De-Repression of PDGFRβ Transcription Promotes Acquired Resistance to EGFR Tyrosine Kinase Inhibitors in Glioblastoma Patients

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Acquired resistance to tyrosine kinase inhibitors (TKI) represents a major challenge for personalized cancer therapy. Multiple genetic mechanisms of acquired TKI resistance have been identified in several types of human cancer. However, the possibility that cancer cells may also evade treatment by co-opting physiologically regulated receptors has not been addressed. Here, we show the first example of this alternate mechanism in brain tumors by showing that EGFR receptor (EGFR)-mutant glioblastomas (GBMs) evade EGFR TKIs by transcriptionally de-repressing platelet-derived growth factor receptor β (PDGFRβ). Mechanistic studies show that EGFRvIII signaling actively suppresses PDGFRβ transcription in an mTORC1- and extracellular signal-regulated kinase-dependent manner. Genetic or pharmacologic inhibition of oncogenic EGFR renders GBMs dependent on the consequently de-repressed PDGFRβ signaling for growth and survival. Importantly, combined inhibition of EGFR and PDGFRβ signaling potently suppresses tumor growth in vivo. These data identify a novel, nongenetic TKI resistance mechanism in brain tumors and provide compelling rationale for combination therapy.

SIGNIFICANCE: These results provide the first clinical and biologic evidence for receptor tyrosine kinase (RTK) “switching” as a mechanism of resistance to EGFR inhibitors in GBM and provide a molecular explanation of how tumors can become “addicted” to a nonamplified, nonmutated, physiologically regulated RTK to evade targeted treatment. Cancer Discov; 3(5): 534–47. © 2013 AACR.

INTRODUCTION

The EGFR receptor, EGFR, is commonly amplified and/or mutated in many types of solid cancer, including a variety of epithelial cancers and glioblastoma (GBM) (1-3). Despite compelling evidence for EGFR addiction in experimental models, the clinical benefit of most EGFR tyrosine kinase inhibitors (TKI) has been quite limited. Multiple genetic mechanisms of acquired resistance to tyrosine kinase inhibitors (TKI) represent a major challenge for personalized cancer therapy. Multiple genetic mechanisms of acquired TKI resistance have been identified in several types of human cancer. However, the possibility that cancer cells may also evade treatment by co-opting physiologically regulated receptors has not been addressed. Here, we show the first example of this alternate mechanism in brain tumors by showing that EGFR receptor (EGFR)-mutant glioblastomas (GBMs) evade EGFR TKIs by transcriptionally de-repressing platelet-derived growth factor receptor β (PDGFRβ). Mechanistic studies show that EGFRvIII signaling actively suppresses PDGFRβ transcription in an mTORC1- and extracellular signal-regulated kinase-dependent manner. Genetic or pharmacologic inhibition of oncogenic EGFR renders GBMs dependent on the consequently de-repressed PDGFRβ signaling for growth and survival. Importantly, combined inhibition of EGFR and PDGFRβ signaling potently suppresses tumor growth in vivo. These data identify a novel, nongenetic TKI resistance mechanism in brain tumors and provide compelling rationale for combination therapy.

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RESULTS

EGFR Inhibition Promotes PDGFRβ Upregulation in Glioma

To better understand how malignant glioma acquires resistance to EGFR inhibitors in vivo, U87 glioma cells expressing the EGFRvIII gain-of-function mutation (designated U87-EGFRvIII herein) were placed in the flanks of severe combined immunodeficient mice. After 2 weeks, tumors were xenotransplanted into athymic mice and allowed to regrow. Tumors were then treated with gefitinib (10 mg/kg) or vehicle for 2 weeks. Tumor extracts were used to measure PDGFRβ immunoblotting. The results showed that PDGFRβ expression was significantly increased in gefitinib-treated tumors compared to vehicle-treated tumors. These data identify a novel physiologic EGFR TKI resistance mechanism in GBM and suggest a clinically actionable approach to suppress it.

ABSTRACT

Acquired resistance to tyrosine kinase inhibitors (TKI) represents a major challenge for personalized cancer therapy. Multiple genetic mechanisms of acquired TKI resistance have been identified in several types of human cancer. However, the possibility that cancer cells may also evade treatment by co-opting physiologically regulated receptors has not been addressed. Here, we show the first example of this alternate mechanism in brain tumors by showing that EGFR receptor (EGFR)-mutant glioblastomas (GBMs) evade EGFR TKIs by transcriptionally de-repressing platelet-derived growth factor receptor β (PDGFRβ). Mechanistic studies show that EGFRvIII signaling actively suppresses PDGFRβ transcription in an mTORC1- and extracellular signal-regulated kinase-dependent manner. Genetic or pharmacologic inhibition of oncogenic EGFR renders GBMs dependent on the consequently de-repressed PDGFRβ signaling for growth and survival. Importantly, combined inhibition of EGFR and PDGFRβ signaling potently suppresses tumor growth in vivo. These data identify a novel, nongenetic TKI resistance mechanism in brain tumors and provide compelling rationale for combination therapy.

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immunodefficient (SCID) mice. EGFRvIII, the most common EGFR mutation in GBM, arises from an in-frame genomic deletion of exons 2 to 7, resulting in a persistently active, highly oncogenic protein (10). Tumor-bearing mice were gavaged with the EGFR inhibitor erlotinib (150 mg/kg) or vehicle control, and tumor growth was assessed over 18 days (Fig. 1A). As expected, erlotinib treatment slowed U87-EGFRvIII tumor growth relative to control; however, tumors retained a substantial growth rate despite continued erlotinib treatment (Fig. 1B). Immunoblots of tumor lysates confirmed
that erlotinib treatment of mice significantly reduced EGFR signaling in xenografts to a level comparable with that of U87 tumor cells expressing kinase-dead EGFR-VIII (Fig. 1C). Thus, U87-EGFRvIII-expressing tumors maintain growth despite a significant reduction in EGFR activity when treated with erlotinib, phenocopying the results observed in human trials (2).

We considered the possibility that erlotinib-treated U87-EGFRvIII tumors maintain growth by acquiring neo-RTK activity. To directly address this, we conducted a phospho-RTK array on control and erlotinib-treated tumor lysates. As expected, control U87-EGFRvIII tumors expressed significant levels of phospho-EGFR that were reduced in erlotinib-treated mice (Fig. 1D). Erlotinib-treated U87-EGFRvIII tumors also had considerable phospho-PDGFRβ expression (Fig. 1D). Heightened activation of the RTK AXL was also noted, albeit to a lesser extent than PDGFRβ. Immunoblotting of tumor lysates confirmed upregulation and activation of PDGFRβ in response to pharmacologic or genetic inhibition of EGFRvIII (Fig. 1C). Parental U87 tumors expressed significant PDGFRβ (Fig. 1C), which was suppressed by EGFRvIII. Furthermore, erlotinib treatment markedly upregulated PDGFRβ expression in orthotopic U87-EGFRvIII GBM xenografts (Fig. 1E), suggesting that EGFRvIII signaling actively represses PDGFRβ.

To determine if the reciprocal relationship could be extended into other patient-derived glioma models endogenously expressing EGFRvIII, or high levels of wild-type EGFR, we examined phospho-EGFR and PDGFRβ expression in a panel of low passage patient-derived GBM neurospheres (11). Uniformly, erlotinib treatment resulted in upregulation PDGFRβ expression in EGFRvIII-expressing GBM neurospheres, as well as GBM neurospheres expressing high levels of EGFR (Fig. 1F). In addition, GBM39 cells that developed erlotinib resistance in long-term culture maintained suppression of EGFR phosphorylation and concomitant PDGFRβ upregulation (Supplementary Fig. S1A). Of note and in contrast to PDGFRβ, erlotinib treatment had no effect on PDGFRα expression. To assess whether erlotinib treatment similarly elevated PDGFRβ levels in GBMs endogenously expressing EGFRvIII in vivo, we examined PDGFRβ expression up to 10 days of treatment with erlotinib at 150 mg/kg. Consistent with our proposed model, erlotinib treatment resulted in elevated phosphorylated and total levels of PDGFRβ (Fig. 1G and Supplementary Fig. S1B). Single-nucleotide polymorphism array analysis showed that PDGFRβ was not amplified in these tumors, either at baseline or after erlotinib treatment (data not shown). Taken together, these data indicate that EGFRvIII/EGFR signaling negatively regulates PDGFRβ expression in glioma models, and that inhibition of EGFRvIII/EGFR signaling results in upregulation of PDGFRβ.

An RTK Switch to PDGFRβ Occurs in Lapatinib-Treated Patients

Intratumoral heterogeneity of RTK expression is a common feature of malignant gliomas, but it remains unclear if this heterogeneity reflects co-amplification of RTKs within a given tumor cell or differences in RTK expression among tumor cells. To distinguish between these possibilities, we examined glioma tissue microarrays (TMA) for EGFR and PDGFRβ expression. Similar to our model system studies, we observed a strong inverse correlation between EGFR (total and phosphorylated tyrosine 1086) and PDGFRβ expression in patient glioma tissues (Fig. 2A; \( P = 0.02 \)). To determine if RTK expression was fixed within a given tumor, we used patient tissues from a cohort of patients enrolled in a biopsy-treat-biopsy study, in which patients underwent 7 to 10 days oral treatment with another EGFR TKI, lapatinib, as part of a phase II clinical trial (12). Post-lapatinib biopsy samples were divided into EGFR-on and EGFR-off groups following immunoblot analysis and show striking inverse correlation between phospho-EGFR status and PDGFRβ protein expression (Fig. 2B; \( P = 0.04 \)). Immunohistochemical (IHC) analysis of one patient was available before and after lapatinib treatment and showed significant reduction of phospho-EGFR after treatment, with concomitant PDGFRβ expression in the tumor (Fig. 2C). These clinical data support a model where highly active EGFR signaling negatively regulates PDGFRβ expression in primary brain tumors and indicate that pharmacologic inhibition of EGFR signaling results in an RTK switch to PDGFRβ.

Suppression of PDGFRβ Expression Is Dependent on the AKT/mTOR Signaling Pathway

EGFRvIII and, to a lesser extent, wild-type EGFR have been shown to potently activate phosphoinositide 3-kinase (PI3K) signaling in GBM, resulting in phosphorylation of AKT and its downstream effector mTORC1 (12–17). Therefore, we set out to determine whether EGFRvIII inhibits PDGFRβ expression in AKT and mTORC1 signaling. To examine whether EGFRvIII inhibits PDGFRβ expression in AKT, U87-EGFRvIII cells were transfected with the constitutively active AKT1 E17K allele (18). Ectopic expression of AKT1 E17K fully abrogated the upregulation of PDGFRβ in response to erlotinib, confirming that EGFRvIII inhibits PDGFRβ expression in AKT (Fig. 3A). Previous work has identified mTOR as a negative regulator of PDGFRβ expression in mouse embryonic fibroblasts (19), leading us to hypothesize that EGFRvIII signaling to AKT suppresses PDGFRβ expression through mTORC1. To test this, we determined PDGFRβ expression in U87-EGFRvIII cells transiently transfected with siRNA targeting the mTORC1 proteins Raptor and Rictor. Immunoblot analysis of U87-EGFRvIII cells transiently transfected with siRNA targeting the mTORC1 proteins Raptor and Rictor indicated that the inhibition of mTORC1, and to a lesser extent mTORC2, led to increased levels of PDGFRβ expression (Fig. 3B). Conversely, transfection of a constitutively active mTOR (S2215Y) allele (20) abrogated erlotinib-dependent upregulation of PDGFRβ (Fig. 3C). Furthermore, genetic depletion of the mTORC1 effector p70 S6Kinase by siRNA knockdown similarly upregulated PDGFRβ (Fig. 3D). Confirming mTOR-dependent repression of PDGFRβ, rapamycin robustly upregulated PDGFRβ protein expression in GBM cell lines in vitro and in vivo (Fig. 3E and F). These results show that EGFR signals through AKT and mTORC1 to suppress PDGFRβ.

EGFR Signaling Represses Transcription of the PDGFRβ Gene

Next, we sought to determine if the influence of mTOR signaling on PDGFRβ expression was regulated at the transcriptional level. To that end, U87-EGFRvIII cells were treated with erlotinib or vehicle, and mRNA was collected up to
36 hours after treatment. Quantitative real-time PCR (qRT-PCR) showed that PDGFRβ mRNA was upregulated by 8 hours after the addition of erlotinib, and expression progressively increased over a 24-hour period (Fig. 4A; \( P < 0.001 \)).

To determine if the increase in PDGFRβ expression was a function of increased transcription at the PDGFRβ gene locus, we assessed the expression levels of PDGFRβ primary transcripts. qRT-PCR studies revealed that the expression pattern of PDGFRβ primary transcript mirrored that of PDGFRβ mRNA following EGFR inhibition (Fig. 4A; \( P < 0.001 \)). Treatment and washout studies revealed that PDGFRβ primary transcript was dynamically regulated by the addition or removal of erlotinib, further suggesting that expression of PDGFRβ is an active transcriptional process (Fig. 4B). Correspondingly, transcriptional reporter studies using the PDGFRβ promoter upstream of luciferase indicated that knockdown of EGFR or Raptor significantly increased luciferase activity in U87-EGFRvIII cells (Fig. 4C; \( P < 0.001 \)). Finally, chromatin immunoprecipitation (ChIP) experiments revealed that rapamycin (5 nmol/L) treatment results in recruitment of RNA polymerase II to both the transcriptional start site and exon 1 of PDGFRβ (Fig. 4D; \( P < 0.01 \)). Taken together, these studies support a model where EGFR signaling dynamically regulates transcription of PDGFRβ in an mTOR-dependent manner. However, we cannot rule out the possibility that additional factors such as increased stability of the PDGFRβ mRNA pool or heightened translation also contribute to PDGFRβ upregulation.

ERK Signaling Contributes to the Regulation of PDGFRβ

The mitogen-activated protein kinase (MAPK) pathway is also activated by EGFRvIII signaling (Fig. 5A); thus, we investigated whether the MAPK signaling pathway also contributes to the regulation of PDGFRβ expression. The MAP–ERK kinase (MEK) inhibitor U0126 upregulated PDGFRβ...
Figure 3. EGFRvIII suppresses PDGFRβ through AKT and mTORC1 signaling. A, immunoblot of PDGFRβ and indicated proteins in U87-EGFRvIII cells expressing constitutively active AKT1 (E17K) treated with erlotinib (5 μmol/L) for 24 hours. B, immunoblot of lysates from U87-EGFRvIII cells with transient knockdown of mTOR complex proteins Raptor or Rictor and treated with erlotinib (5 μmol/L) as indicated. C, immunoblot of U87-EGFRvIII cells expressing constitutively active (S2215Y) or wild-type mTOR and treated with erlotinib (5 μmol/L) for 24 hours as indicated. D, immunoblot of PDGFRβ levels in response to transient knockdown of EGFRvIII, or S6 kinase 1 in U87-EGFRvIII cells. E, PDGFRβ levels in U87-EGFRvIII and U251 cells treated with vehicle or rapamycin (5 nmol/L) for 24 hours. F, IHC of PDGFRβ in intracranial U251 GBM tumors following 3 days of rapamycin (2 mg/kg/d) or vehicle treatment.

expression, although to a lesser extent than erlotinib (Fig. 5A), which was not abrogated by overexpression of wild-type S6K1 or constitutively active S6K1 or S6K1 and S6K2 alleles (Fig. 5B). Taken together, these results show the presence of a parallel pathway by which EGFRvIII/EGFR signaling regulates PDGFRβ through a MAPK (Fig. 5C). Other RTKs such as MET have been shown to engage P38K signaling to confer resistance to erlotinib in GBM (7). Therefore, we asked whether MET signaling, which can activate both AKT/mTORC1 and MAPK pathways, could similarly promote PDGFRβ upregulation. In U87-EGFRvIII or GBM39 neurospheres, the MET inhibitor PHA-665752 (PHA, 0.05–4 μmol/L) was not sufficient to promote PDGFRβ upregulation as erlotinib did (Fig. 5D and E). However, the addition of exogenous hepatocyte growth factor (HGF) ligand promoted AKT and ERK phosphorylation and suppressed erlotinib-mediated upregulation of PDGFRβ in a dose-dependent fashion (Fig. 5F). These results suggest that HGF-mediated activation of MET can also repress PDGFRβ by engaging AKT/mTOR and MAPK signaling.

PDGFRβ Is Dispensable for EGFRvIII-Driven GBM Growth but Becomes Required for the Growth of EGFRvIII-Inhibited Tumors

Next, we asked if PDGFRβ signaling influences proliferative capacity in EGFR-inhibited glioma. To that end, U87-EGFRvIII cells were cultured with erlotinib or vehicle and PDGF-BB (0–20 ng/mL/d) for 4 days. The addition of PDGFRβ
ligand to untreated U87-EGFRvIII cells had little effect on proliferative capacity (Fig. 6A). As expected, treating U87-EGFRvIII cells with erlotinib alone significantly reduced both EGFR signaling (Supplementary Fig. S2A) and proliferation (Fig. 6A). The addition of PDGF-BB to cultures restored proliferative capacity of erlotinib-treated cells (Fig. 2A) in a receptor-specific (Supplementary Fig. S2B) and dose-dependent manner (Supplementary Fig. S2C). Similarly, the addition of PDGFRβ ligand to cultures significantly restored the proliferative capacity of U87-EGFRvIII cells transfected with siRNA-targeting EGFRvIII (Fig. 6B and Supplementary Fig. S2D).

Next, we asked whether PDGFRβ signaling was required for tumor growth in vivo in GBM cells expressing EGFRvIII, and whether abrogation of EGFRvIII rendered these tumor cells PDGFRβ-dependent. To that end, U87-EGFRvIII and U87-EGFRvIII-kinase dead cells were stably transduced with short hairpin RNAs (shRNA) targeting PDGFRβ or control shRNA and implanted in the flanks of SCID mice. Consistent with our in vitro studies, the silencing of PDGFRβ had little effect on U87-EGFRvIII tumor growth (Fig. 6C). In contrast, silencing PDGFRβ significantly attenuated the growth of tumors expressing kinase dead-EGFRvIII (Fig. 6D). Immunoblots of xenograft lysates confirmed an inverse relationship between PDGFRβ and EGFR activation in tumors (Fig. 6E).

To determine whether PDGFRβ signaling could abrogate the growth-inhibitory effects of erlotinib in GBM cells endogenously expressing EGFRvIII or high levels of wild-type EGFR, we examined the effect of PDGFRβ signaling on the proliferative capacity on patient-derived GBM neurospheres. The PDGFR kinase inhibitor AG1295 (2 μmol/L) alone had no antiproliferative effect on GBM39 (EGFRvIII positive, PTEN intact) or HK250 (high-level wild-type EGFR, PTEN-deficient) cells (Fig. 6F and G). In contrast, in the presence of erlotinib, the addition of the
Figure 5. ERK signaling contributes to the regulation of PDGFRβ. A, immunoblot of PDGFRβ and indicated proteins from whole-cell lysates of U87-EGFRvIII cells treated with MEK inhibitor U0126 (5 μmol/L), erlotinib (5 μmol/L), or vehicle for 24 hours. B, determination of PDGFRβ protein levels from U87-EGFRvIII cells transfected with empty vector (pcDNA), wild-type S6K1, or constitutively active S6K1 (T412D) and S6K2 (T401D). In addition, cells were treated with MEK inhibitor U0126 (10 μmol/L) or vehicle for 24 hours as indicated. C, a schematic of the signals downstream of EGFRvIII regulating PDGFRβ protein expression. D and E, PDGFRβ protein levels from U87-EGFRvIII cells (D) or patient-derived neurosphere GBM39 (E) treated with erlotinib (5 μmol/L) or MET inhibitor PHA at the indicated dose. F, immunoblot of PDGFRβ and indicated proteins from U87-EGFRvIII cells treated with erlotinib and MET ligand HGF (50–100 ng/mL) for 24 hours as indicated.
Figure 6. PDGFRβ is dispensable for EGFRvIII-driven GBM growth but is required for the optimal growth of EGFR-inhibited tumors. A, proliferation of U87-EGFRvIII cells over 4 days treated with erlotinib (5 μmol/L) or PDGF-BB ligand (20 ng/mL) alone or in combination as indicated. Erlotinib was added on day 0 of culture, and PDGF-BB was added daily at 20 ng/mL thereafter. B, growth of U87-EGFRvIII cells transiently transfected with control scrambled siRNA or siEGFRvIII and treated with PDGF-BB as described in A and C, growth curve of xenografts subcutaneously implanted with U87-EGFRvIII, U87-EGFRvIII/shPDGFRβ, U87-EGFRvIII kinase dead, or U87-EGFRvIII kinase dead/shPDGFRβ cells as indicated. E, immunoblot of phospho-PDGFRβ and EGFR from lysates harvested on day 24 from tumors as described in C and D. F and G, proliferation of EGFRvIII-expressing patient-derived neurospheres GBM39 and HK-250 treated with erlotinib (5 μm) and PDGFRβ inhibitor AG1295 (3 μmol/L) alone or in combination as indicated. **, P < 0.01; ***, P < 0.001. n.s., no statistical significance.

PDGFR kinase inhibitor AG1295 significantly suppressed tumor cell proliferation (Fig. 6F and G; P < 0.01). Of note, and in contrast to our studies on U87-EGFRvIII-engineered cells, the patient-derived neurosphere cultures did not require the addition of exogenous PDGFR ligand, consistent with the role of autocrine and paracrine PDGF signaling in GBM (21, 22). Taken together, these data suggest a physiologic RTK switch to the PDGFRβ to maintain the growth of EGFRvIII/EGFR-activated GBMs in response to EGFR TKIs (Fig. 7).

DISCUSSION

Acquired drug resistance presents a significant challenge for personalized cancer therapy. In principle, upfront sequencing may guide successful combination TKI therapy by defining
An RTK Switch in Malignant Glioma

Figure 7. Model of proposed RTK-switch. Under conditions of heightened growth receptor signaling (e.g., EGFRVIII mutation), PDGFRβ expression is repressed by downstream ERK and mTOR activity. Inhibition of these growth pathways, as with EGFR or mTOR inhibitors, results in the transcription of the PDGFRβ gene and the upregulation of PDGFRβ receptor.

both the druggable kinase mutations and the potential “seeds” of resistance—second site mutations, downstream effector mutations, and cocompliation of multiple RTKs. However, nongenetic adaptive resistance mechanisms complicate this paradigm. Identifying the ways that cancer cells “rewire” their circuitry through pathway cross-talk and release of inhibitory feedback loops to evade treatment may be critical for developing more successful combination approaches. By integrating studies of cells, mice, and tumor tissue from patients treated with EGFR inhibitors in a clinical trial, we provide the first experimental evidence that EGFR TKI resistance can be mediated by transcriptional de-repression of another, physiologically regulated RTK: PDGFRβ.

TKI-mediated release of inhibitory feedback loops is emerging as a frequent, nongenetic mechanism of targeted cancer drug resistance. In colorectal cancer cells bearing the BRAF V600E mutation, resistance to the BRAF inhibitor PLX-4032 (vemurafenib) is mediated by reactivation of EGFR signaling through the MAPK pathway (23, 24), although upregulation of EGFR itself does not seem to be involved. In breast cancer cells, AKT and mTOR inhibition reactivates PI3K signaling through release of an inhibitory feedback loop in a process that seems to involve multiple RTKs (25, 26). Most recently, transcriptional upregulation of the RTK AXL has been shown to promote erlotinib resistance in non-small cell lung cancer (9), although the mechanism underlying AXL upregulation is not known. It is interesting to note that we too observed AXL upregulation in response to erlotinib treatment (Fig. 1), suggesting that it may also play a role in mediating erlotinib resistance in GBM. However, we focused on PDGFRβ because of the dominance of its signal across all in vitro and in vivo models and in all patient samples we studied.

In contrast to the recognized importance of PDGFRα alterations (7, 27–29), the role of PDGFRβ in malignant gliomas has not been clearly defined. PDGFRβ amplifications and/or mutations are exceedingly rare events in GBM (27). In mouse genetic models, PDGF-B ligand overexpression can promote gliomagenesis by enhancing cellular proliferation (30–34). Recently, PDGFRβ has been shown to promote glioma stem cell self-renewal, suggesting a more definitive role in tumorigenesis and/or maintenance (35). In addition, a PDGF signaling class of GBMs, characterized by PDGFRβ phosphorylation and a lack of EGFR signaling, among other features, has been identified (36). Yet the contribution of PDGFRβ signaling to drug resistance remains incompletely understood.

Here, we provide the first demonstration that mTORC1 inhibition mediates EGFR TKI resistance in GBM through transcriptional regulation of PDGFRβ, a mechanism which could also be active in other cancer types. PDGFRβ has been shown to mediate vemurafenib resistance through transcriptional upregulation in melanoma (37). However, the mechanism underlying this event is not known. In mouse embryonic fibroblasts, PDGFRβ was shown to be a target of mTOR-dependent negative transcriptional downregulation (19). However, its role in mediating EGFR and/or mTOR TKI resistance has not previously been recognized. In addition, we identify a parallel pathway (38) by which ERK signaling also suppresses PDGFRβ. Our data definitively show that EGFR inhibitors de-repress PDGFRβ transcription, providing a potent mechanism underlying RTK switching. These findings have broad implications for understanding acquired resistance to EGFR TKIs, and potentially mTOR inhibitors as well, across multiple cancer types. Future studies will be necessary to address this possibility more fully. In addition, future studies will be needed to identify the transcriptional machinery linking mTOR/S6K with PDGFRβ.

EGFR amplification and mutation presents perhaps the most compelling druggable target in GBM. Genetic and/or functional PTEN loss (2, 39), cooccurrence of c-MET and PDGFRα gene amplification (7, 28, 29), and pharmacokinetic considerations (40) all contribute to EGFR TKI resistance, indicating a broad repertoire of resistance mechanisms that can be cotargeted. However, the role of nongenetic
“rewiring” in mediating drug resistance remains to be defined. Here, we have identified a transcriptional repressive mechanism by which EGFRvIII regulates PDGFRβ, shown that EGFR-inhibited GBMs become PDGFRβ-dependent for survival through mTOR-dependent transcriptional de-repression, and showed that abrogation of EGFRvIII and PDGFRβ stop tumor growth, providing a strong rationale for combination therapy. These results provide the first clinical and biologic evidence for the concept of RTK “switching” as an EGFR TKI resistance mechanism in GBM, and provide a molecular explanation for how tumors can become “addicted” to a nonamplified, nonmutated, physiologically regulated RTK to evade targeted treatment.

METHODS

Cell Lines and Media

U87 and isogenic U87-EGFRvIII, U87-EGFRvIII kinase dead, U87-EGFRvIII/shPDGFRβ, and U87-EGFRvIII kinase dead shPDGFRβ cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Cellgro) supplemented with 10% FBS (Omega Scientific) and penicillin streptomycin-glutamine (PSQ; Invitrogen) in a humidified atmosphere of 5% CO₂ at 37°C. U87-EGFRvIII kinase dead cells were a gift from W.K. Cavenee (Ludwig Institute for Cancer Research, UCSF). U87-EGFRvIII-shPDGFRβ cells were generated by transgenic-mediated transfection of shPDGFRβ into U87-EGFRvIII cells followed by selection for stable clones. Neurosphere cell lines (GBM6, GBM12, GBM39, HK296, HK242, and HK250) were cultured in DMEM/F12 (Cellgro) supplemented with EGF, fibroblast growth factor, heparin (Sigma), GlutaMax, and PSQ (Invitrogen). Long-term erlotinib-resistant GBM39 neurospheres were cultured in the presence of erlotinib for 30 days until cells were resistant.

Isogenic human malignant glioma cells were implanted into immunodeficient SCID/Beige mice for subsequent xenograft studies as follows. SCID/Beige mice were bred and kept under defined-flora pathogen-free conditions at the Association for Assessment of Laboratory Animal Care–approved Animal Facility of the Division of Experimental Radiation Oncology, UCLA. U87 tumor cells (or indicated isogenic U87-EGFRvIII, U87-EGFRvIII kinase dead, U87-EGFRvIII/shPDGFRβ, and U87-EGFRvIII kinase dead shPDGFRβ) or GBM39 xenografts, single-cell suspensions were injected subcutaneously at 600,000 cells/150 μL in a solution of Dulbecco’s PBS (dpBS) and Matrigel (BD Biosciences). Tumor growth was monitored with calipers by measuring the perpendicular diameter of each subcutaneous tumor. For intracranial xenograft studies, U251 cells were injected intracranially in rats as described previously (41). Rapamycin was administered for 1.5 hours (5% CO₂, 37°C) after the addition of tetrazolium salt WST-1 [2-(4-tiodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, monosodium salt], and the absorbance was then measured in a microplate reader (Bio-Rad) at 450 nm, with a background reading at 650 nm subtracted. For assays using erlotinib or AG1295, small molecules or dimethyl sulfoxide (DMSO) vehicle were added at the indicated doses on day 0 of assays. In assays with PDGF-BB ligand, cultures were stimulated daily with PDGF-BB ligand at 20 ng/mL for the indicated days. Neurospheres were plated in laminin-coated 96-well dishes in neurobasal media supplemented with 5% charcoal-stripped FBS (Omega Scientific) and treated with indicated drug as above. For transient siRNA knockdown of EGFRvIII, cells were incubated overnight with transient siRNA of EGFRvIII (Ambion) or scrambled siRNA (Ambion) at 10 nmol/L with RNAiMax Lipofectamine reagent (Invitrogen) and Opti-MEM (Invitrogen). Cells were then plated in 12-well plates and were stimulated with PDGF-BB ligand or vehicle in medium containing 2% charcoal-stripped FBS (Omega Scientific) and counted as above.

Knockdown Studies

Transgenic knockdown of EGFRvIII, S6K1, Raptor, and Rictor (Ambion) were conducted as follows. siRNA was diluted to a final concentration of 10 nmol/L in Opti-MEM and 7.5 μL Lipofectamine RNAi-max, in serum-containing, PSQ-free media overnight in a final volume of 6 mL in a 60-mm dish. Media was changed the following morning, and cells were incubated for 24 hours before lysis collection. For the generation of stable knockdown cell lines, cells (5 × 10⁶) were seeded in 12-well plates and maintained for 24 hours, after which the medium was replaced with fresh 5% FBS medium including polypeptide (5 μg/mL; Sigma), and shRNA lentivirus was added to cells followed by incubation for 24 hours.

Transient Transfection

Plasmids used were pcDNA control, wild-type mTOR, mTOR S2215Y, and AKT E17K [mTOR constructs were a gift from F. Tamanoi (UCLA), E17K was a gift from Ingo Mellinghoff (Memorial Sloan-Kettering Cancer Center), and pcDNA control was from Addgene]. Empty vector, mTOR constructs, and AKT E17K were diluted to 2,500 ng in Opti-MEM and incubated with Lipofectamine PLUS and LTX reagents (Invitrogen) according to the manufacturer’s instructions. Cells were plated in 1 mL of 5% FBS, and 1 mL of Opti-MEM/plasmid/Lipofectamine was added to each plate. Media was changed the next day to serum-free media, and cells were incubated with erlotinib for 24 hours before lysates were collected. For S6 kinase wild-type and constitutively active forms, plasmids were diluted to 16 μg in Opti-MEM and incubated with Fugene6 (Roche) according to the manufacturer’s instructions. Cells were plated in 5 mL of 10% FBS, and 1 mL of Opti-MEM/plasmid/Fugene6 was added to each plate. Media was changed to serum-free media the following morning, and cells were incubated with U0126 for 24 hours. Lysates were then collected for immunoblotting. Plasmids used were pcDNA control, E6S6K1, and T412D/T401D S6K1/2 (S6 kinase plasmids were a gift from Ivan Gout, University College London).

Immunoblots

Cultured cells or snap-frozen tissue samples were lysed and homogenized with radioimmunoprecipitation assay buffer (buffer, Boston Bioproducts; protease and phosphatase inhibitor, Thermo-scientific). Protein concentration was determined via BCA Assay (reagents A and B, Thermo-scientific; standards, Bio Rad) and samples were subjected to 4% to 12% gradient SDS-PAGE and then transferred to a nitrocellulose membrane (Bio-Rad Laboratories). The membrane was then probed with indicated primary antibodies, followed by secondary antibodies conjugated to horseradish peroxidase.

**Immunohistochemistry**

Xenografts were excised from mice treated with vehicle or erlotinib as described earlier. A portion of the tumor was fixed in paraformaldehyde and ethanol and sent to the Department of Pathology and Laboratory Medicine at UCLA tissue core for slicing and staining as required.

**Phospho-RTK Array**

U87-EGFRvIII tumors from vehicle- or erlotinib-treated mice were harvested and homogenized in NP40-containing lysis buffer, then loaded at 2,000 μg per RTK array membrane, according to the manufacturer’s instructions (R&D Systems). For U87-EGFRvIII cells in culture, lysates were generated and loaded on RTK array as described earlier.

**Pilot Study of Lapatinib**

North American Brain Tumor Consortium trial 04-01 titled “A biomarker and phase II study of GW 572016 (lapatinib) in recurrent malignant glioma” enrolled consented patients from UCLA, UCSF, Dana-Farber Cancer Center (Boston, MA), Memorial Sloan-Kettering Cancer Center (New York, NY), University of Pittsburgh, (Pittsburgh, PA), and Duke University (Durham, NC; ref. 12). Adult patients who had a Karnofsky performance score equal to or greater than 60, who were not on enzyme-inducing antiepileptic agents, and who had normal hematologic, metabolic, and cardiac function were eligible for this study. In addition, patients must have been candidates for surgical re-resection at the time of enrollment. Patients were administered 750 mg of lapatinib orally twice a day for 7 to 10 days (depending on whether treatment interval fell over a weekend) before surgery, the time to steady state. Blood and tissue samples were obtained at the time of resection. After recovery from surgery, patients resumed lapatinib treatment at the neoadjuvant dose of 750 mg twice a day until clinical or radiographic evidence for tumor progression was found. The first cohort of patients for whom tissue was available before and after lapatinib (n = 10) were included in this study.

**Tissue Microarrays**

TMAs were used to analyze PDGFRα and p-EGFR Tyr1068 IHC staining in 140 GBM patient samples. Two GBM TMAs were constructed with a 0.6-mm needle to extract 252 representative tumor tissue cores and 91 adjacent normal brain tissue cores from the paraffin-embedded tissue blocks of 140 patients with primary GBM (12). These cores were placed in a grid pattern into 2 recipient paraffin-embedded tissue blocks of 140 patients with primary GBM (12). These cores were placed in a grid pattern into 2 recipient paraffin-embedded tissue blocks of 140 patients with primary GBM (12).

**Real-Time PCR**

In vitro, U87-EGFRvIII cells were incubated in serum-free media with and without erlotinib for 32 hours, as well as in 10% serum with and without rapamycin for 24 hours. At each time point, cells were lysed in Trizol for RNA extraction. RT-PCR analysis was conducted using primers designed to amplify either the primary transcript of PDGFRα or the mRNA transcript. Primer sequences for PDGFRα mRNA are AGGACACGCCAGGAGGTGTCAT (forward) and TTCTGCGAAACAGCATGATG (reverse). Primer sequences for PDGFRα primary transcripts are CACTGTGACAAAAACCATTTG (forward) and ACTTTGCTTCTGCTGACATC (reverse). For the washout, U87-EGFRvIII cells were plated in 10% FBS-containing media. The media was changed to serum-free media and erlotinib (5 μmol/L) was added at t = 0 hours. Media was changed at 24 hours, and erlotinib (5 μmol/L) was added again at 48 hours. At each time point, cells were lysed in Trizol for RNA extraction as described earlier. mRNA and primary transcripts were normalized against 36B4.

**Luciferase Assay**

U87-EGFRvIII cells were transfected with Switchgear genomics PDGFRα promoter plasmid concurrently with control cytomegalovirus plasmid promoter Luciferase and Renilla and Firefly Luciferase control. Luciferase assay was conducted using Promega Dual Luciferase Reporter Assay System.

**Chromatin Immunoprecipitation**

ChIP assays were conducted in U87-EGFRvIII cells with or without rapamycin (5 μmol/L) for 24 hours. Cells in 2 15-cm plates were pooled for each replicate. ChIP was conducted as previously described (42) with minor modifications. Briefly, cells were cross-linked for 5 minutes in 1% formaldehyde in PBS. After sonication (15 minutes total sonication time in 30-second pulses), soluble chromatin from each replicate was split 4 ways for overnight immunoprecipitations with 2 μg of the following antibodies: mouse immunoglobulin G (IgG; Millipore cat #12-371) antibody against polymerase II (Millpore clone CTD-4H13, cat #05-623, positive control). Five microliter of chromatin was used as control. DNA–protein complexes were pulled down by incubation for 2 hours with protein G-sepharose, washed, and eluted with 1% SDS buffer. Resulting chromatin was de-cross-linked with heat and protein digested with proteinase K, along with input controls. Genomic DNA (gDNA) was assayed by quantitative PCR (qPCR) with primers amplifying PDGFRα transcriptional start site (TSS) and a fragment upstream of the TSS. qPCR values were normalized against the input gDNA content for each replicate. qPCR primers are available upon request.

**ERK and MET Studies**

For treatment with erlotinib and the MEK inhibitor U0126 or the MET inhibitor PHA-665752, adherent cells were plated in 10% FBS. GBM39 cells were plated on plates coated with laminin (Sigma) as described earlier in complete neurosphere media. The following day, media was changed to serum-free media (U0126), 2% FBS-containing media (U87-EGFRvIII, PHA-665752), or DMEM/F12 (GBM39, PHA-665752). Cells were then incubated with drug for 24 hours before being lysed for immunoblot analysis.

**Statistical Analysis**

Fisher’s exact test was used to assess correlation between EGFR and PDGFRα in clinical samples. All other comparisons of cell proliferation, transcript level, and tumor volume were conducted using one-way or two-way ANOVA with Tukey’s Honestly Significant Difference test as required. All results are shown as mean ± SD.

**Disclosure of Potential Conflicts of Interest**

P.S. Mischel and T.F. Cloughesy served on an advisory board for Celgene’s mTOR kinase inhibitor program and collaborated with Celgene and Sanoﬁ through research contracts on their mTOR kinase and PI3K/mTOR kinase inhibitor clinical trials. H.I. Kornblum collaborated with Celgene on a research contract for the mTOR kinase inhibitor program. No potential conflicts of interest were disclosed by the other authors.
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