InsR/IGF1R pathway mediates resistance to EGFR inhibitors in glioblastoma

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**Purpose:** Aberrant activation of epidermal growth factor receptor (EGFR) is a hallmark of glioblastoma. However, EGFR inhibitors exhibit at best modest efficacy in glioblastoma. This is in sharp contrast to the observations in EGFR-mutant lung cancer. We examined whether activation of functionally redundant receptor tyrosine kinases (RTKs) conferred resistance to EGFR inhibitors in glioblastoma.

**Experimental Design:** We collected a panel of patient-derived glioblastoma xenograft (PDX) lines that maintained expression of wild type or mutant EGFR in serial xenotransplantation and tissue cultures. Using this physiologically relevant platform, we tested the abilities of several RTK ligands to protect glioblastoma cells against an EGFR inhibitor, gefitinib. Based on the screening results, we further developed a combination therapy co-targeting EGFR and insulin receptor (InsR)/insulin-like growth factor 1 receptor (IGF1R).

**Results:** Insulin and IGF1 induced significant protection against gefitinib in the majority of EGFR-dependent PDX lines with one exception that did not expression InsR or IGF1R. Blockade of the InsR/IGF1R pathway synergistically improved sensitivity to gefitinib or dacomitinib. Gefitinib alone effectively attenuated EGFR activities and the downstream MEK/ERK pathway. However, repression of AKT and induction of apoptosis required concurrent inhibition of both EGFR and InsR/IGF1R. A combination of gefitinib and OSI-906, a dual InsR/IGF1R inhibitor, was more effective than either agent alone to treat subcutaneous glioblastoma xenograft tumors.

**Conclusions:** Our results suggest that activation of the InsR/IGF1R pathway confers resistance to EGFR inhibitors in EGFR-dependent glioblastoma through AKT regulation. Concurrent blockade of these two pathways holds promise to treat EGFR-dependent glioblastoma.
Translational Relevance

Glioblastoma is frequently affected by aberrantly activated EGFR. However, unlike EGFR-dependent epithelial cancers, glioblastoma exhibits intrinsic resistance to EGFR inhibitors. Functionally redundant RTKs are widely implicated in resistance to anti-EGFR therapy. Yet, it remains to be determined which RTK(s) is accounted for clinical failure of EGFR inhibitors in glioblastoma. Our recent study unveiled frequent activation of InsR and the related family member IGF1R in glioblastoma. Here, we demonstrated that the activation of the InsR/IGF1R pathway is associated with resistance to EGFR inhibitors in the majority of EGFR-dependent glioblastoma samples with few exceptions that did not express InsR or IGF1R. Targeting InsR/IGF1R synergistically improved glioblastoma response to EGFR inhibitors in vitro and in vivo. Therefore, for glioblastoma patients affected by both EGFR aberration and InsR/IGF1R activation, dual inhibition of InsR/IGF1R and EGFR is a promising therapy.
INTRODUCTION

Aberrantly activated EGFR affects a wide range of human cancers, particularly lung cancer, colorectal cancer, head and neck squamous carcinoma (HNSCC), and glioblastoma. Among all these cancer types, glioblastoma has the highest rate of EGFR gene alteration. It is estimated that EGFR is activated in more than half of glioblastoma tumors through genetic events, such as amplification, rearrangement, point mutations, and in-frame deletion (1-4). However, despite extensive preclinical and clinical efforts, EGFR inhibitors, either as monotherapy or in combination with other therapeutic modalities, fail to produce significant clinical benefits in glioblastoma (5). Many challenges remain to be addressed to effectively target EGFR-dependent glioblastoma. For example, the majority of glioblastoma trials for EGFR inhibitors are performed in unselected patient populations without adequate characterization of EGFR status (5). Currently available EGFR inhibitors often fail to achieve adequate inhibition of EGFR in tumors due to sub-optimal brain distribution (6). EGFRvIII, the most common form of mutant EGFR in glioblastoma, is relatively refractory to currently available EGFR inhibitors (7). Additionally, two recent studies suggest that glioblastoma are enriched for mutations in the extracellular domain (ECD) of EGFR, which are refractory to the first-generation EGFR kinase inhibitors, such as erlotinib (8, 9). Conversely, lung cancer-type EGFR mutations are frequently found in the catalytic domains that confer sensitivity to gefitinib and erlotinib (8, 9).

An alternative model to interpret resistance to EGFR-targeted therapy is activation of functionally redundant RTKs (10). Preclinical and clinical evidence supporting this model has been extensively documented (11, 12). However, primary RTKs that mediate resistance to EGFR-targeted therapy in glioblastoma remains to be characterized. Recurrent aberrations of several functionally overlapping RTKs, such as ERBB2, MET and PDGFR, have been reported in glioblastoma (1-3). Non-genetic mechanisms may also contribute to compensatory activation of additional RTKs. Our group recently demonstrated that insulin receptor (InsR) and the closely related family member insulin-like growth factor 1 receptor (IGF1R) are frequently activated in glioblastoma (13). Both InsR and IGF1R are rarely
mutated or amplified in glioblastoma. Rather, activation of the InsR/IGF1R pathway appears to be primarily mediated through ligands produced via endocrine mechanisms. Genetic or pharmacological targeting of InsR or IGF1R has significant impact on AKT signaling in glioblastoma (13), suggesting potential implications in resistance to EGFR inhibitors. Exactly which RTK pathway(s) play significant roles in glioblastoma resistance to EGFR inhibitors remains an open question. In the current study, we screened several growth factors that activate RTKs implicated in gliomagenesis. Our results demonstrated that activation of the InsR/IGF1R pathway induced potent resistance to EGFR inhibitors in the majority of EGFR-dependent glioblastoma PDX lines and that inhibition of InsR/IGF1R significantly improved response to EGFR inhibitors. These findings provide the proof-of-principle to co-target these two RTKs pathways for treating glioblastoma.

MATERIALS AND METHODS

Glioblastoma samples and cell culture

Primary glioblastoma xenograft lines were originally derived from surgical specimens and serially transplanted in immunocompromised mice as previously described (14, 15). Samples with a “GBM” prefix were established at Mayo Clinic, T08-320 and T08-322 were generous gifts from Jeremy Rich at Cleveland Clinic, VU10369 and VU10827 are established at Vanderbilt University Medical Center. All glioblastoma ex vivo cultures were maintained for less than 8 passages in Neurobasal medium (Life Technologies) supplemented containing B-27 Supplement Minus Vitamin A (Life Technologies), 20 ng/ml EGF (Peprotech , #AF-100-15 ) and 20 ng/mL bFGF (#AF-100-18B ), 1% penicillin-streptomycin, 2mM L-glutamine and 1mM sodium pyruvate. The human non-small cell lung cancer cell line PC9 were maintained in RPMI-1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Life Technologies). All cells were maintained at 37°C in a 5% CO2-humidified incubator.
Plasmids, antibodies and other reagents

Lentiviral expression for Myr-AKT1 was previously described (15). Antibodies specific to p-IGFIRβ/InsRβ (#3024), IGFIRβ (#3018), pY1068-EGFR (#3777), EGFR (#4267), pY705-STAT3 (#9145), STAT3 (#9139), pS473-AKT (#4060), AKT (#2920), p-ERK (#4370), ERK (#4696) and cleaved-caspase 3 (#9661) were purchased from Cell Signaling Technologies. Monoclonal antibody against InsRβ (sc-57342) was purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody against actin (#MAB1501) was purchased from Millipore.

Gefitinib (G-4408) and OSI-906 (L-5814) was purchased from LC Laboratories. BMS-754807 (CT-BMS75) was purchased from Chemitek. Dacomitinib (S2727) was purchased from Selleckchem. Recombinant Human IGF1 (AF-100-11), Heregulin-β1 (#100-03), HGF (#100-39), PDGF-AB (100-00AB), were from Peprotech. Insulin (#12585-014) was from Life technology.

Cell viability assay and caspase activation assay

To determine drug-induced changes in cell viability, glioblastoma cells were aliquoted into 96-well plate at 5,000 cells per well in triplicates. Drugs were added by 2-fold serial dilutions. Cell number was measured using the Sensolyte Cell Viability and Proliferation Assay Kit (AnaSpec) after a 5-day incubation and normalized to corresponding vehicle-treated groups. Dose-response curves were generated using GraphPad Prism 5 software following a three-parameter nonlinear regression model.

Activation of caspase-3/7 was measured by the Caspase-Glo 3/7 Assay Kit (Promega) according to the manufacturer's instructions. Values of caspase activities were normalized to the corresponding cell titers measured by CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega) to determine the relative caspase-3/7 activities.
Genome sequencing

Multiplexed targeted resequencing assays were performed by Vanderbilt Technologies for Advanced Genomics using the Illumina TruSeq Amplicon – Cancer Panel following the manufacturer's instructions. Samples were sequenced on the Illumina HiSeq 2500 platform. Data were processed through Illumina’s CASAVA v1.8.2 pipeline.

Polymerase chain reaction (PCR)

PCR distinguishing full-length EGFR and EGFRvIII was performed using cDNA. First, total RNA was isolated using the Illustra RNAspin kit (GE Healthcare) and reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). EGFR transcripts were then was amplified by two sets of primers. The first primer set (EGFRF1 + EGFRR1) generated an amplicon of 1044 bp from full-length EGFR and an amplicon of 243 bp from EGFRvIII. EGFRF1: 5’-CTTCGGGGAGCAGCGATGCGAC and EGFRR1: 5’-ACCAATACCTATTCCGTAC. The second primer set (EGFRF2 + EGFRR1) utilizes sequences deleted in EGFRvIII, thus only recognized full length EGFR, generating a 478-bp amplicon. EGFRF2: 5’-TTTACAGGGCCAAAAGTGAT. PCR reactions comprised 30 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 90 seconds at 72°C. Plasmid encoding EGFRvIII (Addgene, #20737) was used as control (16).

Immunohistochemistry

Immunohistochemical staining of xenograft tumor sections were performed with primary antibody against Ki67 (#VP-K451, Vector Laboratories, Inc., Burlingame, CA) at a 1:2000 dilution or cleaved Caspase-3.
(# 9664, Cell Signaling) at a 1:300 dilution. Stainings were visualized by the Bond Polymer Refine detection system.

**Xenograft tumor assays**

All animal experiments were performed in accordance with a protocol approved by the Vanderbilt University Institutional Animal Care and Use Committee. Female athymic nude mice were used for all experiments. One million GBM76 cells or 0.25 million GBM39 cells were suspended in 50 µl phosphate buffered saline, mixed with an equal volume of Matrigel (BD Biosciences), and implanted into flank sites. Subcutaneous xenograft tumors were established for both sides of each mouse. Tumor volumes were calculated as length × width × width/2. When tumors reached approximately 50 mm³, mice were treated with 50 mg/kg OSI-906 suspended in 25 mM tartaric acid, 50 mg/kg gefitinib in 1% tween 80, or drug combination once per day by oral gavage.

**Statistical Analysis**

GraphPad Prism 5.0 was used to determine statistical significance. When p values are smaller than 0.05, the results are considered statistically significant. Combination index (CI) values were calculated using the Compusyn software. CI values smaller than 1 indicate synergistic drug interaction.

**RESULTS**

**Insulin and IGF1 protect glioblastoma cells in the presence of gefitinib**

Therapeutic response to EGFR inhibitors in glioblastoma has been mostly modeled using established cell lines. However, aberrant activation of EGFR and expression of EGFRvIII is progressively lost during
prolonged culture (17-19). As such, ectopic EGFR or EGFR mutants are often introduced into glioblastoma cell lines to study therapeutic response to EGFR inhibitors (7). More recently, it has been demonstrated that glioblastoma PDX lines and primary cultures maintain expression of EGFR and EGFRvIII in serial xenotransplantation and serum-free culture systems (19-21). We have collected a panel of glioblastoma PDX lines carrying various EGFR aberrations to model therapeutic response to EGFR inhibitors. EGFR point mutations in these PDX lines were determined by targeted re-sequencing (Supplementary Table 1). Expression of EGFRvIII and full length EGFR in these PDX lines maintained in serum-free growth factor-supplemented medium was determined by PCR and immunoblotting (Supplementary Fig. 1A-1C). EGFRvIII was the dominating form in some lines, such as GBM39, GBM59, VU10369 and VU10827, while others primarily expressed wild type EGFR or full-length EGFR with point mutations, such as GBM6, GBM26, GBM46 and T08-320. Expression of EGFRvIII in GBM6 and GBM46 had been shown in xenotransplantation but was not maintained in culture (22). GBM76 expressed both full-length EGFR and EGFRvIII in culture. In contrast, EGFR expression was barely detected in two control lines, GBM44 and T08-322. Five of these PDX lines carrying representative EGFR aberrations were selected for subsequent screening.

To identify mechanisms mediating resistance to anti-EGFR therapy, we asked whether activation of additional RTK pathways by recombinant ligands might protect glioblastoma cells in the presence EGFR inhibitors (Fig. 1A). We selected a representative subset of six growth factors because their corresponding receptors are frequently upregulated in glioblastoma, including MET, InsR/IGF1R, HER3/4, and PDGFR. Gefitinib was used for this screening because it has exhibited modest efficacy in a small subset of glioblastoma patients and resulted in significant dephosphorylation of EGFR in some patient tumors (23, 24). Glioblastoma cells dissociated from subcutaneous xenograft tumors were briefly cultured in vitro, starved overnight in growth factor-free medium, and exposed to serially diluted gefitinib in the presence of absence of 100 ng/ml ligands. Cell viability was measured to determine sensitivity to gefitinib. This
screening showed that recombinant EGF promoted cell growth at low nanomolar concentrations of gefitinib, when EGFR was not effectively blocked. However, the growth promoting effect of EGF was dose-dependently abolished by gefitinib (Fig. 1B, 1C, Supplementary Fig. 2A-2C). Of note, EGF did not significantly promote growth in GBM12, probably due to lung cancer-type of point mutations in the kinase domain that render EGFR constitutively active in this line (Supplementary Table 1). In contrast to EGF, Insulin and IGF1 significantly increased cell viability in a manner that was not sensitive to gefitinib (Fig. 1B and Supplementary Fig. 2A-2C). Additionally, PDGF moderately protected GBM12 and T08-320 cells against gefitinib, suggesting that the PDGFR pathway may also contribute to resistance to EGFR inhibitors in a subset of glioblastoma (Fig. 1A, Supplementary Fig. 2D-2F). GBM46 was not affected by insulin or IGF1 (Fig. 1C), which was consistent with our recent findings that GBM46 did not express InsR or IGF1R (13). These results indicate that activation of the InsR/IGF1R pathway represents an important mechanism in resistance to EGFR inhibitors in glioblastoma.

Inhibition of InsR/IGF1R sensitizes glioblastoma cells to EGFR inhibitors

The ability of insulin and IGF1 to protect glioblastoma cells against gefitinib suggests that co-targeting the InsR/IGF1R pathway may improve tumor response to EGFR inhibitors. To test this hypothesis, we assessed the efficacy of gefitinib either alone or in combination with OSI-906, an InsR/IGF1R dual kinase inhibitor (25). Our results showed that the combination of gefitinib and OSI-906 was significantly more effective than either agent alone in all lines that we have tested (Fig. 2A, 2B, and Supplementary Fig. 3A, 3B). Loss of cell viability following treatment of both gefitinib and OSI-906 was associated with potent activation of caspase 3 in both GBM39 and GMB76 cells (Fig. 2C and 2D). Using the Chou-Talalay statistical method, we demonstrated that the CI values of gefitinib and OSI-906 were significantly lower than 1 at concentrations that ranged over two orders of magnitudes, indicating strong synergism (Fig. 2A, 2B, and Supplementary Fig. 3A, 3B). Similar synergistic interactions were showed using an irreversible EGFR inhibitor, dacomitinib, and a highly potent InsR/IGF1R inhibitor, BMS-754807 (Supplementary Fig. 3C-3F), suggesting that the synergistic interaction could be applied to additional compounds in these
two categories of RTK inhibitors. The patterns of EGFR aberrations did not significantly affect response to this combination therapy. EGFRvIII-expressing GBM39 and VU10369 were sensitive to the combination therapy. GBM76 expressed both full-length EGFR and EGFRvIII. In addition, 10% of the full-length EGFR allele in GBM76 harbors a glioblastoma-type A289V mutation, while EGFR in GBM12 harbors a lung cancer-type G719A mutation. Yet, both GBM12 and GBM76 were only modestly responsive to EGFR inhibitors but were highly sensitive to the combination therapy (Fig. 2B, Supplementary Fig. 3A, 3C, and 3F). In contrast, the InsR/IGF1R-negative GBM46 lines only responded to gefitinib or dacomitinib, but not OSI-906 or BMS-754807, indicating that the actions of OSI-906 and BMS-754807 were specifically mediated through inhibition of InsR and IGF1R (Supplementary Fig. 3G and 3H). These results collectively suggest that concurrent inhibition of EGFR and InsR/IGF1R has the potential to induce significant response in EGFR-dependent glioblastoma tumors.

**Blockade of both InsR/IGF1R and EGFR pathways results in potent AKT inhibition**

To interrogate the crosstalk between EGFR and InsR/IGF1R, we asked how turning on and off these two pathways affected key downstream signaling molecules, namely AKT, ERK and STAT3. GBM39 cells were starved in growth factor-free medium overnight and stimulated with insulin, IGF1 or EGF in the presence or absence of gefitinib. This experiment showed that gefitinib decreased AKT phosphorylation in the absence of insulin or IGF1 (Fig. 3A). However, administration of insulin or IGF1 induced potent AKT activation that was resistant to gefitinib (Fig. 3A), suggesting that the InsR/IGF1R pathway is the dominating RTK regulator for AKT. In contrast, the ability of gefitinib to repress STAT3 and MEK/ERK was not affected by activation of the InsR/IGF1R pathway (Fig. 3A). In InsR/IGF1R-negative GBM46, insulin and IGF1 did not stimulate AKT or alter the ability of gefitinib to repress AKT (Supplementary Fig. 4A). These results suggest that the InsR/IGF1R pathway functions as the key RTK regulator of the PI3K/AKT signaling axis in glioblastoma tumors in which the receptors are expressed.
We further showed that blockade of the InsR/IGF1R pathway by OSI-906 significantly repressed AKT activity in all tested lines with the exception of GBM46 (Fig. 3B, 3C, and Supplementary Fig. 4A-4C), underscoring the role of the InsR/IGF1R pathway in maintaining AKT activities in glioblastoma. While gefitinib as monotherapy had modest impact on AKT, the combination of OSI-906 and gefitinib was more effective than either agent alone to repress AKT (Fig. 3B, 3C and Supplementary Fig. 4B). Gefitinib decreased STAT3 signaling in EGFRvIII-expressing lines, GBM39 and GBM76 (Fig. 3B and 3C), but not in GBM12 that expressed EGFR<sup>G719A</sup> (Supplementary Fig. 4B). This was in concordance with the function of EGFRvIII as a direct regulator of STAT3 (26). Inhibition of EGFR increased phosphorylation of InsR/IGF1R in GBM12 (Supplementary Fig. 4B), suggesting a feedback crosstalk between these two RTK pathways in glioblastoma. However, this mechanism was not showed in other EGFR-dependent lines (Fig. 3B and 3C). In InsR/IGF1R-negative GBM46, gefitinib alone not only repressed STAT3 and ERK, but also inhibited AKT (Supplementary Fig. 4A and 4C), indicating that EGFR may function as the primary RTK regulator of the PI3K/AKT signaling when InsR and IGF1R do not compete with it. To further assess the role of AKT in regulating responsiveness of glioblastoma to EGFR inhibitors, a constitutively active Myr-AKT1 was stably expressed in GBM39 or GBM12 by lentivirus (Supplementary Fig. 5). Expression of Myr-AKT1 essentially restored cell viability in the presence of gefitinib and OSI-906, either alone or in combination (Fig. 3D). Finally, we showed that either MK-2206, an AKT inhibitor, or PF-04691502, a PI3K/mTOR dual kinase inhibitor, also increased GBM76 sensitivity to gefitinib (Supplementary Fig. 6). Thus, effective repression of AKT is essential to induce significant cytotoxicity by EGFR inhibitors. Collectively, our findings suggest that the InsR/IGF1R pathway plays a prominent role in regulation of AKT signaling in a substantial percentage of glioblastoma and that inhibition of InsR/IGF1R compromises resistance to EGFR inhibitors through repression of AKT activity.

We next compared how inhibition of EGFR affected these downstream signaling molecules in EGFR-dependent lung cancer. PC9 cells have a deletion in exon 19 of EGFR typical to lung cancer, which
activates EGFR and confers sensitivity to EGFR inhibitors. Like GBM46, PC9 cells only responded to gefitinib but not OSI-906 (Supplementary Fig. 7A). Gefitinib potently decreased phosphorylation of both AKT and ERK in this line (Supplementary Fig. 7B). In contrast, OSI-906 had limited impact on AKT and other downstream signaling pathways, despite expression of both InsR and IGF1R (Supplementary Fig. 7B). Therefore, in lung cancer, EGFR may function as the predominating regulator of the PI3K/AKT signaling axis, irrespective of the status of InsR and IGF1R. Another difference between PC9 and glioblastoma lines was that gefitinib induced a significant feedback activation of STAT3 in PC9 cells (Supplementary Fig. 6B), potentially due to activation of additional RTKs, such as the FGFR pathway and the IL-6/JAK pathway as shown by Lee and colleagues (27). These results clearly indicate that oncogenic RTKs, such as EGFR and InsR/IGF1R, crosstalk with downstream signaling pathways through distinct mechanisms in glioblastoma and lung cancer. This discrepancy has significant implications in response to therapeutics targeting these RTKs.

**A combination of gefitinib and OSI-906 effectively represses glioblastoma xenograft tumors**

The *in vivo* therapeutic potential of the combination of EGFR inhibitors and InsR/IGF1R inhibitors was examined using subcutaneous glioblastoma xenograft models, due to a lack of brain-penetrating InsR/IGF1R inhibitors. GBM76 was selected as it was not sensitive to either gefitinib or OSI-906 alone in culture. In addition to EGFRvIII, this line carries an EGFR^A289T^ mutation that is thought to reduce sensitivity to the first generation EGFR inhibitors (8, 9). Expression of PTEN in GBM76 culture was not detected by immunoblotting (Supplementary Fig. 1C). Additionally, two PTEN point mutations, N69D (9%) and I253N (95%), were identified in GBM76 (Supplementary Table 1), which are expected to attenuate PTEN functions (28). Accordingly, gefitinib, OSI-906 or the combination was less effective to repress AKT in this line compared with PTEN^wt^ lines, such as GBM12 and GBM39 (Fig. 3B, 3C and Supplementary Fig. 4B). Mice bearing GBM76 xenograft tumors were treated following a 5-days-on/2-days-off schedule, which did not induce significant weight loss during the entire course of treatment (Supplementary Fig. 8A). While gefitinib and OSI-906 as monotherapy modestly reduced tumor growth,
the combination was significantly more effective than either compound alone (Fig. 4A). The combination therapy was also examined in a PTENwt/EGFRvIII-expressing PDX line, GBM39. This line is sensitive to EGFR inhibition. Our previous study has demonstrated that erlotinib modestly extended survival of mice bearing intracranial GBM39 tumors (21). In this study, we showed that median tumor size in the gefitinib-treated arm increased by approximately 2 folds after the 16-day treatment compared with a 13-fold increase in the vehicle-treated arms (Fig. 4B). While OSI-906 alone did not have any efficacy, the combination of gefitinib and OSI-906 resulted in approximately 50% reduction in median tumor size (Fig. 4B). Thus, co-targeting InsR/IGF1R further augmented the efficacy of EGFR inhibitors in sensitive tumors. A continuous daily administration schedule was employed in this experiment. At the end of the 16-day course of treatment, the median weight of animals in the combination therapy-treated arm was approximately 15% lower than the vehicle-treated arm, but all animals remained stable and active (Supplementary Fig. 8B). Immunoblotting of GBM39 tumor lysates 4 hours after a single-dose drug administration showed that EGFR and InsR/IGF1R were effectively dephosphorylated by gefitinib or OSI-906, respectively (Fig. 4C). The combination therapy induced more effective reduction of AKT activity than either agent alone (Fig. 4C). Additionally, the combination was more potent than monotherapies to induce apoptosis and inhibit proliferation, as shown by immunohistochemical staining of cleaved caspase 3 and Ki67 (Fig. 4D). Collectively, our in vivo observations recapitulated in vitro results, suggesting that concurrent inhibition of InsR/IGF1R has the potential to significantly improve the efficacy of EGFR inhibitors for treating EGFR-mutant glioblastoma, even in tumors carrying mutations in EGFR and PTEN that are associated with resistance to EGFR inhibitors.

DISCUSSION

Although anti-EGFR therapies have achieved significant clinical success in several epithelial cancers, outcomes in glioblastoma are disappointing. Why is the cancer type affected by the highest rate of aberrant EGFR activation not responsive to EGFR inhibitors? An emerging theory explains this discrepancy by comparing EGFR-mutants commonly found in glioblastoma with lung cancer-type EGFR
mutants (8, 9). EGFR mutations in lung cancer are enriched in the kinase domain, making lung cancer sensitive to the first generation EGFR inhibitors. Conversely, mutations in glioblastoma predominately affect the extracellular region of EGFR, including deletion at exon 2-7 and point mutations at amino acid residues A289 and R108. These ECD mutants release the first generation EGFR inhibitors at a more rapid rate compared with wild type EGFR, thus they are less sensitive (8). However, lung cancer-type EGFR mutations are not common in colorectal cancer and HNSCC. Therefore, the efficacy of anti-EGFR therapy in these cancer types cannot be explained by the mutational patterns of EGFR. In addition, amplification of wild type EGFR is a major mechanism that activates this pathway in glioblastoma, which is expected to be sensitive to the first generation EGFR inhibitors (1). In a small scale clinical trial, Hegi et al. found that gefitinib treatment induced apparent dephosphorylation of EGFR in patient tumors (24). However, downstream signaling molecules, components of the AKT pathway in particular, were not significantly repressed (24), suggesting that blockade of EGFR alone may not be sufficient to inhibit key signaling molecules in glioblastoma. Collectively, these observations suggest that resistance to EGFR inhibitors in glioblastoma cannot be fully explained by the mutational status pattern of EGFR alone.

Functionally redundant RTKs pathways are known to induce intrinsic and acquired resistance to anti-EGFR therapy in epithelial cancers. Activation of MET, AXL, ALK and additional EGFR family members are repetitively identified in cancer cell lines or patient tumors resistant to EGFR inhibitors (29-34). Overexpression of IGF1R has been also found to confer resistance to EGFR inhibitors in glioblastoma cell lines (35). Stommel and colleagues described multiplexed activation of RTKs in glioblastoma cell lines, xenograft tumors, and patient specimens (10). Specifically, active InsR and/or IGF1R were identified in 9 out of 14 primary glioblastoma specimens. Our group recently demonstrated expression of InsR and IGF1R in glioblastoma surgical specimens and PDX lines (13). Both receptors were implicated in AKT regulation, as knocking down either one of them reduced AKT activity (13). In the current study, we demonstrated that activation of the InsR/IGF1R pathway conferred resistance to EGFR inhibitors in the majority of glioblastoma tumors affected by EGFR aberration. Inhibition of
InsR/IGF1R increased sensitivity to gefitinib in vivo and in vitro. AKT is a key downstream target of InsR/IGF1R mediating this phenotype, as expression of a constitutively active AKT fusion protein abolished the activities of OSI-906, either alone or in combination with gefitinib. Our data further showed that gefitinib modestly reduced AKT signaling in EGFR-mutant glioblastoma and that addition of insulin or IGF1 activated AKT and abolished the ability of gefitinib to repress AKT signaling. In contrast to glioblastoma, EGFR appears to be the preferential RTK regulator of AKT signaling in lung cancer. Engelman and colleagues found that gefitinib reduced AKT activity in gefitinib-sensitive lung cancer cell lines but not in the resistant lines (36). Our results also showed that gefitinib alone was sufficient to almost completely block AKT signaling in PC9 lung cancer cells despite expression of both InsR and IGF1R. These findings suggest an additional level of discrepancy between glioblastoma and lung cancer with respect to how the PI3K/AKT pathway is controlled by upstream RTKs. In glioblastoma, full activation of the PI3K/AKT signaling in glioblastoma involves signal inputs of multiple RTKs, in addition to other perturbations, such as loss of PTEN. In EGFR-mutant lung cancer and possibly other cancer types that are sensitive to anti-EGFR monotherapy, the PI3K/AKT pathway is predominantly regulated by EGFR. However, prolonged treatment may induce ‘oncogenic shift’ and lead to acquired resistance by activating additional RTKs (11). Conversely, ‘oncogenic shift’ is a pre-existing condition in glioblastoma. As such, concurrent inhibition of both EGFR and InsR/IGF1R is necessary to effectively attenuate AKT signaling and induce significant response in glioblastoma. These findings collectively suggest that differential crosstalk between RTKs and the PI3K/AKT signaling axis have important indications in therapeutic response to RTK-targeted therapies. A better understanding of these mechanisms may lead to innovative and more effective therapeutic strategies.

It is important to identify patient subpopulations that have the best chance to benefit from the combination therapy of EGFR inhibitors and InsR/IGF1R inhibitors. Our current study suggests that the efficacy of EGFR inhibitors can be enhanced by InsR/IGF1R inhibitors in EGFR-dependent glioblastoma tumors that express InsR, IGF1R or both. InsR and IGF1R are not frequently mutated or amplified in glioblastoma
according to the Cancer Genome Atlas data (<2%, retrieved via cBioPortal) (37). However, active InsR and/or IGF1R have been identified in the majority of glioblastoma surgical specimens (10). Our recent study also demonstrated that both InsR and IGF1R could be detected in glioblastoma patient specimens by immunohistochemical staining or immunoblot. Neurobiological functions of insulin and IGF1 in the central nervous system have been extensively documented (38, 39). Thus, the InsR/IGF1R pathway is potentially activated through endocrine mechanisms in a substantial portion of glioblastoma. Alternatively, IGF2 overexpression has been identified in a minor subset of glioblastoma tumors and is associated with elevated AKT phosphorylation and poor survival (41). In addition, insulin is routinely used in glioblastoma patients affected by hyperglycemia, suggesting potential risk of stimulating the InsR/IGF1R pathway in tumors (42). Taken together, expression of InsR and IGF1R or their ligands in glioblastoma may be utilized to guide use of InsR/IGF1R inhibitors. Antibodies that can selectively detect active InsR and IGF1R in formalin fixed and paraffin embedded tissues would have important diagnostic values.

In conclusion, our study provides the proof-of-concept of a combination therapy of InsR/IGF1R inhibitors and EGFR inhibitors to treat EGFR-dependent glioblastoma. However, while novel brain-penetrating EGFR inhibitors have recently emerged (43, 44), the lack of InsR/IGF1R inhibitors with favorable brain distribution remains a significant obstacle for clinical translation. Additionally, generation of significant tumor response may require nearly complete and sustained blockade of both RTKs in tumors. Because InsR and IGF1R have important functions in normal metabolism, prolonged blockade of this pathway may be associated with significant adverse effects. As such, a therapeutic window needs to be carefully defined. Compounds that can penetrate the blood-brain-barrier and enrich in tumor tissues are highly desired to improve efficacy without inducing significant side effects.

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Use of cell lines

The glioblastoma PDX lines used in this study were originally derived from surgical specimens obtained at Cleveland Clinic, Mayo Clinic and Vanderbilt University in accordance of the protocols approved by Institutional Review Boards. These lines were passaged in immunocompromised mice through serial xenotransplantation without tissue culture. The identities of these lines were determined by targeted re-sequencing completed in 2013 as described in methods. Cells used in this study were derived from xenograft tumors passaged less than 2 times in mice after authentication. Cells were cultured no more than 8 messages in vitro for this study.
References

Legends

Figure 1. Ligands of InsR/IGF1R confer resistance to gefitinib in EGFR-mutant glioblastoma ex vivo cultures. (A) Summarized response to RTK ligands at 100 ng/ml. The screening used glioblastoma short term ex vivo cultures derived from freshly collected subcutaneous xenograft tumors. Rescue: ligands significantly increased cell viability with gefitinib at concentrations > 500 nM; Growth promoting: ligands significantly increased cell viability only when gefitinib at concentrations < 100 nM; Blank: no significant response. HGRβ1: Heregulin-β1. (B) Representative response demonstrating rescue against gefitinib in an insulin/IGF1-sensitive line, GBM39, and absence of rescue in an insulin/IGF1-irresponsive line, GBM46. Error bars represent standard deviation derived from 3 technical replicates unless otherwise indicated.

Figure 2. Inhibition of the InsR/IGF1R pathway synergistically increases sensitivity to EGFR inhibitors. (A) GBM39 or (B) GBM76 cells were plated in 96-well plates and treated with gefitinib ± OSI-906 following a 2-fold serial dilution. The combination contained a fixed equal molar ratio of gefitinib and OSI-906. Cell viability was determined 5 days after treatment. The combination index values were calculated for each data point by the Chou-Talalay method and presented as green crosses. (C) GBM39 or (D) GBM76 cells were treated with gefitinib ± OSI-906 at indicated concentrations. Three days after treatment, activities of caspase 3 were determined by Promega Caspase3/7-Glo assay and normalized to the corresponding cell titers.

Figure 3. Concurrent inhibition of InsR/IGF1R and EGFR effectively represses AKT activation. (A) GBM39 cells were starved in growth factor-free medium overnight and stimulated with 100 ng/ml insulin, IGF1, or EGF in the presence or absence of 1 µM gefitinib as indicated. Cells were then collected for immunoblotting. In this figure and subsequent immunoblot figures, the numbers represent relative densities of pS473-AKT bands quantified using ImageJ and normalized to the corresponding total AKT bands. A value of 1 was assigned to the control groups. (B) GBM39 or (C) GBM76 cells were treated
with gefitinib ± OSI-906 at indicated concentrations for 24 hours before lysed for immunoblotting. (D) GBM12 and GBM39 cells were infected with lentivirus directing expression of AKT1 fused to an N-terminal myristoylation sequence, selected by puromycin for 2 days. Cellular response to gefitinib ± OSI-906 was then determined as described in Figure 2A.

**Figure 4. The combination of gefitinib and OSI-906 induces superior efficacy against subcutaneous glioblastoma xenograft tumors related to single agents.** (A) GBM76 subcutaneous xenograft tumors (n=7) were treated with 50 mg/kg gefitinib ± 50 mg/kg OSI-906 once a day by oral gavage following a 5-days-on/2-days-off schedule. (B) GBM39 subcutaneous xenograft tumors (n=7) were treated daily with the same doses. Data shown are median tumor volume with error bars representing interquartile range. *: p<0.05, combination arm vs. gefitinib alone arm, calculated by Student’s t-test. (C) Mice bearing GBM39 tumors were treated with a single dose of 50 mg/kg gefitinib ± 50 mg/kg OSI-906. Four hours later, animals were sacrificed and tumors were resected, lysed and subjected to immunoblotting for indicated targets. (D) Mice bearing GBM39 tumors with sizes at approximately 500 mm³ were treated for 5 days as described above. Tumors were then resected, fixed in formalin, and stained for Ki67 or cleaved caspase-3.
Figure 1

A

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<th></th>
<th>IGF1</th>
<th>Insulin</th>
<th>EGF</th>
<th>HRGβ1</th>
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<tr>
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</tbody>
</table>

- R: Rescue
- G: Growth promoting
- Green: No response

B

GBM39, rescue

- Vehicle
- IGF1
- Insulin
- EGF

C

GBM46, no rescue

- Vehicle
- IGF1
- Insulin
- EGF

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Figure 2

A

B

C

D

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Figure 4

A

B

C

D

Ki67

Cleaved caspase-3

Veh Gef OSI Combo

p-IGFIRβ/InsRβ

IGFIRβ

InsRβ

pY1068-EGFR

EGFR

pS473-AKT

AKT

pERK

ERK

Actin

Vehicle

Gefitinib

OSI-906

Combination
Clinical Cancer Research

InsR/IGF1R pathway mediates resistance to EGFR inhibitors in glioblastoma

Yufang Ma, Nan Tang, Reid Carleton Thompson, et al.

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