Development of Resistance to EGFR-Targeted Therapy in Malignant Glioma Can Occur through EGFR-Dependent and -Independent Mechanisms

Stefan Klingler¹, Baofeng Guo¹, Jun Yao², Haiyan Yan³, Ling Zhang¹, Angelina V. Vaseva¹, Sida Chen¹, Peter Canoll⁴, James W. Horner⁵, Y. Alan Wang⁵, Ji-Hye Paik⁶, Haoqiang Ying², and Hongwu Zheng¹

Abstract

Epidermal growth factor receptor (EGFR) is highly amplified, mutated, and overexpressed in human malignant gliomas. Despite its prevalence and growth-promoting functions, therapeutic strategies to inhibit EGFR kinase activity have not been translated into profound beneficial effects in glioma clinical trials. To determine the roles of oncogenic EGFR signaling in glioma genesis and tumor maintenance, we generated a novel glioma mouse model driven by inducible expression of a mutant EGFR (EGFR⁰). Using combined genetic and pharmacological interventions, we revealed that EGFR⁰-driven gliomas were insensitive to EGFR tyrosine kinase inhibitors, although they could efficiently inhibit EGFR⁰ autophosphorylation in vitro and in vivo. This is in contrast with the genetic suppression of EGFR⁰ induction that led to significant tumor regression and prolonged animal survival. However, despite their initial response to genetic EGFR⁰ extinction, all tumors would relapse and propagate independent of EGFR⁰. We further showed that EGFR⁰-independent tumor cells existed prior to treatment and were responsible for relapse following genetic EGFR⁰ suppression. And, the addition of a PI3K/mTOR inhibitor could significantly delay relapse and prolong animal survival. Our findings shed mechanistic insight into EGFR drug resistance in glioma and provide a platform to test therapies targeting aberrant EGFR signaling in this setting. Cancer Res; 75(10); 2109–19. ©2015 AACR.

Introduction

Malignant glioma is the most common and lethal type of brain tumor (1, 2). The current standard care for malignant glioma includes maximal surgical resection, followed by radiation with adjuvant chemotherapy for the residual infiltrative tumor component. Despite such aggressive treatment efforts, the disease invariably returns. In its most aggressive form, glioblastoma (GBM) has a median survival of only 12 to 15 months after initial diagnosis (2, 3). Unfortunately, refinements of available therapeutic modalities, including microneurosurgery, radiation, and chemotherapy, in the last several decades have not substantially improved patient survival.

Although clinical progress has been slow, the past decade has witnessed major advances in our understanding of malignant glioma molecular pathogenesis. Detailed genetic and genomic characterization of malignant gliomas has yielded a comprehensive atlas of genomic rearrangements, genetic mutations, and epigenetic alterations that drive disease pathogenesis and biology (4–8). These analyses also defined clinically relevant malignant glioma molecular subtypes (8). Specifically, genetic alterations of IDH1/PDGFRα and NF1 define proneural and mesenchymal subtypes, whereas the classical subtype is highly enriched for epidermal growth factor receptor (EGFR) amplification/mutation and loss of PTEN and CDKN2A (4). These revelations underscore the selective cooperativity among different genetic and genomic alterations during malignant glioma formation.

EGFR gene amplification occurs in approximately 30% to 50% of malignant gliomas and is often associated with gene rearrangements (9). Among them, a constitutively activated mutant form of EGFR with in-frame deletion of 2–7 exons (referred to hereafter as EGFR⁰) is the most common, and presents in approximately 40% to 50% of EGFR-amplified malignant gliomas (4, 10–12). The high-level EGFR amplifications often comprise extrachromosomal double minutes and are heterogeneously distributed within the tumors (13–16). To date, abundant experimental evidences, including mouse models, have firmly established the causal role of aberrant EGFR amplification/mutation and its overexpression on glioma pathogenesis (10, 17–20), making it a compelling candidate for targeted therapies. However, despite the remarkable therapeutic benefits of EGFR tyrosine kinase inhibitors (TKI) on non–small cell lung cancer (NSCLC) patients carrying EGFR mutations (21–23), these agents have showed meager efficacy in malignant glioma clinical trials (24–26). The clinical observations have raised questions about whether EGFR signaling is a viable...
therapeutic target for malignant glioma treatment. In this study, we present a novel inducible glioma mouse model to interrogate the role of oncogenic EGFR signaling on glioma maintenance.

Materials and Methods

Mice

All mouse manipulations were approved and performed under the guidelines of the Institutional Animal Care and Use Committee of the Cold Spring Harbor Laboratory (CSHL; Cold Spring Harbor, NY). The conditional Ink4a/Arf (27), Pten (28), transgenic tetO-EGFRF (29), Nestin-CreER2T2 (30), and hGFAP-tTA mice (from The Jackson Laboratory; ref. 31) have been described previously. All combinations of compound mice were generated by interbreeding and maintained on FvB/C57BL/6 hybrid background in specifically pathogen-free conditions at CSHL. The breeding pairs and neonatal pups until 4-week-old age were kept continuously on doxycycline (Dox) containing drinking water (2 g/L) unless otherwise indicated. Genotypes were confirmed using PCR. To induce glioma formation, 4-week-old compound mice containing Nestin-CreERT2 transgene were injected intraperitoneally with tamoxifen (124 mg/kg body weight) dissolved in sunflower oil daily for 5 consecutive days. Mice were monitored daily for signs of ill-health, and euthanized and necropsied when moribund following NIH guidelines.

Reagents

Erlotinib, gefitinib, crizotinib, and Bez-235 were purchased from LC Laboratories. Doxycycline was ordered from Research Products International. Tamoxifen was purchased from Sigma. d-Luciferin was ordered from Goldbio Technology. The antibodies used in this study are described in Supplementary Experimental Procedures.

Histology and immunohistochemistry

At the time of sacrifice, mice were perfused with 4% paraformaldehyde (PFA), and brains were dissected, followed by overnight post fixation in 4% PFA at 4°C. Tissues were processed and embedded in paraffin by CSHL Research Pathology Core. Serial sections were prepared at 5 μm for paraffin sections with every tenth slide stained by hematoxylin and eosin (H&E). All slides were examined by S. Klingler, tumor grading was determined by H. Zheng assisted by P. Canoll based on the WHO grading system (1). IHC and immunofluorescence analyses were performed as previously described (28). Images were captured using an Olympus BX53 or a Zeiss 710 LSM confocal microscope.

Cell culture

Primary tumor cells were isolated from tumor regions of affected mice using a stereo-dissection microscope (Zeiss). Single-cell suspensions made from enzymatically dissociated tissues were cultured in neurobasal media supplemented with EGF (20 ng/mL) and basic fibroblast growth factor (bFGF; 10 ng/mL) as previously described (28). Murine astrocytes were prepared as previously described (32) and maintained in DMEM supplemented with 10% fetal bovine serum (FBS). For EGFR TKI treatment, control or EGFR-mutant transduced astrocytes were seeded in equal cell numbers and serum starved for 24 hours before treatment. EGFR TKIs erlotinib (250 nmol/L) and gefitinib (50 nmol/L) in dimethyl sulfoxide (DMSO) were added to the cells for 4 hours before collection.

Quantitative real-time PCR

Total RNAs were extracted from tissues using RNeasy (Qiagen) and first-strand cDNAs were prepared with SuperScript VIILO cDNA Synthesis Kit (Applied Biosystems, ABI). Quantitative real-time PCR (qPCR) was performed using QuantiTect SYBR Green PCR Kit (Qiagen) on Applied Biosystems StepOne. The primer sequences used in this study are described in Supplementary Experimental Procedures.

Grafting experiments and in vivo inhibitor treatments

For orthotopic grafting, 10,000 primary mouse glioma cells transduced with either luciferase or GFP-expressing vector were injected into front-lobe caudate nucleus of 4- to 6-week-old nu/nu mice (Charles River) using a stereotaxic frame as previously described (32, 33). For subcutaneous grafting, 200,000 cells were injected into flanks of 4- to 6-week-old nu/nu mice. Mice were monitored daily and subjected to weekly bioluminescent imaging (BLI) for tumor development. In vivo treatments were initiated at the indicated time (for orthotopic transplants) or tumor volume (~200 mm3). The tumor-bearing mice were randomized into different groups and treated with doxycycline (2 g/L in drinking water), or daily oral administration of control vehicle, erlotinib (50 mg/kg/d), gefitinib (150 mg/kg/d), crizotinib (25 mg/kg/d), Bez-235 (45 mg/kg/d), alone or in combination as indicated. Mice were monitored daily, and sacrificed at onset of symptoms. At the time of sacrifice, tumor tissues were processed for further experiments.

In vivo bioluminescent imaging

Mice were given intraperitoneal injections of d-luciferin (150 mg/kg) and imaged 5 to 8 minutes after injection using the Caliper Xenogen IVIS Spectrum imaging system following the manufacturer’s instructions.

Expression analysis

Expression Profiling was performed at the CSHL Microarray Core facility using the Mouse Genome 430 2.0 Array (Affymetrix). The Student t test was used to identify differentially expressed genes according to expression profiles. The Ingenuity Pathway Analysis application was performed according to the manufacturer’s instruction (Ingenuity Systems). In brief, differentially expressed genes with threshold P ≤ 0.05 were mapped into Ingenuity Pathways Analysis database as input. Complete profiles are deposited on the Gene Expression Omnibus (GEO) website under accession GSE64751.

Statistical analysis

Animal survivals were analyzed using Graphpad Prism5. Statistical analyses were performed using the log-rank (Mantel–Cox) test. The Student t test was used for comparisons of experiments from two groups. For all experiments with error bars, standard deviation was calculated to indicate the variation within each experiment and data, and values represent mean ± SD.

Results

EGFR TKIs effectively block EGFR+ autophosphorylation in murine astrocytes

The poor response of glioma patients carrying EGFR amplification/mutation to EGFR TKIs is in stark contrast to their remarkable therapeutic benefits for lung cancer patients harboring...
EGFR lysates were prepared and subjected to immunoblot analysis. Note, the molecular weight of EGFR (wt) and EGFR-L858R is approximately 170 kD and indicated EGFR mutants were serum-starved for 24 hours followed by 4-hour treatment of vehicle (DMSO), 250 nmol/L erlotinib, or 50 nmol/L gefitinib. The lung cancer mutants, cellular domain mutants respond to EGFR TKIs differently than EGFR activated mutants. Although the glioma and lung cancer EGFR mutants both exhibit ligand-independent autophosphorylation and activation, the locations of the mutations are conspicuously different—lung cancer mutations generally reside in the EGFR intracellular kinase domain, whereas glioma mutations mainly cluster in the extracellular domain (such as EGFR<sup>E545L</sup>; refs. 34, 35). To investigate whether the extracellular domain mutants respond to EGFR TKIs differently than the lung cancer mutants, EGFR<sup>+</sup> and a TKI-sensitive lung cancer mutant EGFR-L858R were transduced individually into murine astrocytes. To analyze their autophosphorylation, control and mutant EGFR-L858R-transduced astrocytes were serum-starved before being subjected to treatment with two clinically approved small-molecule EGFR TKIs—erlotinib or gefitinib. Immunoblot analysis of phosphorylation of tyrosine residues Y1068 and Y1173 indicated that the inhibitors could effectively block autophosphorylation in both EGFR-L858R and EGFR<sup>+</sup> (Fig. 1A). Similar results were observed when measuring total phosphotyrosine content of the immunoprecipitated EGFR receptors or total EGFR in the immunoprecipitated phosphotyrosine protein (Fig. 1B and C). These observations indicated that the EGFR autophosphorylation could be inhibited comparably as the TKI-sensitive lung cancer–mutant EGFR-L858R TKI treatment also resulted in visible inhibition of EGFR downstream signaling pathways as evidenced by decreased phospho-Akt and phospho-Mapk levels, although to a lesser extent in erlotinib-treated EGFR<sup>+</sup>-transduced samples (Fig. 1A). Similar results were also obtained for two other glioma-specific EGFR extracellular domain mutants—A289V and G398V (Supplementary Fig. S1). Together, these data indicate that autophosphorylation of the brain cancer EGFR mutants can be efficiently suppressed by EGFR TKIs.

EGFR<sup>+</sup> induction alone is insufficient to promote gliomagenesis in adult mice

Because EGFR TKIs could effectively inhibit autophosphorylation of the glioma-specific EGFR mutants, we reasoned that the poor response to EGFR TKIs observed in glioma patients might be due to inefficient drug penetration through the blood–brain barrier, or the possibility that aberrant EGFR signaling might not be required for glioma maintenance. To address these questions, we generated compound transgenic mice that harbored a tetracycline-inducible EGFR<sup>+</sup> (tetO-EGFR<sup>+</sup>) allele driven by hGFAP-tTA, which targets tTA expression to neural progenitor cells and cortical astrocytes (Fig. 2A; ref. 31). Because early induction of EGFR<sup>+</sup> gene expression caused profound brain developmental abnormalities that led to embryonic or early postnatal death in bitransgenic hGFAP-tTA tetO-EGFR<sup>+</sup> mice, the breeding pairs and neonatal pups were maintained on doxycycline-containing drinking water to repress transgene expression.

To evaluate EGFR<sup>+</sup> transgene inducibility, we first compared EGFR<sup>+</sup> mRNA and protein expression of 12-week-old off-Dox bitransgenic mice (n = 2) with their on-Dox littermate controls (n = 3). qPCR and immunoblot analysis demonstrated strong EGFR<sup>+</sup> induction in the off-Dox animal brains (Fig. 2B and Supplementary Fig. S2A). Immunofluorescence analysis confirmed that EGFR<sup>+</sup> expression in the off-Dox brains was confined to Gfap-positive cells across all central nervous system (CNS) regions (Supplementary Fig. S2B). Besides neurogenic...
subventricular zone (SVZ)/subgranular (SGL) regions, EGFR/C3 expression was induced in a subset of astrocytes characterized by stellate morphology and Gfap protein expression. As control, bitransgenic mice kept on doxycycline showed no discernible EGFR/C3 protein expression, indicating that doxycycline could penetrate the blood–brain barrier in adult mice and enable efficient repression of EGFR/C3 transgene induction. Despite abundant EGFR/C3 induction, gross examination of whole brains from 12-week-old mice that were switched to off-Dox at 4 weeks of age revealed no major CNS developmental abnormalities. One exception was noticed in the neurogenic SVZ region, where we found off-Dox mice harbored a modestly expanded Gfap-positive neural precursor cell (NPC) population with strong EGFR/C3 induction. Occasional EGFR-positive cells were seen migrating out of SVZ into the adjacent sub-striatum of cortical white matter (Supplementary Fig. S2C). However, despite the elevated NPC proliferation, none of the mice \((n = 18)\) kept off-Dox from 4 weeks of age developed brain tumors up to 18 months of age, indicating that EGFR/C3 induction alone is not sufficient to induce glioma formation in adult mice.

Figure 2. EGFR/C3 induction cooperates with conditional Ink4a/Arf and Pten deletions to induce malignant glioma formation. A, schematic of inducible EGFR/C3 allele under regulation of human GFAP promoter element. B, EGFR/C3 induction was efficiently repressed by doxycycline (Dox) administration. Brain tissue lysates were prepared from littermate single- or bitransgenic mice maintained on (+) or off (−) doxycycline and subjected to immunoblot analysis for EGFR and actin. C, the Kaplan–Meier brain tumor-free survival analysis of tamoxifen-treated mouse cohorts consisting of tetO-EGFR/cPteni (iEIP) maintained off-Dox \((n = 25)\), tetO-EGFR/cPteni (iEIP) on-Dox \((n = 5)\), tetO-EGFR/cInkL (iEI) off-Dox \((n = 6)\), tetO-EGFR/cInkL (iEI) off-Dox \((n = 5)\), tetO-EGFR/cPteni (iEP) off-Dox \((n = 7)\), and cPteni (cIP) off-Dox \((n = 8)\). D, iEIP gliomas displayed high mitotic indices and low levels of apoptosis, H&E and IHC staining against Ki67 and activated caspase-3 (Act-Cas3) were performed using sections from normal mouse brains or iEIP malignant gliomas. E, sections of normal mouse brain tissue or iEIP tumors were stained with antibodies against Pten and EGFR. Arrowheads, embedded Pten-positive endothelial cells in the tumors. F, shown are representative images of IHC staining against phosphorylated Akt (P-Akt), Mapk (P-Mapk), and Stat3 (P-Stat3) performed on sections from normal mouse brains and iEIP malignant gliomas. Scale bars, 50 \(\mu\)m.

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EGFR/C3 induction cooperates with conditional Ink4a/Arf and Pten deletions to induce malignant glioma formation

Malignant glioma pathogenesis is driven by the accumulation of genetic and epigenetic alterations (5, 7). In human gliomas, EGFR amplification/mutation is frequently associated with deletions of PTEN and CDKN2A (encoding for both p16INK4A and p14ARF) tumor-suppressor genes (8). To model these cooperative genetic events, we crossed the hGFAP-tTA/tet-EGFR/C3 mice to conditional knockout alleles of Ink4a/Arf (cInk4a/ArfLox) and Pten (cPtenLox), together with a tamoxifen-activated Nestin-CreER\(^{lox}\) allele that allowed tamoxifen-activated Cre activation in NPCs.
at various developmental stages (30). Nestin and Gfap proteins were coexpressed in a subset of SVZ/SGL NPCs but not in differentiated astrocytes (Supplementary Fig. S3). As the result, were coexpressed in a subset of SVZ/SGL NPCs but not in differentiated astrocytes (Supplementary Fig. S3). As the result, were coexpressed in a subset of SVZ/SGL NPCs but not in differentiated astrocytes (Supplementary Fig. S3). As the result, were coexpressed in a subset of SVZ/SGL NPCs but not in differentiated astrocytes (Supplementary Fig. S3).

To induce glioma formation, compound mice were administered with tamoxifen to transiently activate the Cre recombinase at 4 weeks of age and subsequently kept off-Dox to initiate iEIP induction. Between 16 and 45 weeks of age, 23 of 25 (92%) of induction and inactivation of Ink4a/Arf gliomas. Stronger EGFR expression was found at invasive edges of tumor periphery compared with relatively solid tumor centers. Scale bars, 50 μm. C, shown are representative immunofluorescent images of iEIP gliomas with interspersed EGFR-high and -low tumor cells. Tumor cells were distinguished by their negative Pten expression compared with embedded Pten-positive normal cells (green).

Malignant gliomas are heterogeneous. A, iEIP glioma cells display multilineage differentiation. Normal mouse brain and iEIP glioma sections were stained with H&E or antibodies against indicated lineage markers. B, IHC staining against EGFR was performed on sections from different regions of three independent iEIP gliomas. Stronger EGFR expression was found at invasive edges of tumor periphery compared with relatively solid tumor centers. Scale bars, 50 μm. C, shown are representative immunofluorescent images of iEIP gliomas with interspersed EGFR-high and -low tumor cells. Tumor cells were distinguished by their negative Pten expression compared with embedded Pten-positive normal cells (green).

For the methodologies and in vivo glioma initiation, see Materials and Methods. A classical feature of human malignant glioma is its high degree of inter- and intratumoral histologic heterogeneity, hence the moniker of GBM "multiforme." Similar phenotypic and molecular heterogeneity were mirrored in the iEIP tumors. As illustrated in Fig. 3A, the iEIP glioma cells expressed an assortment of stem or lineage progenitor markers commonly observed in human gliomas, including the stem/progenitor marker Nestin (N), astrocytic lineage marker Gfap (G), neuronal lineage marker Tuj1 (Tuj), oligodendroglial progenitor marker Olig2 (O), but not mature neuronal and oligodendrocyte markers, such as NeuN and MBP (Supplementary Fig. S5). Importantly, a heterogeneous pattern of intratumoral EGFR expression was also observed in the tumors despite EGFR+ induction was required for iEIP glioma initiation. Specifically, the tumor periphery and infiltrating borders tended to have high EGFR protein expression, whereas cancer cells in the tumor centers generally showed lower levels of EGFR expression (Fig. 3B), reminiscent of the heterogeneous EGFR gene amplification/expression pattern observed in human malignant glioma samples (13–16). In some regions, glioma cells, distinguished by their negative Pten staining, displayed mosaic EGFR expression pattern with interspersed EGFR-high and -low tumor cells (Fig. 3C). Co-immunofluorescent staining further revealed that the EGFR-low tumor cell population also contained K67-positive proliferative cells (Fig. 3D), suggesting that not all tumor cells depended on high EGFR+ levels for survival and propagation.
iEIP gliomas are sensitive to genetic suppression of EGFR\textsuperscript* transgene ablation but resistant to EGFR TKI treatment

Tumor cells often retain their dependence on an initiating oncogene even after serial passages (36). To determine whether EGFR\textsuperscript* induction and its protein phosphorylation were required for tumor maintenance, a cohort of glioma-bearing iEIP transgenic mice were randomly assigned to receive control vehicle (\(n = 6\)), erlotinib (\(n = 4\)), or doxycycline (\(n = 6\)). Consistent with previous clinical observations (24), erlotinib treatment only marginally prolonged median survival of the affected mice compared with the vehicle-treated group despite markedly attenuated EGFR phosphorylation in the tumors (Fig. 4A and B). This minor effect of erlotinib was in contrast to doxycycline treatment, which elicited a universal response within 3 to 5 days. And the affected mice exhibited generally alleviated symptoms thereafter, indicating EGFR\textsuperscript* expression was required for tumor maintenance. However, this regression was not durable and all the mice under continuous doxycycline treatment succumbed to relapse within 5 to 18 weeks. IHC staining confirmed the absence of EGFR\textsuperscript* induction in all doxycycline-treated relapsed tumors (\(n = 6\); Fig. 4B), indicating relapse was not caused by escape from doxycycline-mediated EGFR\textsuperscript* repression or from compensatory mechanism(s) that upregulated endogenous EGFR gene.

To further analyze the development of resistance, we used an orthotopic transplantation model by using freshly isolated primary iEIP glioma cells. Compared with the primary tumors, the orthotopic model provided a flexible and more controlled system to investigate therapeutic response and resistance development in a large group of animals. To facilitate in vivo imaging, isolated glioma cells were transduced with luciferase-expressing vector. In general, animals carrying grafted iEIP glioma cells started to develop measurable BLI signals by 4 to 6 weeks. At week 8, the mice were randomly assigned to four groups to receive vehicle control (\(n = 4\)), erlotinib (\(n = 5\)), gefitinib (\(n = 3\)), or doxycycline (\(n = 7\)) after tumors were established. Day 0 represents the day when treatment was initiated. Representative bioluminescence images of animals subjected to the indicated treatment from C. E, EGFR\textsuperscript* phosphorylation but not EGFR protein levels were diminished in tumors treated with gefitinib or erlotinib. Representative tumor sections from C were stained with H&E, anti-EGFR, or anti-p-EGFR. Scale bars, 50 \(\mu\)m.
tumor relapse would later resume and it eventually proved fatal. The response to doxycycline nevertheless translated into significant overall survival benefit compared with erlotinib or gefitinib treatment.

To fully exclude possibility that the poor response to EGFR TKIs might be caused by inefficient drug penetration through the blood–brain barrier, the treatment was repeated in a cohort of immunocompromised nu/nu mice subcutaneously transplanted with iEIP glioma cells. Again, erlotinib only transiently slowed down tumor progression compared with doxycycline treatment, which caused robust initial tumor regression followed by an extended period of stasis before the eventual relapse (Supplementary Fig. S6A). IHC staining and immunoblot analysis confirmed the inhibition of EGFR phosphorylation (Supplementary Fig. S6B and S6C). Collectively, our data indicate that EGFR protein expression is important for the iEIP tumor maintenance, but its phosphorylation level might not be an accurate indicator of its tumor maintenance functions.

**EGFR**-independent glioma cells preexisted

Although doxycycline treatment elicited robust initial response and glioma regression, tumors invariably relapsed under continued suppression of **EGFR** induction. To examine whether the resistant tumor cells were present before treatment or acquired resistance during treatment, we conducted a serial time-course analysis to define the acute response following genetic **EGFR** suppression. To facilitate tumor cell tracking, the freshly isolated iEIP glioma cells were transduced with a GFP-expressing vector before being orthotopically transplanted into recipient mice. At 8 weeks after transplantation, the tumor-bearing mice were switched to doxycycline drinking water and sacrificed at 0, 2, 4, 6, 8, and 10 days, respectively. As illustrated in Fig. 5A, doxycycline treatment caused rapid **EGFR** protein downregulation and completely silenced **EGFR** transgene induction by 4 days after treatment. The suppression of **EGFR** induction was accompanied by markedly enhanced apoptosis (activated caspase-3) and progressively decreased tumor cellularity and phospho-Akt staining (Fig. 5A and Supplementary Fig. S7). Despite pronounced cell death, however, doxycycline treatment did not completely eradicate the tumor cell population. Immunofluorescent staining revealed the retention of residual GFP-positive but **EGFR**-negative tumor cells following 4- and 10-day treatment (Fig. 5B). The presence of mitotically active tumor cells suggests that these **EGFR**-independent tumor cells existed prior to treatment and likely fueled the later relapse.

**Hgf/Met signaling is activated in relapsed tumors refractory to **EGFR** ablation**

In an effort to identify the molecular mechanism underlying the tumor relapse, we performed a gene expression analysis comparing doxycycline-treated relapsed tumors with untreated controls. The Ingenuity Pathway Analysis of the two groups identified “hepatic fibrosis/hepatic stellate cell activation” as the most enriched canonical pathway (Supplementary Fig. S8). qPCR revealed that hepatocyte growth factor (Hgf), and to a lesser extent its receptor Met, was significantly upregulated in the doxycycline-treated relapsed tumors (Fig. 6A). IHC further confirmed markedly enhanced regional Met activation in the relapsed tumors compared with their untreated controls (Fig. 6B). Notably, the Met-activated cells were not distributed evenly within the relapsed tumors, but instead, were focally patched and generally comprised less than 10% of total tumor cell population.

MET activation, either by amplification or overexpression of its ligand Hgf, has been shown to cause *de novo* resistance to EGFR TKIs in human NSCLC (37, 38). To determine its impact on tumor relapse in our model, we next examined the function of a Met inhibitor, alone or in combination with doxycycline, on in vivo iEIP glioma growth. Freshly isolated iEIP glioma cells were subcutaneously transplanted into the flanks of recipient nu/nu mice. As tumor volumes reached a palpable size (~200 mm³), mice were randomly assigned to four groups to receive vehicle (*n* = 5), Met inhibitor crizotinib (*n* = 5), doxycycline (*n* = 5), or a combination of doxycycline + crizotinib (*n* = 5). Consistent with the minor presence of Met-activated tumor cells in the iEIP gliomas, crizotinib treatment alone only modestly slowed tumor growth compared with the vehicle control (Fig. 6C and D). The combination of doxycycline + crizotinib treatment elicited a slightly deeper initial tumor regression response but still failed to significantly delay tumor relapse when compared with doxycycline treatment alone. These data suggest that besides the Met–Hgf signaling axis, other survival signaling pathways might have evolved to sustain **EGFR**-independent relapsed tumors.

**Combined PI3K/mTOR and **EGFR** repression delay tumor relapse and prolong survival**

The focal presence and clonal nature of Met-activated cells within the relapsed tumors suggested that multiple independent survival pathways might be activated to compensate **EGFR**—
repression. We therefore reasoned that inhibition of single or even multiple RTK signaling might not elicit a durable cure. And a combinatorial blockade of EGFR/C3 and major downstream signaling outputs might represent a better option to counter relapse. By examining common downstream pathways, we found that while the phospho-Mapk was significantly diminished, the relapsed tumors still maintained activated PI3K/mTOR signaling as evidenced by their strong phospho-Akt staining (Fig. 7A). These findings suggested that activated PI3K/mTOR signaling, but not Mapk pathways, might play an important role in relapse development. To determine whether the addition of a dual PI3K/mTOR inhibitor (Bez-235) could prevent relapse, we evaluated the effect of a regimen with either single or combined doxycycline and Bez-235 on subcutaneous iEIP tumor growth. Despite the fact that it significantly inhibited PI3K/mTOR signaling as evidenced by marked diminished Akt and S6 phosphorylation (Fig. 7B and Supplementary Fig. S9), Bez-235 treatment alone had limited effect on iEIP tumor growth (Fig. 7C). In contrast, combined doxycycline + Bez-235 significantly delayed relapse and prolonged animal survival compared with doxycycline treatment alone. Notably, focally patched P-Met cells again were found in both doxycycline and doxycycline + Bez-treated relapsed tumors, confirming that Met activation likely acted as one source of resistance against EGFR suppression. Nevertheless, the synergistic therapeutic effect of combined EGFR/C3 and PI3K/mTOR inhibition suggests that the combination might be useful for treatment of aberrant EGFR signaling-driven malignant gliomas in clinic.

**Discussion**

In this study, we have described a novel malignant glioma mouse model driven by inducible EGFR/C3 expression and provided genetic evidence that oncogenic EGFR/C3 serves a tumor maintenance role in fully established EGFR/C3-driven malignant gliomas. Genetic suppression of EGFR/C3 in affected animals led to significant tumor regression. This was in contrast to treatment using two different EGFR TKIs, despite the fact that they could efficiently inhibit EGFR/C3 auto-phosphorylation both in vitro and in vivo. Our findings therefore suggested that poor response to EGFR TKIs in glioma clinical trials might not be due to their inability to inhibit EGFR phosphorylation but rather glioma’s less dependence on EGFR kinase activity relative to lung cancer. In addition to the EGFR-dependent resistance mechanism, our studies also uncovered an EGFR-independent mechanism in which preexisting resistant tumor cells persist through EGFR ablation and lead to tumor relapse. The addition of a PI3K/mTOR inhibitor together with genetic EGFR/C3 suppression could significantly delay relapse and prolong animal survival. These observations have significant implications for our understanding of glioma biology as well as future therapeutic development.
EGFR TKIs have emerged as effective therapeutic entities for lung cancer patients carrying EGFR kinase domain mutations (21–23). But perplexingly, the same TKIs were ineffective in glioma patients with EGFR amplification/mutation (24–26, 39). This clinical observation is also confirmed in our current studies using an iEIP animal model. However, despite their marginal effect on glioma progression, we and others have shown that EGFR TKIs could efficiently inhibit EGFR\(^\ast\) autophosphorylation both in vitro and in vivo (40–42). In addition, treatment of subcutaneously grafted iEIP tumors with EGFR TKIs in our studies also excluded the possibility of inefficient drug penetration through the blood–brain barrier as the main cause of the poor response, suggesting that glioma maintenance, unlike lung cancer, might not require high-level EGFR kinase activity. Intriguingly, glioma-specific EGFR mutants have a very unique mutation pattern compared with other cancer types. For example, the EGFR intracellular kinase domain mutations frequently seen in lung cancers are conspicuously absent in gliomas (40, 42). Instead, the glioma-specific mutations mainly cluster in EGFR extracellular domain (such as EGFR\(^\ast\)). Whether this tissue-specific mutational pattern reflect glioma’s less dependence on EGFR kinase activity remains to be determined.

Two recent reports proposed that distinct conformational requirements and/or kinase-site occupancy rates may contribute to the different sensitivities to EGFR TKIs observed in brain versus lung cancer EGFR mutants (40, 42). It is plausible that low levels of EGFR kinase activity might be sufficient for glioma maintenance. As such, more potent or selective EGFR TKIs could theoretically overcome the resistance. However, because a substantially higher dose of EGFR TKIs is generally required to achieve comparable cell death in cultured mutant expressing-glioma versus -lung cancer cells (40, 42, 43), deeper target inhibition might not be feasible owing to potential toxicity to normal tissues.

In addition, besides the much studied kinase-dependent features, EGFR kinase-independent function was also shown to endow tumor cells with increased survival capacity against EGFR TKIs by maintaining the basal intracellular glucose level (44). Therefore, in order to achieve optimal efficacy against EGFR-driven gliomas, the next generation of targeted therapeutics might need to consider other EGFR functional domains in addition to its kinase activity.

Studies in multiple genetically engineered mouse models indicate that tumor maintenance is often dependent on the driver oncogene that initiates tumor development (36). Indeed, genetic suppression of EGFR\(^\ast\) induction in our study elicited rapid cell death and tumor regression. However, the gliomas did not regress fully, and a small population of residual tumor cells persisted through EGFR\(^\ast\) suppression and likely formed the basis for later relapse. The incomplete remission was consistent with the heterogeneous EGFR\(^\ast\) expression pattern observed in fully established iEIP gliomas, as one would expect that tumor cells with diverse EGFR\(^\ast\) expression would have varied degrees of dependence on oncogenic EGFR signaling. Considering that EGFR\(^\ast\) induction is required for iEIP glioma initiation, our findings support a model directly linking glioma plasticity with therapeutic resistance (Fig. 7D). In this model, EGFR\(^\ast\) induction cooperates with Ink4a/Arf and Pten inactivation to initiate tumor formation. But later on, a glioma progression process amplifies diverse lineages of tumor cells that vary in their molecular characteristics and dependence on oncogenic EGFR signaling. Genetic EGFR\(^\ast\) suppression generates initial tumor regression by eradicating the bulk of EGFR\(^\ast\)-dependent glioma cells before the relapse, during which the previous minor or even dormant EGFR\(^\ast\)-independent tumor cells take over. Due to the high tumor plasticity and clonal nature of relapsed tumors, it is quite possible that different resistant tumor cell clones carrying varied EGFR-independent...
survival signaling pathways can evolve in parallel within the same lesion. If so, one would expect that therapies targeted single or even combinations of RTKs will not be able to suppress all types of relapsed tumor cells. Indeed, this study identified Met activation as one source of resistance against EGFR$^{\text{a}}$ ablation in relapsed gliomas. However, the addition of a Met inhibitor to genetic EGFR$^{\text{a}}$ suppression induced only slightly deeper initial tumor regression without significantly delaying tumor relapse. In contrast, combined EGFR$^{\text{a}}$ ablation with administration of a P38K/mTOR inhibitor significantly delayed tumor relapse, suggesting that combinatorial blockade of key downstream outputs together with EGFR suppression might represent a better therapeutic approach to overcome innate resistance.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: S. Klingler, Y.A. Wang, H. Ying, H. Zheng

Development of methodology: S. Klingler, H. Yan

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Klingler, B. Guo, H. Yan, L. Zhang, S. Chen, P. Canoll, J.W. Horner, Y.A. Wang

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Klingler, J. Yao, S. Chen

Writing, review, and/or revision of the manuscript: S. Klingler, J.-H. Paik, H. Ying, H. Zheng

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.V. Vaseva, S. Chen, H. Ying

Study supervision: H. Zheng

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