

**EGFRvIII Deletion Mutations in Pediatric High-Grade Glioma and Response to Targeted Therapy in Pediatric Glioma Cell Lines**

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**Abstract Purpose:** The epidermal growth factor receptor (EGFR) is amplified and overexpressed in adult glioblastoma, with response to targeted inhibition dependent on the underlying biology of the disease. EGFR has thus far been considered to play a less important role in pediatric glioma, although extensive data are lacking. We have sought to clarify the role of EGFR in pediatric high-grade glioma (HGG).

**Experimental Design:** We retrospectively studied a total of 90 archival pediatric HGG specimens for EGFR protein overexpression, gene amplification, and mutation and assessed the *in vitro* sensitivity of pediatric glioma cell line models to the small-molecule EGFR inhibitor erlotinib.

**Results:** Amplification was detected in 11% of cases, with corresponding overexpression of the receptor. No kinase or extracellular domain mutations were observed; however, 6 of 35 (17%) cases harbored the EGFRvIII deletion, including two anaplastic oligodendrogliomas and a gliosarcoma overexpressing EGFRvIII in the absence of gene amplification and coexpressing platelet-derived growth factor receptor  $\alpha$ . Pediatric glioblastoma cells transduced with wild-type or deletion mutant EGFRvIII were not rendered more sensitive to erlotinib despite expressing wild-type PTEN. Phosphorylated receptor tyrosine kinase profiling showed a specific activation of platelet-derived growth factor receptor  $\alpha/\beta$  in EGFRvIII-transduced pediatric glioblastoma cells, and targeted coinhibition with erlotinib and imatinib leads to enhanced efficacy in this model.

**Conclusions:** These data identify an elevated frequency of *EGFR* gene amplification and *EGFRvIII* mutation in pediatric HGG than previously recognized and show the likely necessity of targeting multiple genetic alterations in the tumors of these children. (Clin Cancer Res 2009;15(18):5753–61)

Amplification, overexpression, and/or mutation of the epidermal growth factor receptor (EGFR) represent a compelling set of molecular genetic indicators for targeted therapy in adult glioblastoma. About 40% of glioblastomas show amplification of the *EGFR* gene locus, and about half of these tumors express a mutant receptor (EGFRvIII) that is constitutively active due to an in-frame truncation within the extracellular ligand-binding domain (1). In addition, novel missense mutations have been

reported in the extracellular domain of tumors and cell lines (2), and recently, additional mutations have been described outside of these regions (e.g., in the transmembrane domains), although the significance of these is not yet clear (3).

EGFRvIII is caused by deletion of exons 2 to 7, resulting in a protein that lacks a ligand-binding domain and is constitutively activated and is further resistant to down-regulation due to a low rate of receptor endocytosis (4). EGFRvIII has been shown

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### Translational Relevance

Dysregulation of epidermal growth factor receptor (EGFR) is common in adult glioblastoma but has been considered less so in the pediatric setting, although unequivocal data have been lacking. This article details the largest and most comprehensive study of EGFR gene alterations in high-grade glioma of childhood yet undertaken. In addition to an elevated frequency of gene amplification in comparison with previous smaller series, we have identified for the first time the presence of EGFRvIII deletions in pediatric cases. By generating stably EGFRvIII-overexpressing pediatric glioblastoma cell lines, we show that the deletion is not sufficient to confer increased sensitivity to the EGFR inhibitor erlotinib due to the specific coactivation of platelet-derived growth factor receptor  $\alpha/\beta$  in these cells. Thus, we provide evidence for a novel, pediatric-specific mechanism for erlotinib resistance in glioblastoma.

to confer enhanced tumorigenicity both in human glioma cells *in vitro* and in xenografts in immunodeprived mice (5, 6), and in a xenograft model, tumorigenicity is directly proportional to EGFRvIII receptor load (7). EGFRvIII has also been shown to confer resistance to both radiotherapy and chemotherapy (8, 9). Constitutive activation by EGFRvIII stimulates proliferation and inhibits apoptosis predominantly through stimulation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway but also via the Ras/mitogen-activated protein kinase (MAPK) signal transduction pathway (10, 11). These data may explain the observation that patients with EGFRvIII-expressing tumors have a shorter interval to clinical relapse and poorer survival than patients with EGFRvIII-negative tumors (12).

Erlotinib (Tarceva, OSI-774) is a selective inhibitor of the EGFR tyrosine kinase and has shown activity in adult high-grade glioma (HGG) patients (13). A differential therapeutic response has been reported in these cases, with a variety of factors reported to be predictive for treatment efficacy, including activating mutations of *EGFR* and a wild-type *PTEN* (14, 15). Published preclinical data show equivocal results in adult glioblastoma model systems engineered to overexpress the deletion mutant. Erlotinib has been shown to inhibit EGFRvIII, blocking constitutive EGFRvIII kinase activity and the growth of EGFRvIII-transformed cells (15, 16). Furthermore, long-term exposure to erlotinib decreased EGFRvIII expression in transformed cells and selectively down-regulated the EGFRvIII-mediated induction of oncogenes that drive invasion in transformed glioblastoma cells (17). By contrast, other reports have shown that *EGFR* amplification/mutation even in the presence of functional *PTEN* did not render sensitivity to EGFR inhibitors (18, 19). This combination of alterations may therefore be necessary but not sufficient for conferring glioblastoma sensitivity to EGFR kinase inhibition (20). Consequently, one could infer the existence of additional determinants of glioblastoma sensitivity to EGFR kinase inhibition yet to be identified. For instance, activating mutations of *PIK3CA*, as recently shown in adult and pediatric glioblastomas, could account for tumor insensitivity to EGFR inhibition.

HGGs in children seem to be clinically and biologically distinct from their adult counterparts. Glioblastomas in adults are classified as primary or secondary based on progression from preexisting low-grade lesions and on distinct patterns of molecular abnormalities (21). Although some molecular abnormalities encountered in pediatric cases are reminiscent of secondary adult glioblastoma [*TP53* mutation (22) and platelet-derived growth factor receptor  $\alpha$  (PDGFR $\alpha$ ) overexpression (23)], these neoplasms rarely originate from preexisting low-grade lesions (24). Unfortunately, our understanding of these differences is restricted by the relative paucity of molecular data derived from pediatric tumors.

EGFR has been considered to play a less important role in pediatric HGG, although this issue is compounded by the limited available data. The receptor seems to be frequently overexpressed, usually in the absence of gene amplification, with reports varying from 10% to 80% of pediatric nonbrain stem HGGs (25–29). Interestingly, the primary site of the tumor may affect the type of genetic changes involved, with data indicating that *TP53* mutation and *EGFR* overexpression are more frequent in diffuse intrinsic pontine glioma than in supratentorial sites in children (25).

We have sought to clarify the role of EGFR in pediatric HGG and to assess the *in vitro* sensitivity of pediatric glioma cell line models to erlotinib. These data identify a higher prevalence of *EGFR* gene amplification and *EGFRvIII* mutation in pediatric HGG than previously recognized. We further investigated the potential of treatments targeting the receptor in deletion mutant-positive cases.

### Materials and Methods

**Tumor samples.** HGG samples from 90 patients were obtained after approval by Local and Multicenter Ethical Review Committees. The collection consisted of 53 glioblastoma multiforme, 16 anaplastic astrocytomas, 3 anaplastic oligodendrogliomas, 3 brainstem gliomas, and 25 other WHO grade 3 or 4 lesions. All cases were archival formalin-fixed, paraffin-embedded (FFPE) tissues. The presence of tumor tissue in these samples and the tumor type was verified on a H&E-stained section independently by two neuropathologists (D.W.E. and S.A.-S.).

**Immunohistochemistry and chromogenic *in situ* hybridization.** Immunohistochemistry was done on representative 4- or 5- $\mu$ m FFPE sections. EGFR overexpression was assessed using the mouse monoclonal antibody 31G7 (Zymed) at a dilution of 1:50 using the Envision-HRP system (K4006, Dako) as previously described (30). EGFRvIII was assessed using a 1:100 dilution of the mouse monoclonal antibody G100 (Zymed) as a primary and antigen retrieval by microwaving in 10 mmol/L citrate buffer (pH 6.0) for 3  $\times$  5 min (31). PDGFR $\alpha$  was assessed using the polyclonal rabbit primary antibody RB-1691 (LabVision) at 1:150 dilution with 15 min antigen retrieval in 10 mmol/L citrate buffer (pH 6.0; ref. 32). Chromogenic *in situ* hybridization (CISH) was done using Spot-Light amplification probes for *EGFR* (Zymed) according to the manufacturer's protocol and as reported earlier (30). Cases were considered to be amplified for EGFR when >50% of the neoplastic cells harbored (a) more than five signals per nuclei or (b) large gene copy clusters. Immunohistochemical and CISH analyses were done with observers blinded to the results of *EGFR* and sequencing data.

**Mutation analysis.** Genomic DNA was isolated from representative 10- $\mu$ m-thick unstained tissue sections containing >85% tumor cells, as determined from a serial H&E-stained section, using the QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions. Exons 2 to 8, coding for the extracellular domain of EGFR, and exons 18 to 21, coding for the receptor tyrosine kinase (RTK) domain of EGFR, were

amplified by PCR (33). Products were purified using the QIAquick PCR Purification kit and subjected to bidirectional sequencing using BigDye Terminator Mix 3.1 (Applied Biosystems) according to the manufacturer's instructions. Capillary sequencing was done in duplicate on an ABI 3100 genetic analyzer (Applied Biosystems), and sequences were evaluated for the presence of mutations using Mutation Surveyor Software (SoftGenetics LLC). A reverse transcription-PCR (RT-PCR) was developed for detection of the EGFRvIII mutation from FFPE sections. RNA was extracted from representative 10- $\mu$ m-thick unstained tissue sections containing >85% tumor cells, as determined from a serial H&E-stained section, using the Recover-All kit (Ambion). cDNA was synthesized from 200 ng RNA using SuperScript II reverse transcriptase (Invitrogen Ltd.). The deletion region was amplified with primers in exon 1 (5'-GGGCTCTGGAGGAAAAGAAA-3') and exon 9 (5'-CCTCCATCTCAGTAGCTGTCG-3') amplifying a region of 91 bp when the deletion is present and 892 bp in the wild-type. The presence of the EGFRvIII fragment was confirmed in duplicate by direct sequencing.

**Cell culture and generation of isogenic cell lines.** Glioblastoma cell lines U87MG and KNS42 were obtained from the American Type Culture Collection (LGC Promochem) and Japan Cancer Research Resources [s] cell banks, respectively. SF188 was kindly provided by Dr. Daphne Haas-Kogan (University of California at San Francisco, San Francisco, CA), whereas UW479, Res259, and Res186 were kindly provided by Dr. Michael Bobola (University of Washington, Seattle, WA). Cells were grown as monolayers in DMEM/F12 Ham's medium + 10% FCS in 5% CO<sub>2</sub>. EGFR constructs for isogenic cell lines were created by PCR's done using Phusion DNA polymerase enzyme (Finnzymes) to minimize the risk of error. Full-length wild-type EGFR was amplified from A431 cell line cDNA using the forward primer 5'-TCTTCGGCTAGCAACGATGCGACCCCTC-3' and the reverse primer 5'-ATGCGGCCGCTCACTATCCTCCGTGGT-3', which contained *NheI* and *NotI* enzyme restriction sites, respectively. The EGFRvIII mutant was created using splice overlapping extension PCR. Two PCR's were done to generate two fragments with overlapping ends surrounding the deleted region (exon 1: forward, 5'-TCTTCGGCTAGCAACGATGCGACCCCTC-3'; reverse, 5'-TGTCACCACATAATTACCTTTTCITTTCCCTCCAGAGCC-3'; exon 8: forward, 5'-GGAAAAGAAAGGTAATTATGTGGTGACAGATCACGGC-3'; reverse, 5'-GCCGCGTATGATTCTAGATTCTCAAAGGC-3'). These fragments were then combined in a subsequent "fusion" PCR in which the overlapping ends annealed, allowing 3' overlap of each strand to serve as a primer for the 3' extension of the complementary strand. The result was a fragment of 499 bp with EGFRvIII deletion and *NheI* and *XbaI* restriction sites at the 3' and 5' ends. The remaining EGFR cDNA sequence was amplified using forward primer 5'-GAATCTAGAAATCATACGGCGCAGGACC-3' and reverse primer 5'-ATGCGGCCGCTCACTATCCTCCGTGGT-3', which contained *XbaI* and *NotI* enzyme restriction sites. The vector F527 (34) was digested by *NheI* and *NotI* and the PCR fragments by the enzyme corresponding to their restriction sites. The cDNA fragment was then extracted from an agarose gel and purified using the QIAquick Gel Extraction kit (Qiagen). The ligation of inserts and vector F527 was done using T4 DNA ligase (Invitrogen). Electrocompetent XL1 blue *Escherichia coli* (kindly provided by Betsy Julienne, Institut de Cancérologie Gustave Roussy, Villejuif, France) was transformed by electroporation in the presence of the ligation products, and selection was done by bacterial culture on ampicillin LB agar Petri dishes. Colonies showing the correct orientation by restriction digestion were further designated to sequencing. The DNA from plasmid with the correct sequence was then efficiently isolated from *E. coli* using Maxiprep using the PureLink HiPure Plasmid Filter Purification kit (Invitrogen). U87MG and SF188 were then transfected with either the F527 empty vector, or F527-EGFR wild-type or F527-EGFRvIII plasmids using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Stable clones were obtained after 4 wk of antibiotic selection by 1  $\mu$ g/mL puromycin.

**Western blot analysis.** Cells at 80% confluence were trypsinized, washed with PBS, and lysed for 1 h at 4 °C in lysis buffer (Cell Signaling) and a complete mini protease inhibitor cocktail (Roche Diagnostics).

Cells were then centrifuged at 11,000 rpm at 4 °C for 15 min, and protein concentration was determined (bicinchoninic acid assay, Pierce). Total protein extracts (30  $\mu$ g/lane) were separated electrophoretically in 4% to 20% SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Invitrogen). Immunodetection was done using antibodies directed against EGFR, phosphorylated and total extracellular signal-regulated kinase 1/2, phosphorylated/total Akt, and PTEN (all 1:1,000; Cell Signaling) as well as glyceraldehyde-3-phosphate dehydrogenase (1:2,000; Chemicon). Blots were revealed with peroxidase-conjugated secondary anti-rabbit or anti-mouse antibodies (GE Healthcare) followed by enhanced chemiluminescence solution (GE Healthcare).

**Detection of phosphorylated RTKs.** The levels of phosphorylated and total wild-type EGFR were determined from 2.5  $\mu$ g of protein lysate by an electrochemiluminescent immunoassay (MesoScale Discovery) according to the manufacturer's instructions. A phosphorylated RTK assay (R&D Systems) was used to screen multiple RTKs. Cells were lysed in radioimmunoprecipitation assay lysis buffer (Cell Signaling) and a complete mini protease inhibitor cocktail (Roche Diagnostics), as described for Western blot analysis. Phosphorylated PDGFR $\alpha/\beta$  was measured in a sandwich ELISA assay (Cell Signaling) according to the manufacturer's instructions.

**Growth inhibition studies.** Erlotinib (Euroasian Chemicals PVT Ltd.), imatinib (LC Laboratories), the dual PI3K and mammalian target of rapamycin inhibitor PI-103 (provided by Piramed Ltd.), and the MAPK/extracellular signal-regulated kinase kinase (MEK) inhibitor PD325901 (provided by Dundee University, Dundee, United Kingdom) were obtained in powder form and diluted in either DMSO or water at 10 mmol/L stock solutions. Growth inhibition was determined using the sulforhodamine B (SRB) assay as described previously (35). Briefly, 10<sup>3</sup> cells were seeded into 96-well microtiter plates and allowed to attach for 36 h. Compounds at a range of concentrations were added in quadruplicate wells for 6 d (at least three doubling times) in a volume of 200  $\mu$ L/well. Cells were then fixed with ice-cold 10% trichloroacetic acid for 30 min, then washed with tap water, and dried. The plates were subsequently stained with 0.4% SRB in 1% acetic acid for 15 min, and excess stain was washed off with 1% acetic acid. After the plates were dried, the stain was solubilized with 10 mmol/L Tris base, and the absorbance was determined at 570 nm. The IC<sub>50</sub> was calculated as the drug concentration that inhibits cell growth by 50% compared with control growth using GraphPad Prism 4 program (GraphPad Software). All values are given as mean  $\pm$  SD of at least three independent experiments.

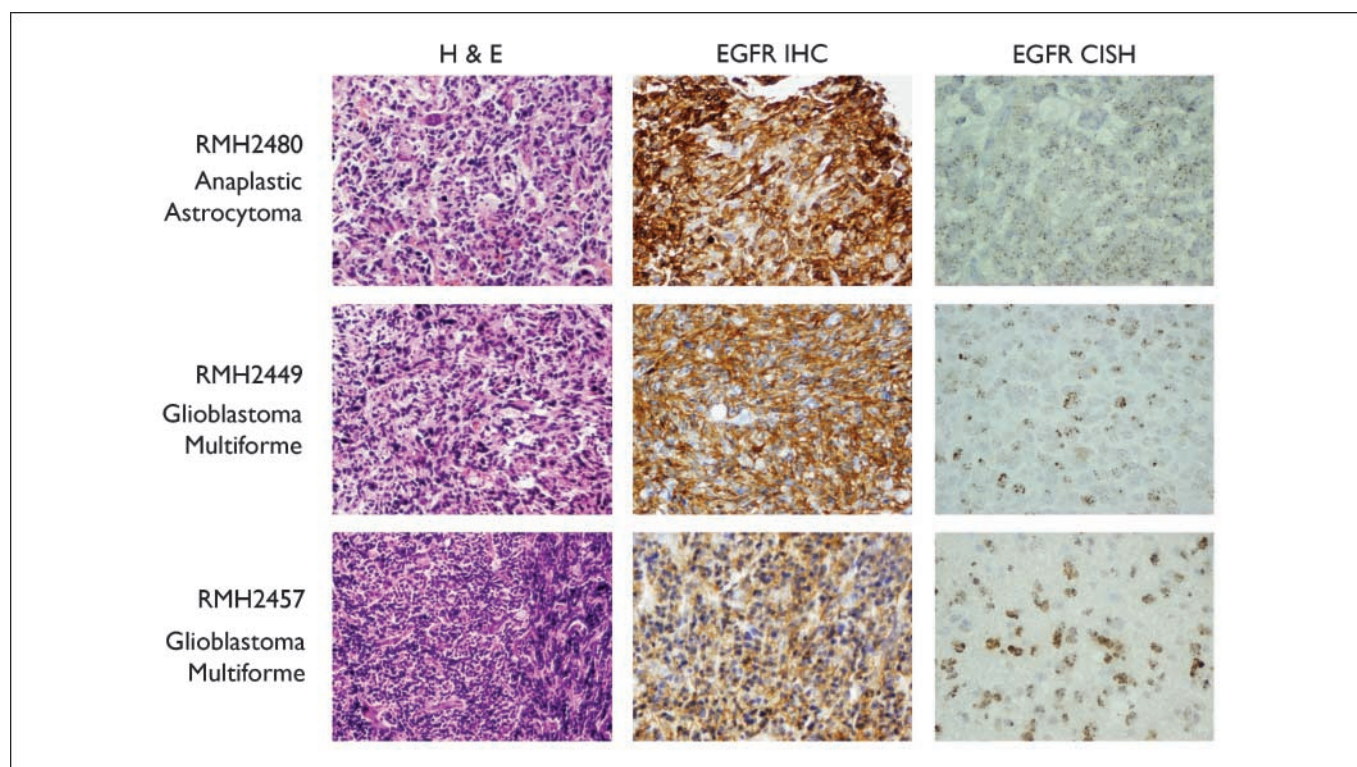
**Statistical analysis.** All statistical tests were done in R2.6.1.<sup>10</sup> Correlations between categorical values were done using the  $\chi^2$  and Fisher's exact tests. Correlations between continuous variables were done using Student's *t* test or the Mann-Whitney *U* test. Cumulative survival probabilities were calculated using the Kaplan-Meier method, with differences between survival rates analyzed with the log-rank test. All tests were two tailed, with a confidence interval of 95%. *P* values of <0.05 were considered statistically significant.

## Results

**EGFR is amplified and overexpressed in pediatric HGGs.** We collected a series of 90 HGGs from patients under the age of 19 years (median, 11.3 years) for molecular analysis. All samples were archival FFPE pathology specimens, with variable quantity and quality of material available as sections for immunohistochemistry or DNA/RNA extraction. To evaluate gene dosage and receptor expression of EGFR, we used CISH and immunohistochemistry on tissue sections.

We observed gene amplification in 8 of 74 (11%) evaluable cases. These consisted of 6 glioblastomas (6 of 43, 14%) and 2 anaplastic astrocytomas (2 of 11, 18%). No amplifications were

<sup>10</sup> <http://www.r-project.org/>



**Fig. 1.** EGFR expression and amplification in pediatric HGG. H&E-stained sections, immunohistochemistry for wild-type EGFR, and EGFR CISH for three representative cases of pediatric HGG. Amplifications were present either as discrete signals at a copy number of 9 to 10 per nucleus (RMH2480), in scattered cells throughout the tumor (RMH2449), or in dense clusters in the vast majority of the tumor cells (RMH2457).

seen in any other high-grade lesion. Polysomy of chromosome 7 was noted in an additional 14 of 74 (19%) cases and included 10 cases of glioblastoma, 3 anaplastic astrocytoma, and a gliosarcoma.

These amplifications were mostly present as dense clusters of nuclear signals representing a high-level DNA copy number gain in the vast majority of the tumor cells (Fig. 1, RMH2457); however, additional patterns were also observed. These included discrete signals at a copy number of 9 to 10 per nucleus, observed in a case of anaplastic astrocytoma (Fig. 1, RMH2480), or high-density clusters, present only in scattered cells throughout the tumor, in a glioblastoma (Fig. 1, RMH2449).

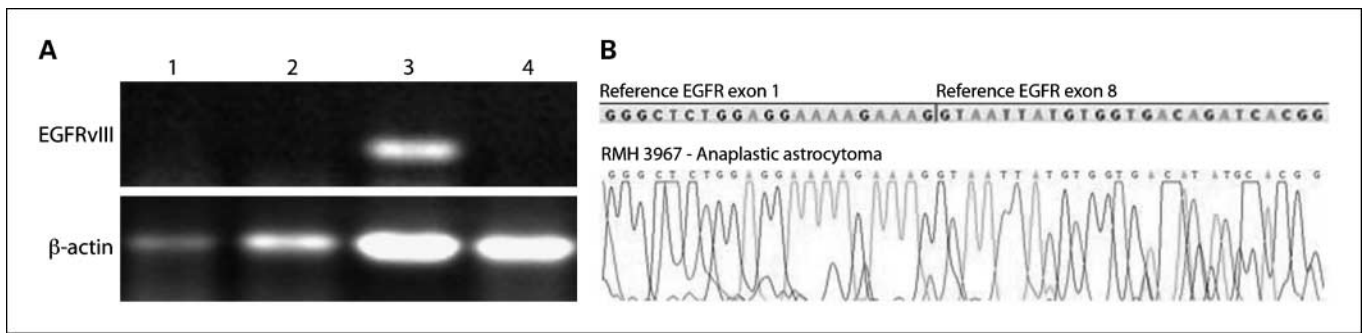
All cases of pediatric HGG harboring *EGFR* amplification were found to express the protein at high levels (Fig. 1). There was no association between *EGFR* amplification and clinical outcome ( $P = 0.457$ , log-rank test for overall survival) or age at diagnosis ( $P = 0.591$ , Mann-Whitney *U* test).

**Pediatric HGGs harbor *vIII* deletions but no activating mutations in the extracellular or kinase domains.** We further screened our pediatric HGG cases for mutations in the *EGFR* gene. Previously reported activating mutations in the kinase domain (in non-small cell lung cancer and others) and the extracellular domain (adult glioblastoma) were screened by PCR amplification of exonic sequences and direct sequencing. We observed no mutations in 60 (kinase domain) and 66 (extracellular domain) pediatric cases. Although the lack of kinase domain mutations is consistent with data from adult glioblastoma, the absence of extracellular domain mutations in our pediatric series is significantly discordant with the published frequency of 18 of 132 (13.6%; ref. 2) in adult glioblastoma ( $P = 0.0019$ , Fisher's exact test).

In addition, we developed a RT-PCR assay applicable to FFPE samples to screen for the presence of the *EGFRvIII* deletion mutation. A product of 91 bp spanning exons 1 and 8 was generated from extracted mRNA in cases harboring deletion of exons 2 to 7, which was verified by direct sequencing (Fig. 2). We detected six cases of *EGFRvIII* in 35 assessable cases (17%). These included 1 of 20 (5%) glioblastoma, a single case of gliosarcoma, and an increased frequency in anaplastic astrocytoma (2 of 6, 33%) and anaplastic oligodendroglioma (2 of 3, 67%). Despite the small numbers, these latter cases had a statistically significant increased frequency compared with other pediatric HGG ( $P = 0.036$ , Fisher's exact test). Interestingly, the glioblastoma and anaplastic astrocytoma cases were *EGFR* amplified, whereas the anaplastic oligodendroglioma and gliosarcomas were not. A summary of mutation data is given in Table 1.

**Pediatric glioma cell lines and response to erlotinib.** In addition to the primary tumors, we also screened our panel of pediatric glioma cell lines for *EGFR* mutations as described above. None of five lines harbored any point mutations, deletions, or amplifications nor significant levels of protein or mRNA expression of the receptor (data not shown). Treatment with the small-molecule *EGFR* inhibitor erlotinib revealed a general lack of sensitivity, with an  $IC_{50}$  of  $>50 \mu\text{mol/L}$  for most of the pediatric cell lines. The most sensitive cell line was the glioblastoma SF188 line ( $IC_{50} = 8.3 \pm 0.7 \mu\text{mol/L}$ ) derived from an 8-year-old male patient, which is *PTEN* wild-type and harbors mutant *TP53*.<sup>11</sup>

<sup>11</sup> Bax DA\*, Little SE\*, Gaspar N, et al. Molecular and phenotypic characterisation of paediatric high grade glioma cell lines as models for preclinical drug development PLoS ONE 4: e5209.



**Fig. 2.** Detection of EGFRvIII deletions in pediatric HGG. *A*, lane 3, RT-PCR amplifying a product of 91 bp in EGFRvIII-positive cases. *B*, sequencing of the RT-PCR product established the presence of sequence spanning exons 1 and 8 of the *EGFR* gene.

Therefore, to study the effects of EGFR overexpression and vIII deletion on pediatric glioma cells, we constructed isogenic cell line models using the *PTEN* wild-type pediatric glioblastoma cell line SF188 as well as the *PTEN*-deleted adult glioblastoma line U87MG. We successfully transduced either EGFR wild-type or vIII into both cell lines, resulting in stable protein overexpression (Fig. 3A). Treatment of these cell line models with erlotinib inhibited both PI3K and MAPK pathways and induced a G<sub>1</sub> arrest in all cells (Supplementary Fig. S1) but revealed no statistically significant differences in efficacy between EGFR-transduced (wild-type and vIII) and parental/empty vector for both U87MG and SF188 (Fig. 3B). Isolated clones showed a modest (2-fold) increase in sensitivity when overexpressing the vIII deletion in both cell lines; however, this was not generalizable. This was despite showing that vIII-transduced cells harbored constitutive activation of the receptor, as determined by an electrochemiluminescent immunoassay (detecting transactivation of the endogenous EGFR rather than the mutant) and also phosphorylated RTK array analysis (detecting both wild-type and deletion mutant phosphorylation directly).

**Mechanisms of resistance to erlotinib in SF188 cells.** As EGFR signals through both PI3K and MAPK, we sought to determine whether the presence of either wild-type or mutant *EGFR* would render glioblastoma cells more sensitive to agents inhibiting these pathways. Regardless of *PTEN* status, there was no consistent change in efficacy of EGFR-transduced U87MG or SF188 cells with the dual PI3K/mammalian target of rapamycin inhibitor PI-103 (Fig. 4A; refs. 36, 37) or the MEK inhibitor PD325901 (Fig. 4B).

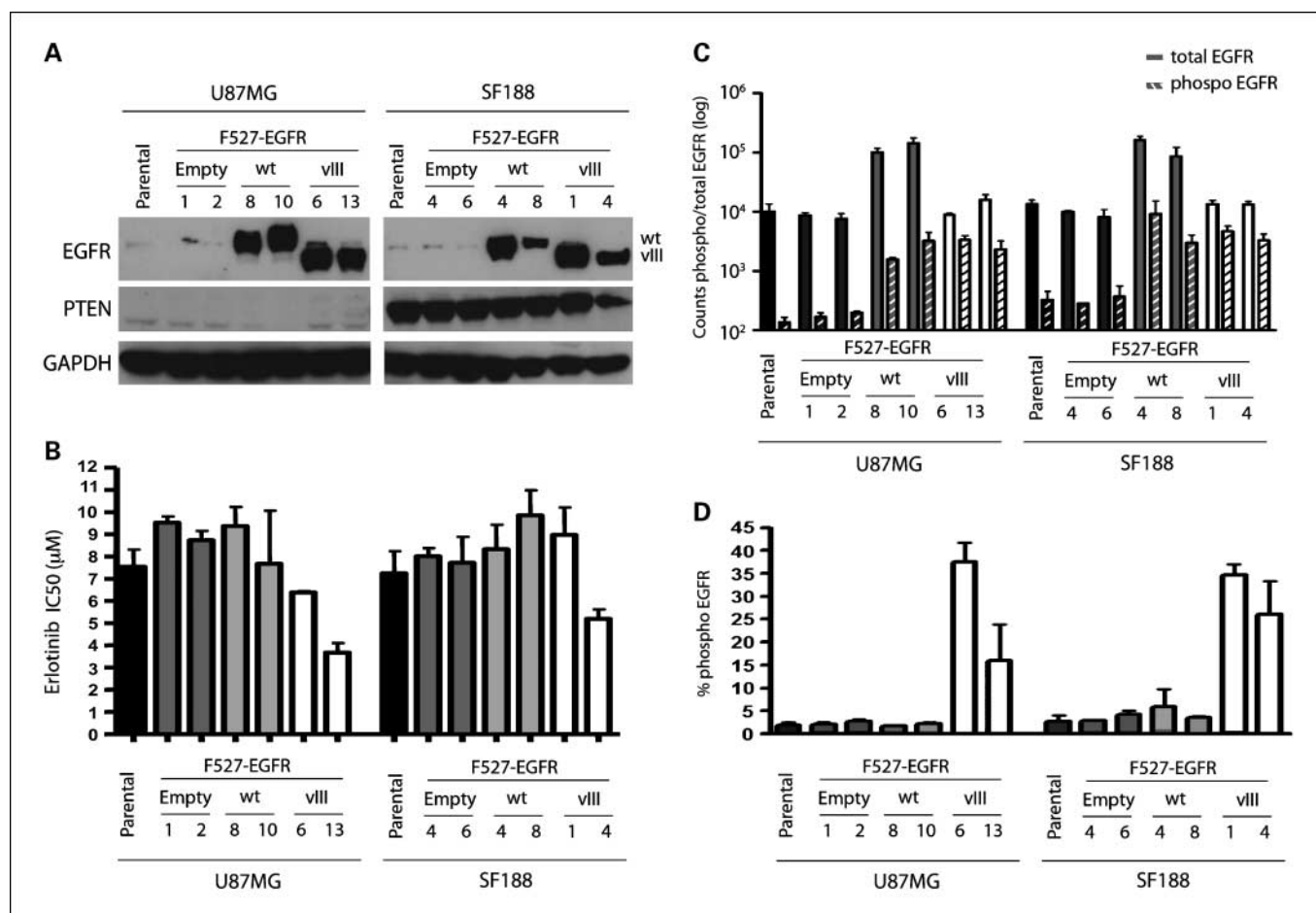
We next focused on the pediatric SF188 line to investigate the possible biological basis for the lack of increased efficacy of erlotinib in EGFR-overexpressing clones despite these cells harboring wild-type *PTEN*. Selecting the most erlotinib-sensitive clones transduced by EGFR, we first showed that there was no difference in response to small-molecule inhibition due to either mutant or wild-type EGFR expression over a period of 72 hours despite a modest diminution of downstream signaling in the EGFR mutant SF188 cells (data not shown).

In U87MG cells, there is coactivation of EGFR and MET, which allows for continued oncogenic RTK signaling in the presence of small-molecule inhibition of EGFR (38, 39). To look for similar association in our pediatric glioma cells, we assayed the SF188 clones with phosphorylated RTK antibody arrays (Fig. 5). There was only very low levels of constitutive EGFR activation in the control cells, reflecting the data from the electrochemiluminescent immunoassay (Fig. 3C and D), and no additional RTK activation. There was an increase in phosphorylated EGFR on transduction with the wild-type receptor and a further significant increase on constitutive activation by the EGFRvIII deletion mutant. In concert with this, we observed a significant up-regulation of phosphorylated PDGFR $\alpha$  and PDGFR $\beta$  as well as a modest increase in activated MER, Tie-2, and Flt-3 (Fig. 5A). The increased levels of phosphorylated PDGFR $\alpha/\beta$  were confirmed by a sandwich ELISA assay in our SF188 model (Supplementary Fig. S2) and were not seen in U87MG:EGFR wild-type or vIII mutant-overexpressing cells (data not shown). Four of the six (50%) pediatric HGG samples positive for EGFRvIII also showed strong expression of

**Table 1.** Summary of EGFR mutations in pediatric HGGs

|                              | WHO grade | Amplification (CISH) | vIII deletion (RT-PCR) | Extracellular domain | Kinase domain |
|------------------------------|-----------|----------------------|------------------------|----------------------|---------------|
| All pediatric HGG            | —         | 8/74 (11%)           | 6/35 (17%)             | 0/66                 | 0/60          |
| Glioblastoma multiforme      | IV        | 6/43 (14%)           | 1/20 (5%)              | 0/34                 | 0/30          |
| Brain stem glioma            | IV        | 0/3                  | 0/1                    | 0/3                  | 0/2           |
| Anaplastic astrocytoma       | III       | 2/11 (18%)           | 2/6 (33%)              | 0/12                 | 0/12          |
| Anaplastic oligodendroglioma | III       | 0/4                  | 2/3 (67%)*             | 0/4                  | 0/4           |
| Other pediatric HGG          | —         | 0/13                 | 1/5 (Gliosarcoma)      | 0/13                 | 0/12          |

\*Significantly higher than that seen in other tumors ( $P = 0.036$ , Fisher's exact test).



**Fig. 3.** Glioblastoma cell lines stably overexpressing the EGFRvIII deletion mutant have constitutive activation but not increased sensitivity to erlotinib. **A**, expression of EGFR and PTEN by Western blot in adult (U87MG) and pediatric (SF188) glioblastoma cell lines stably expressing EGFR wild-type (185 kDa) and vIII deletion mutation (145 kDa). U87MG cells are *PTEN* deleted, and SF188 cells express the wild-type protein. **B**, sensitivity of the isogenic cell lines to erlotinib, as determined by the SRB assay. There were no statistically significant differences in the IC<sub>50</sub> values of any of the cell lines. **C**, constitutive activation of the receptor, in EGFRvIII-overexpressing cell lines. Absolute counts for total and phosphorylated EGFR are plotted on a log<sub>10</sub> scale. **D**, percentage of phosphorylated EGFR to total protein, as determined by an electrochemiluminescent immunoassay, in the isogenic cell line models.

PDGFR $\alpha$  protein by immunohistochemistry (Fig. 5B), a greater frequency than the population as a whole (36%). Intriguingly, these four cases included all those EGFRvIII-positive cases in the absence of gene amplification (both anaplastic oligodendrogliomas and a gliosarcoma) as well as an anaplastic astrocytoma. The number of positive cases was too small to assign a statistical association ( $P = 0.14$ , Fisher's exact test) but shows the cosegregation of these abnormalities in the clinical setting.

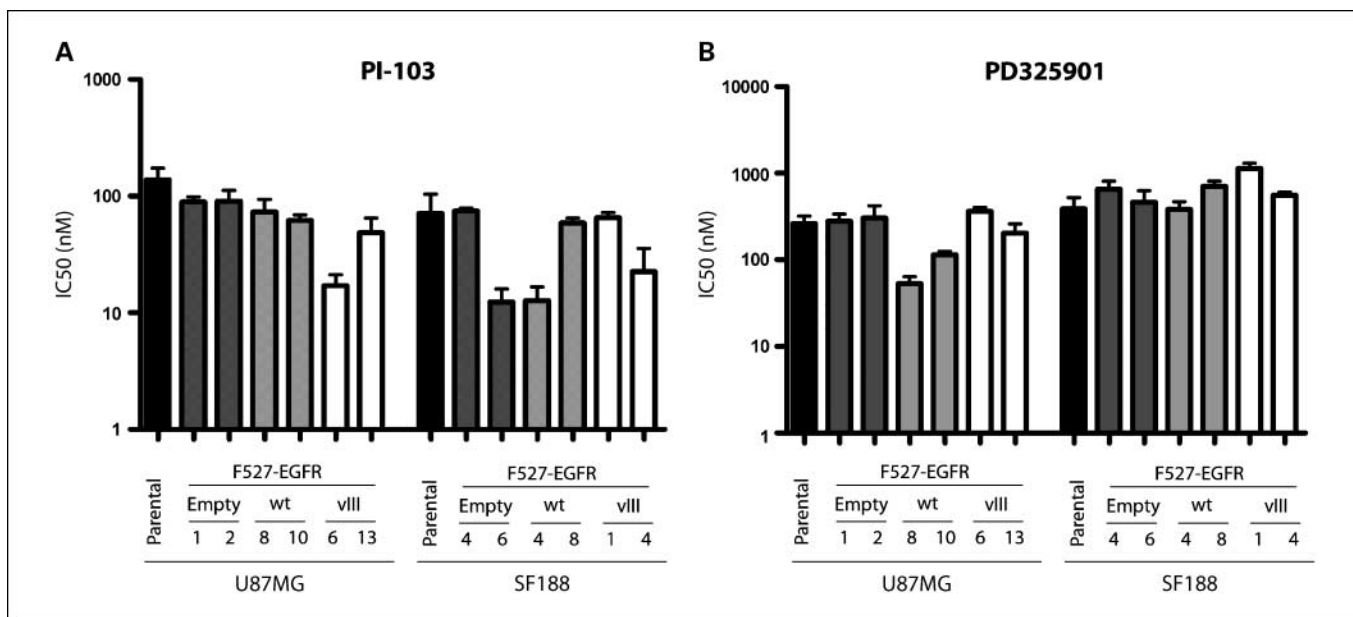
Finally, we tested whether inhibition of PDGFR would enhance the sensitivity of these cells to erlotinib treatment. We measured cell viability of the SF188:EGFRvIII cells treated with a fixed dose of 10  $\mu\text{mol/L}$  erlotinib alone and in combination with 10  $\mu\text{mol/L}$  imatinib. Treatment with a combination of both small molecules showed a significant decrease in cell viability compared with either compound alone ( $P < 0.01$ ,  $t$  test; Fig. 5C).

## Discussion

Although the oncogenic significance of activated EGFR signaling is well established in primary adult glioblastoma, in

the pediatric setting the picture is less clear. We have carried out the largest molecular analysis to date of EGFR in the HGGs of childhood and identified the presence of the EGFRvIII deletion mutation in pediatric cases.

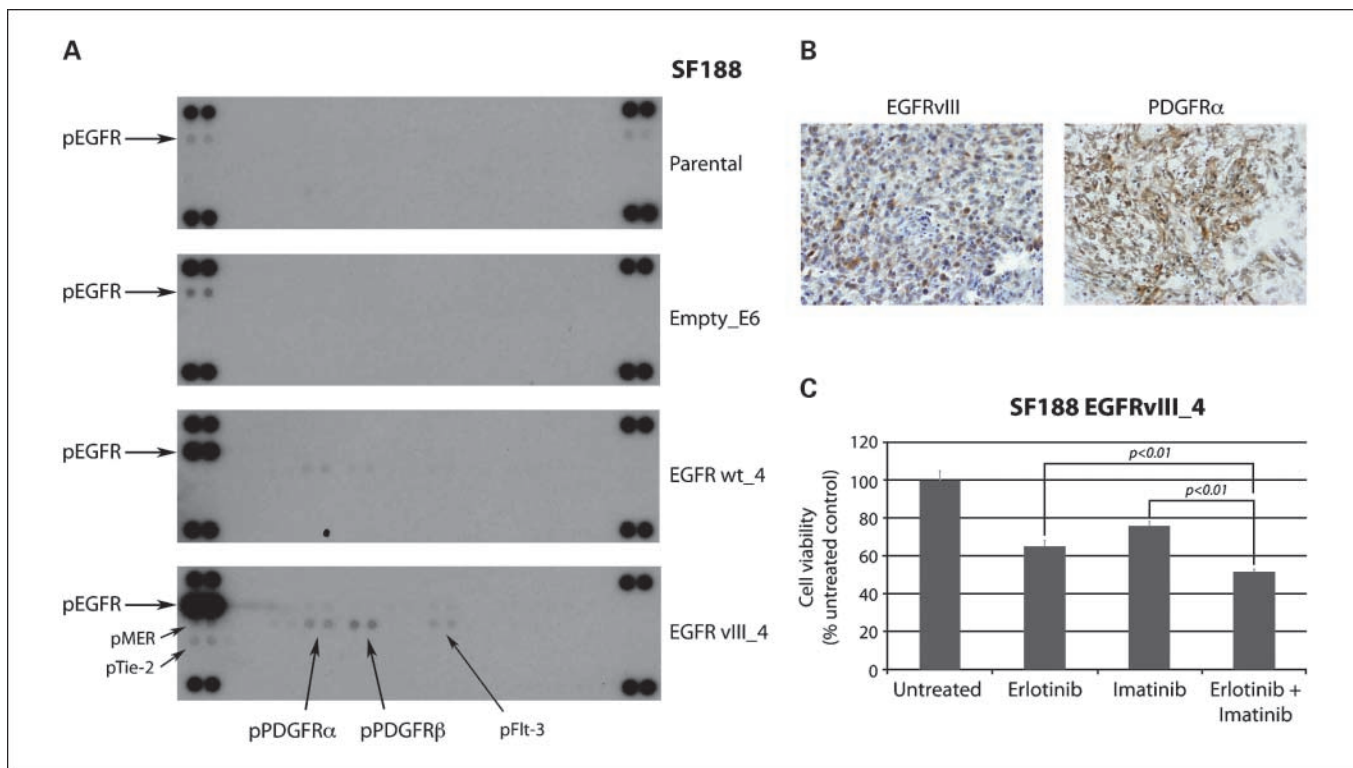
EGFRvIII was noted in WHO grade 4 glioblastoma and WHO grade 3 anaplastic astrocytoma cases in association with gene amplification and receptor overexpression. The few previous reports on EGFR in pediatric gliomas suggested that amplification was less common in pediatric glioblastomas than in adults, although the frequency in these small series ranged from 0% to 25%, measured using a variety of different assays on differing collections of tumor histologies (25–29). We observed an overall frequency of 11% amplification, with up to 14% in glioblastoma and 18% in anaplastic astrocytoma. Of particular interest was the observation that in some cases the high-density clusters of amplified probe signal were seen only in scattered cells throughout the tumor. This may explain the underestimation reported in earlier studies and highlights the benefit of using a technique such as CISH to determine gene amplification, as it allows histologic evaluation concurrent with the molecular analysis.



**Fig. 4.** Pediatric glioblastoma cell lines transduced with wild-type or vlll deletion mutant EGFR were not rendered more sensitive to inhibitors of the PI3K or MAPK pathways. Response of transduced U87MG and SF188 cells to inhibitors of PI3K/mammalian target of rapamycin (PI-103; A) and MEK (PD325901; B), as determined by the SRB assay. There were no statistically significant differences in the IC<sub>50</sub> values of any of the cell lines.

We further observed EGFRvIII expression, in the absence of gene amplification, in two of three WHO grade 3 anaplastic oligodendrogliomas and a single case of WHO grade 4 gliosarcoma. Although there are no reports to our knowledge on the presence

of EGFRvIII in pediatric anaplastic oligodendrogliomas, the deletion has previously been identified in an adult case (40), and more recently, we have observed 2 of 24 adult cases to harbor the deletion (31). Both of these studies further reported that



**Fig. 5.** Coactivation of PDGFR $\alpha/\beta$  in SF188 cells stably expressing EGFRvIII. A, phosphorylated RTK array analysis of isogenic SF188 cells shows low levels of constitutive EGFR activation in the control cells, and no additional RTK activation, in contrast to a large increase on constitutive activation by the EGFRvIII deletion mutant in association with a significant up-regulation of phosphorylated PDGFR $\alpha$  and PDGFR $\beta$  (also seen to a lesser extent with the wild-type receptor) as well as MER, Tie-2, and Flt-3. B, immunohistochemistry for EGFRvIII and PDGFR $\alpha$  in a case of pediatric gliosarcoma (RMH2473) showing coexpression of the receptors in the clinical setting. C, coinhibition of EGFR and PDGFR by erlotinib and imatinib showed a significant decrease in SF188: EGFRvIII cell viability than either compound alone ( $P < 0.01$ , *t* test).

the mutant receptor was amplified, and the significance of our findings of EGFRvIII expression in the absence of amplification is not clear; however, it may be significant that these anaplastic oligodendroglioma cases also had strong PDGFR $\alpha$  expression, as genomic alterations in *EGFR* and *PDGFRA* tend to be mutually exclusive in pediatric glioblastomas (41). Intriguingly, in a mouse model genetically engineered to express both EGFRvIII and Ras under a GFAP promoter, GFAP-V(12)Ha-ras;GFAP-EGFRvIII, but not GFAP-V(12)Ha-ras;GFAP-EGFR(wt) double transgenic mice, had decreased survival and developed oligodendrogliomas and mixed oligoastrocytoma tumors, instead of the fibrillary astrocytomas observed in GFAP-V(12)Ha-ras mice (6). The role of EGFR, and specifically the vIII deletion mutant, remains to be clarified in anaplastic oligodendroglioma, and assessing the translational significance in children is hampered by a lack of cell line models.

Activating mutations in the kinase domain of *EGFR* predict for sensitivity to small molecular inhibitors in tumors such as non-small cell lung cancer; however, these have not been reported in adult glioblastomas, and their absence from our pediatric series was not unexpected. By contrast, the identification of oncogenic point mutations in the extracellular domains in adult glioblastoma, and the resultant efficacy of receptor-targeting strategies, led to the expectation that we may also observe these base changes in childhood cases. The lack of such mutations once again serves to highlight the potential biological differences between gliomas arising at different ages.

Expression of the EGFRvIII mutant in association with wild-type PTEN has been reported to predict for sensitivity to small-molecule EGFR inhibitors, such as erlotinib, in the clinic (15), although in some model systems constitutive EGFR activation did not render the glioblastoma cells sensitive. We sought to clarify the situation in the childhood disease by screening a series of well-characterised<sup>11</sup> glioma cell lines derived from pediatric patients for *in vitro* sensitivity to erlotinib. These lines, consisting of both *PTEN* null and wild-type, all expressed EGFR at only low levels and were relatively insensitive to small-molecule inhibition by erlotinib. Transduction of the *PTEN*-expressing pediatric glioblastoma cell line SF188 with EGFRvIII did not greatly enhance the effects of erlotinib on cell viability nor downstream signaling.

EGFRvIII and the wild-type receptor have recently been shown to differentially activate downstream pathways, suggesting that therapeutic approaches toward tumors expressing these two distinct receptors should be fundamentally different (38). The expectation that targeting the MAPK pathway in wild-type EGFR, and the PI3K pathway in EGFRvIII-expressing cells, was not borne out by our data with engineered SF188 or U87MG cells, although in part this may be explained by the significant transactivation of the wild-type receptor in cells transduced with the mutant.

Previous reports have shown that coexpression of EGFRvIII and PTEN in U87MG cells rendered them highly susceptible to growth arrest by erlotinib compared with controls and U87 cells transfected with *EGFR* wild-type and *EGFRvIII* alone (15). In common with a recent study using a panel of serially propagated glioblastoma xenografts (20), it seems from our experiments with pediatric SF188 cells that the combination of PTEN expression with *EGFRvIII* mutation may be insufficient to confer obligate sensitivity to the small-molecule inhibitor.

The above data strongly suggest the presence of additional determinants of sensitivity to EGFR inhibitors in SF188 cells and possibly to pediatric HGGs in the clinic. The recent discovery of activating mutations in PI3K family member genes, such as *PIK3CA* and *PIK3R1*, in malignant glioma reiterates the importance of multiple mechanisms to increase signaling through this pathway (3, 42). SF188 harbors amplification at chromosome 12q14 of several genes, including *CDK4* and *CENTG1*, also known as the PI3K enhancer *PIKE-A* (43), a ubiquitously expressed GTPase, which binds to and enhances Akt kinase activity in a guanine nucleotide-dependent manner (44). Additional genomic aberrations in SF188 include amplification of *MYC* at 8q24,<sup>11</sup> which can be stabilized by PI3K (45) and provides an additional attenuation of this pathway in SF188 cells. Both amplification events have been reported in pediatric glioblastoma patient samples (43, 46).

Coactivation of RTKs, and the concept of "kinase switching," is an emerging concept that may additionally explain the lack of efficacy of agents targeting activated RTKs, such as EGFR. In adult glioblastoma, EGFR and MET seem frequently to be coactivated, with *in vitro* data supporting cotargeting both receptors (39). Furthermore, in adult glioblastoma models, the MET receptor seems strongly phosphorylated as a function of EGFRvIII levels. This has been hypothesized to be a result of an intermediary signaling component such as Src, as has been postulated for human bladder carcinoma (38). In the pediatric SF188 cells, we observed a significant activation of the closely related PDGFRs PDGFR $\alpha$  and PDGFR $\beta$  on transduction with EGFRvIII (and to a lesser extent with the wild-type receptor). We have previously reported high levels of Src family kinase expression in pediatric glioma cell lines, and SF188 in particular,<sup>11</sup> and the role of these key regulators of signal transduction in the transactivation and/or switching between constitutively activated EGFRvIII and PDGFR $\alpha/\beta$  warrants further investigation.

The identification of *EGFRvIII* deletions in pediatric HGGs and the possible resistance mechanisms to EGFR inhibitors conferred by known oncogenic amplifications have significant translational potential. In particular, PDGFRs are overexpressed at high frequency in pediatric glioblastomas, and specifically with three of six EGFRvIII-positive cases in this study, and this may be due to a differential, amplification-independent mechanism of action in anaplastic oligodendrogliomas. Coupled with evidence of enhanced efficacy from our *in vitro* model systems, these data provide a strong rationale for cotargeting with small-molecule inhibitors of both RTKs, potentially overcoming resistance to single agents in appropriate patient cohorts (47).

### Disclosure of Potential Conflicts of Interest

P. Workman is employed by, has received a commercial research grant from, has an ownership interest in, and is a consultant for Piramed Pharma.

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### **Correction: EGFRvIII Deletion Mutations in Pediatric High-Grade Glioma and Response to Targeted Therapy in Pediatric Glioma Cell Lines**

In this article (Clin Cancer Res 2009;15:5753–61), which was published in the September 15, 2009 issue of *Clinical Cancer Research* (1), the disclosure of potential conflicts of interest statement was incorrect. The correct statement is, as follows:

Dorine Bax, Nathalie Gaspar, Suzanne Little, Lynley Marshall, Lara Perryman, Marta Viani-Pereira, Raisa Vuononvirta, Swee Sharp, Jorge Reis-Filho, Andrew Pearson, Paul Workman, and Chris Jones are or were employees of The Institute of Cancer Research, which has a commercial interest in the development of PI3K inhibitors and operates a rewards-to-inventors scheme. Paul Workman and his team have been involved in a commercial collaboration with Yamanouchi (now Astellas Pharma) and with Piramed Pharma, and intellectual property arising from the program has been licensed to Genentech. Paul Workman was a founder of, consultant to, Scientific Advisory Board member of, and stockholder in Piramed Pharma, which was acquired by Roche, and was formerly an employee of AstraZeneca.

#### **Reference**

1. Bax DA, Gaspar N, Little SE, et al. EGFRvIII deletion mutations in pediatric high-grade glioma and response to targeted therapy in pediatric glioma cell lines. Clin Cancer Res 2009;15:5753–61.

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