). The order of the tumor samples is arranged on the basis of the UHC analysis using 1,000 most variable probe sets, which revealed two major expression subgroups HC1 and HC2 (Supplementary Fig. S7). The histology of each tumor is indicated above the heatmap: anaplastic astrocytoma (purple), anaplastic oligoastrocytomas (green), glioblastoma (brown), oligoastrocytoma (yellow), and white marks tumors with no diagnosis available. Untransduced p53-null PMA cultures are marked with black.
mutations were more common in pediatric HGG arising outside the brainstem, although this was not statistically significant (14%, 13 of 90 nonbrainstem HGG vs. 5%, 2 of 43 DIPG, \(P = 0.14\); refs. 4, 5). Moreover, all six samples with concomitant amplification and mutation of PDGFRA were from nonbrainstem tumors. Given the higher frequency of wild-type amplification in DIPGs, it is possible that wild-type PDGFRA requires a more specific or alternative cell of origin to drive transformation compared with the mutants, which may not be adequately recapitulated in our experimental system. These effects can vary among oncogenic mutations. For example EGFRIII drives tumorigenesis equivalently from Ink-4a/Arf-null cortical astrocytes or neural stem cells, whereas BRAF duplications show a differential growth response in cerebellar neural stem cells compared with astrocytes, and in neural stem cells from the cerebellum compared with those from neocortex (50, 51). Furthermore, comparison of DIPG and nonbrainstem 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 nonbrainstem 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 PDGFRA-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 (oligodendrocytic) phenotype. Because tumors were generated with virally transduced astrocytes, this suggests that in a subset of tumors, signaling activated by mutated PDGFRA triggered astrocyte dedifferentiation as described for tumors induced by PDGF 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 into the brain induced gliomas that had a predominantly proneural signature, perhaps driven in part by the endogenous cells that expressed PDGF and responded 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 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 PDGFRA.

Because 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 HGGs 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 EGFRI 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 PDGFRA 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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