Pathway Analysis of Glioblastoma Tissue after Preoperative Treatment with the EGFR Tyrosine Kinase Inhibitor Gefitinib—A Phase II Trial

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Abstract

Amplification of the epidermal growth factor receptor (EGFR) gene is one of the most common oncogenic alterations in glioblastoma (45%) making it a prime target for therapy. However, small molecule inhibitors of the EGFR tyrosine kinase showed disappointing efficacy in clinical trials for glioblastoma. Here we aimed at investigating the molecular effects of the tyrosine kinase inhibitor gefitinib on the EGFR signaling pathway in human glioblastoma. Twenty-two patients selected for reoperation of recurrent glioblastoma were treated within a phase II trial for 5 days with 500 mg gefitinib before surgery followed by postoperative gefitinib until recurrence. Resected glioblastoma tissues exhibited high concentrations of gefitinib (median, 4.1 μg/g), 20 times higher than respective plasma. EGFR-pathway activity was evaluated with phosphorylation-specific assays. The EGFR was efficiently dephosphorylated in treated patients as compared to a control cohort of 12 patients. However, no significant effect on 12 pathway constituents was detected. In contrast, in vitro treatment of a glioblastoma cell line, BS-153, with endogenous gefitinib reached high concentrations in the tumor tissue and efficiently dephosphorylates similar to the human glioblastoma. Taken together, gefitinib reaches high concentrations in the tumor tissue and efficiently dephosphorylates its target. However, regulation of downstream signal transducers in the EGFR pathway seems to be dominated by regulatory circuits independent of EGFR phosphorylation.

Introduction

The epidermal growth factor receptor (EGFR) offers a particularly attractive target in glioblastoma therapy, because it is overexpressed in 60% of glioblastoma patients. EGFR amplification and EGFRvIII expression is usually associated with high-level amplification of the EGFR gene (1, 2). EGFR activation initiates signal transduction through RAS/MAPK and PI3K/AKT pathways associated with cell proliferation and survival (3). Small molecule drugs such as gefitinib have been developed to specifically target the catalytic tyrosine kinase domain of the EGFR to prevent downstream signaling (4). In non–small lung cancer (NSCLC), particularly good response to EGFR tyrosine kinase inhibitors (TKI) was associated with mutations in the EGFR located around the ATP-binding pocket (5, 6). In glioblastoma, however, such sensitizing mutations have not been found (7). In contrast, missense mutations have been identified in the extracellular domain of a fraction of cases (14%) with potentially activating properties (8), and were usually associated with amplification of the locus. The most common alteration (20%) is the truncation mutant lacking exons 2 to 7 (EGFRvIII) affecting the extracellular domain involved in dimerization and ligand binding that has been associated with constitutive phosphorylation of the receptor conferring an oncogenic potential (9, 10).

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A first publication (11) on a phase II trial testing the EGFR-inhibitor gefitinib in recurrent glioblastoma reported that response to treatment was not correlated with expression of the EGFR, although the authors had not excluded insufficient drug penetration of the tumor. As in most clinical trials for glioblastoma, enzyme inducing antiepileptic drugs (EIAEDs) were allowed in this study that have been shown to reduce systemic availability of TKIs (12). In the meantime, further phase II trials have been reported testing erlotinib or gefitinib in recurrent or progressive glioblastoma, summarized in Yung and colleagues (13), or in newly diagnosed glioblastoma as an addition to combined chemoradiotherapy with temozolomide (14), overall with disappointing efficacy.

The occasional responses incited several studies to search for predictive molecular markers in the diagnostic tissue of the initial surgery (reviewed in ref. 15) and human glioblastoma xenograft models (16) to allow future patient selection. Several sets of markers with predictive value for response to TKIs were proposed, comprising expression of EGFR, amplification of the EGFR gene, lack of elevated levels of AKT phosphorylation, and absence of EGFRvIII expression (17), whereas another study suggested better response of tumors with expression of EGFRvIII and expression of PTEN (18). The markers proposed in these small studies could not be confirmed in subsequent trials, including a randomized phase II trial (12), although low p-AKT showed a trend for association with better outcome. The difficulty to successfully target one of the most commonly activated oncogenic pathways operative in glioblastoma has drastically revealed the complexity of the regulation of receptor tyrosine kinase (RTK) signaling that requires further investigations.

Here we present results of a phase II clinical trial designed to elucidate potential reasons for the unexpected low response rates of glioblastoma to EGFR inhibitors in previous clinical studies, by addressing the following questions: (i) does the drug reach the tumor, (ii) is the EGFR dephosphorylated by the drug, and (iii) what are the effects on downstream signaling. To this end, patients selected for surgery for recurrent glioblastoma were offered participation in a trial with the EGFR TKI gefitinib, comprising 5-day preoperative treatment, followed by postoperative treatment until recurrence or undue side effect. Molecular profiling was done on the human glioblastoma samples obtained from the patients enrolled, and a control set. These efforts were complemented by an experimental in vitro and in vivo model using a tumorigenic glioblastoma cell line with endogenous EGFR amplification and expression of EGFRvIII. This model allowed direct investigation of the treatment effect to aid interpretation of the data obtained from human tumors, where no tumor sampling before and after therapy is ethically feasible.

Materials and Methods

Trial design

Uncontrolled phase II open label study of pre- and postoperative use of gefitinib (www.clinicaltrials.gov, NCT00250887) with translational research. The primary objective was investigation of effects of preoperative treatment of gefitinib on EGFR pathway signaling in the glioblastoma tissue obtained at resection and penetration of gefitinib into glioblastoma tissue. Secondary endpoints comprised survival and safety.

Patients and tumor samples

The following eligibility criteria applied: male or female patients with histologically confirmed glioblastoma and recurrent disease as shown by MRI scan, for whom reoperation was planned, age 18 years or older, fresh frozen sample obtainable, written informed consent for translational biomarker research; exclusion criteria comprised enzyme inducing antiepileptic drugs. The study was done at the University Hospitals in Zurich, and the Inselspital Berne, Switzerland, in accordance with the Declaration of Helsinki, Good Clinical Practice, and International Conference on Harmonisation recommendations. The study protocol and informed consent form were approved by ethics committees at both sites in accordance with local legislation. Written informed consent was obtained from patients before study entry. Archived fresh frozen samples from 12 patients reoperated at the University Hospital Zurich between 2002 and 2007 for recurrent glioblastoma were used as comparators for molecular analyses, approved as part of the protocol by the ethics committee. All tumor samples underwent central pathology review to confirm diagnosis and quality of samples. Frozen tissue samples with a tumor cell content below 50% were excluded for molecular analyses. One frozen tissue sample was available for each patient at re-resection that was used to do all analyses, unless stated differently, including drug dosage in the treated patients. The amount of frozen tissue available for molecular analyses ranged from 45 to 1,500 mg (median 450 mg).

Treatment

Patients were treated for at least 5 days with 500 mg gefitinib before surgery, followed by postoperative daily use, continuously until tumor progression or occurrence
of intolerable side effects. Patients on cytochrome P450 isoenzyme CYP3A4-inducing antiepileptic drugs (EIAEDs) were changed to a non–enzyme-inducing drug before entering the trial.

**Drug concentrations**

Gefitinib was quantified in the frozen tumor tissue and plasma samples by high-performance liquid chromatography coupled to tandem mass spectrometry as described previously (refs. 19 and 20; Eurofins Medinet B.V.). The blood samples were collected during surgery. The gefitinib concentrations in the BS-153 xenografts and the mouse serum were determined at the quantitative mass spectrometry facility at the Lausanne University Hospital, using the same technology with minor modifications. The tumor tissue homogenates were prepared at 200 mg of wet weight/mL in phosphate buffer using a Fast-Prep homogenizer (MP Biomedicals).

**Cell line and xenograft model**

The human glioblastoma cell line BS-153 (21) was cultured in low serum (0.5% FCS) and was either stimulated with 50 ng/mL EGF (PEPROTECH) or treated with 0, 1, 5, 7 mg/kg/day gefitinib [suspended in 1% (w/v) Tween 80] or an equivalent volume of drug vehicle orally for 5 days. Tumor tissues were harvested 4 hours after the last dose and snap frozen or embedded in paraffin for further analysis. The animal experiments were approved by the local authorities (protocol VS_1181.3). The authentication of glioblastoma cell line BS-153 was done by short tandem repeat profiling using the PowerPlex 16 HS Kit (Promega; Bady and colleagues, manuscript in preparation) at the Centre d’identité génétique forensique of the Centre universitaire de médecine légale of the University of Lausanne in September 2010.

**Tissue microarray and immunohistochemistry**

A tissue microarray was constructed from paraffin embedded tumor blocks available from patients and the BS-153 xenografts in nude mice. Immunohistochemistry (IHC) for p-mTOR (#1500694) on films or on a bioluminescence image reader (LAS-4000, Fuji). Tot-Erk1/2 (#9102) and tubulin (Sigma #T5168) served as loading controls.

**Evaluation of EGFR copy number by FISH and/or quantitative PCR**

FISH for determination of EGFR copy number was done using commercially available probes. LSI EGFR labeled with spectrum orange and centromeric probe to chromosome 7 labeled with spectrum green (Vysis, Abbott Laboratories) were mixed with chromosome 12 (P12H8) labeled with Cy5 (Amersham Biosciences) and prepared as described previously (12). The EGFR copy number was normalized by the centromeric probe on chromosome 12 to compare the result to quantification by quantitative PCR (qPCR).

DNA was derived from macrodissected paraffin sections as described previously (22) and subjected to EGFR copy number analysis using the relative qPCR comparative Ct (2^−ΔΔCt) method using DNMI L (12p11.21) as reference gene (primer sets: EGFR-F_669 ATGTCCCGG- GAACACAAAGAC, EGFR-R_670 TTATCTCCCCCTCC- CGGTATC, amplicon size 104 bp; DNMI L-F_671 TCAGATGTAAAGCTGCCATTT, DNMI L-R_672 TCC- CGACGAGATGTCTTCG, amplicon size 101 bp). qPCR was done on a Rotor Gene 6000 Real-Time PCR system (Qiagen) using the Fast Sybr Green Master Mix (Applied Biosystem). DNA from peripheral blood lymphocytes from healthy volunteers served as normal reference, and cell line BS-153 as positive control for high-level amplification.

**Real-time quantitative reverse transcription PCR**

RNA was isolated from frozen tissue using the Qiagen AllPrep DNA/RNA Kit (Qiagen, Cat #80204). Quantitative reverse transcription PCR (qRT-PCR) was used for expression analysis of EGFRwt and EGFRvIII at the Genetics Platform at the University of Geneva as described (23).

**Analysis of phospho-proteins by Western blot and Bio-Plex analysis**

Protein from snap frozen human glioblastoma, xenografts, and cell lines were extracted with the Bio-Plex Cell Lysis Kit (Bio-Rad #171-304011) according to manufacturer’s protocol. The protein concentration was determined (BCA, Pierce #23250). Western blot analysis was done with 20 mg of protein using 7.5% and 10% SDS-PAGE gels, and subsequent transfer to a nitrocellulose membrane (Hybond-C, Amersham Life Science). The phosphorylation status of the following proteins was evaluated using antibodies from Cell Signaling: p-EGFR (#4404), p-AKT (#9271), p-mTOR (#2971), p-elf4G (#2441), and p90RSK (#9341) that were revealed by luminescence (BM Chemiluminescence Blotting Substrate, Roche, #1500694) on films or on a bioluminescence image reader (LAS-4000, Fuji). Tot-Erk1/2 (#9102) and tubulin (Sigma #T5168) served as loading controls.

The Multiplexing Bio-Plex total target and phospho-protein assay (Bio-Rad) was done at the platform of the Center of Integrated Genomics at the University of Lausanne according to Bio-Plex Phosphoprotein detection instruction manual with 0.5 mg/mL protein in 96 wells (duplicate). The following phospho-proteins were measured: pEGFR (pan-phospho), p-AKT (Ser473), p-GSK-3α/β (Ser21/Ser9), p-NFkB p65 (Ser536), p-STAT3 (Tyr205), p-ERK1/2 (Thr202/Tyr204, Thr185/Tyr187), p-MEK1(Ser217/Ser221), p-p38MAPK.
(Thr\textsuperscript{180}/Tyr\textsuperscript{182}), p-p90RSK (Thr\textsuperscript{359}/Ser\textsuperscript{363}), p-p70S6 Kinase (Thr\textsuperscript{421}/Ser\textsuperscript{424}), p-S6 ribosomal Protein (Ser\textsuperscript{235}/Ser\textsuperscript{236}), p-PDGFR-B (Tyr\textsuperscript{751}), and p-SRC (Tyr\textsuperscript{416}). The following total proteins were determined, tot-ERK1/2 (#171-V32238), tot-p38MAPK (#171-V31336), tot-EGFR, tot-MEK1, and tot-AKT. Tot-ERK1/2 was present on all plates and was used for normalization of the data set before log\textsubscript{2}-transformation. The suitability of the technology for phospho-protein analysis of human tumor samples has been reported recently (24).

**Statistical methods**

A sample size of 20 analyzable patients was considered adequate for a first investigation of translational research. For all molecular analyses and outcome, the analysis population is the intent-to-treat population (Table 1). Statistical significance of molecular differences between treatments was evaluated with a non-parametric test, Wilcoxon’s test, and the difference between subgroups, given by combination of treatment and EGFR amplification status, was tested by Kruskal-Wallis test. The set of phospho-proteins was examined by principal component analysis and a Monte-Carlo test on between-group inertia (global test) was done to test the overall difference between treated and untreated patients (25). Dendrograms for the heatmap representations were constructed by the Ward’s algorithm using euclidean distance. The data were scaled and centered by phospho-proteins if not stated otherwise. Survival is summarized by Kaplan-Meier methods. All analyses and graphical representation were done in R (ref. 26; URL http://www.R-project.org) and the R packages gplots and ade4 (27).

**Results**

Twenty-two patients with recurrent glioblastoma selected for second surgery were enrolled between July 2005 and May 2007. Patient characteristics are summarized in Table 1. Patients were treated for a median of 7.5 days (5–150) with 500 mg gefitinib daily before surgery, followed by postoperative gefitinib until recurrence. The median exposure time to gefitinib was 102 days (40–272). Central review confirmed recurrent glioblastoma in all cases. For 3 patients no frozen tissue was available. Five cases in the gefitinib group had to be excluded for molecular analysis, because the available frozen tissue consisted of >80% necroses or showed only reactive changes. For 1 patient (ZH-06), a frozen biopsy became available at 2nd relapse (ZH-06.1). The patient had been treated 11 months with gefitinib, and was then

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Abbreviations: ECOG, Eastern Cooperative Oncology Group; NA, not available; S, surgery; RT radiation therapy; TMZ, temozolomide; RT + TMZ, combined radio-chemotherapy with TMZ.

\(a\)Intent-to-treat 22 patients.

\(b\)For 3 samples the EGFR status was inferred from a previous resection.
reoperated 7 months after the last dose (ZH-06.1). This interesting sample was added in the analysis and included in the nontreatment group.

**EGFR amplification status**

EGFR amplification was identified in 7 of 22 (32%) patients in the gefitinib group and 7 of 12 (58%) patient samples of the control group (Table 1, Fig. 2). For 3 patients we had to infer the EGFR status from the analysis of the glioblastoma tissue from the first resection, as the tissues obtained at reoperation did not comprise enough tumor cells. Overexpression of the wild-type EGFR was associated with amplification of the EGFR gene, but no linear correlation was observed. Expression of the EGFRvIII was detectable in 2 glioblastoma at modest levels not exceeding expression of EGFRwt. BS-153 xenografts displayed high expression of EGFRvIII greater than EGFRwt, similar to the cell line in culture. EGFR amplification, dark green; nonamplified, light green; gefitinib treatment, red; nontreated, blue. For patient 2510, the newly diagnosed glioblastoma 2505 was also included, because no RNA was available for the sample at recurrence.

High-level amplification of the EGFR was measured for the subcutaneous tumors derived from the glioblastoma cell line BS-153, similar to the cell line in culture (FISH analysis on metaphase spreads, Supplementary Fig. S1), and was associated with high-level expression of EGFRvIII in addition to EGFRwt (Fig. 2).
Gefitinib concentrations in tumor tissue and plasma

The median concentration of gefitinib in the tumor tissue was 4.1 μg/g (median, range 0.016–26 μg/g) and 0.153 mg/mL (0.004–0.483 mg/mL) in the plasma (Fig. 3). On average the gefitinib concentration in the resected tissue was 22-fold higher (mean, 95% CI 12–42) than in the respective plasma, similar to breast cancer and NSCLC (42 and 60 folds; refs. 28 and 29). The median time laps between the last drug intake and collection of the tumor tissue and the blood sample was 3 hours 45 minutes (2 hours 30 minutes to >24 hours) and 4 hours 45 minutes (1 hours 30 minutes to >24 hours), respectively (Supplementary Fig. S2). The reported time for maximum plasma concentration ($t_{max}$) is 5 to 7 hours after the last dose (30).

The mean gefitinib concentration in the BS-153 xenografts ($n = 9$) in the mouse was 1.465 μg/g (range 0.457–3.62 μg/g) and 0.252 mg/mL in the serum ($n = 4$).

Figure 4. Effect of gefitinib on EGFR pathway signaling transductors. Fourteen signaling transductors of the EGFR pathway were determined by Bio-Plex technology and normalized to total-ERK1/2. The log₂-intensity of the p-EGFR (A) and p-ERK1/2 (B) measured in the tumor tissues from patients under gefitinib treatment (T1, red; $n = 14$) or an untreated cohort (T0, blue; $n = 10$, plus ZH-06.1 re-resected 7 months after the last dose of gefitinib, see Fig. 5B) are represented in box plots. A significant decrease was found for pEGFR ($P = 0.044$, Wilcoxon test), but not for pERK1/2 ($P = 0.13$). C and D, the treatment effect stratified by the EGFR amplification status (A1, amplified; A0, not amplified) revealed a significant decrease for pEGFR ($P = 0.003$, Kruskal–Wallis test), whereas the enhancement of pERK1/2 did not reach statistical significance ($P = 0.1$). E, histograms (intensities normalized to total ERK1/2) visualize phosphorylation of EGFR and ERK1/2, respectively. EGFR amplification, dark green; nonamplified, light green; gefitinib treatment, red; nontreated, blue. The paired samples, ZH-06 and the corresponding 2nd recurrence, ZH-06.1 are indicated in black. F, overall differences of the 14 measured phospho-proteins between tumors under gefitinib treatment and controls are illustrated with the sample representation on the first vectorial plan of the principal component analysis. Inertia ellipses were used to compare both groups, and their differences were not statistically significant (E). Box plots for the other 12 phospho-proteins are available in Supplementary Fig. S3 and the histograms in Supplementary Fig. S4.
0.065–0.336 μg/mL), with an average ratio of 7.4. The tumors and blood were collected 4 hours after the last treatment.

**Patient outcome**

The median survival after initiation of gefitinib treatment was 8.8 months. No difference was observed between patients with an amplified or a normal EGFR status. However, patients whose resected tissue had to be excluded for molecular analysis due to predominantly necrotic tissue had longer survival (logrank, \( P = 0.004 \)). This may be an indication of pseudo-progression (31). However, the small patient numbers preclude proper analysis and interpretation of this observation.

**Molecular analysis of the EGFR signaling pathway in glioblastoma**

To investigate the effect of gefitinib on activation of the EGFR and respective downstream signaling, a phosphorylation screen for a selected panel of published EGFR pathway signal transducers was done. In addition, it comprised p-PDGFR-B and p-SRC 2 important players in glioblastoma. PDGFR activates elements of the same pathway, is commonly overexpressed in tumor and tumor endothelial cells and pericytes, and has been attributed an important role in glioma angiogenesis (32, 33). SRC has been reported to be an effector of EGFR signaling (34).

Comparison between treated and untreated patients revealed that there was a significant decrease of phosphorylation of the EGFR (\( P = 0.044 \); Wilcoxon test; Fig. 4A). This effect was enhanced when stratifying for the EGFR amplification status (\( P = 0.003 \); Kruskal–Wallis test), indicating efficient dephosphorylation by gefitinib as visualized in Fig. 4. The phosphorylation of the other signaling transducers was not significantly changed. The overall difference between treated and untreated patient samples did not reach statistical significance (\( P = 0.204 \), Monte Carlo test, 999 permutations, Fig. 4E), thus the measured gefitinib treatment effect seems to be mostly restricted to dephosphorylation of the EGFR. The statistical analysis for all phospho-proteins is summarized in Supplementary Table S1 and the respective box plots and histograms are displayed in Supplementary Figs. S3 and S4. Exclusion of 4 cases from the analysis who had an extended pretreatment period (3 cases), or missed drug intake on the day of surgery (1 case) did not reveal other significant factors.

An interaction map of the pathway, indicating the analyzed proteins, and a heatmap of the phosphorylation profiles are shown in Fig. 5. The dendrogram from this unsupervised analysis suggests that EGFR signaling was not dominating the activity of the measured pathway constituents. In fact, EGFR phosphorylation was least related to pathway activation as indicated by the principal component analysis of all measured phospho-proteins (Fig. 5C). The closest correlation was with p-PDGFR-B (Pearson correlation \( r = 0.5 \); Supplementary Fig. S5 shows all pairwise comparisons). However, the amplitude of p-PDGFR-B was much lower (Supplementary Fig. S4). Information on expression of PTEN, CdcD, and p-mTOR was obtained by IHC on the respective tissue microarray included as label to the heatmap (Fig. 5B).

For 1 patient we had paired samples, ZH-06 obtained under gefitinib therapy and the corresponding recurrent tumor ZH-06.1, treated for 11 months, but operated 7 months off gefitinib. This allowed us to compare EGFR phosphorylation on and off gefitinib, respectively. In the sample off treatment, ZH-06.1, the EGFR amplification was retained associated with high EGFR RNA expression (Fig. 2) and EGFR phosphorylation (Figs. 4E and 5B). In contrast, the tumor ZH-06 resected under gefitinib treatment showed low levels of EGFR phosphorylation, suggesting efficient dephosphorylation (Figs. 4E and 5B). The data matrix of the Bio-Plex analysis is available in Supplementary Table S2.

**Effect of gefitinib on EGFR signaling pathway in an in vitro and in vivo model**

In parallel to the human clinical trial we investigated gefitinib modulation of EGFR signaling in an in vitro and in vivo model using the human glioblastoma cell line BS-153. This is one of the rare glioma cell lines retaining endogenous amplification and overexpression of the EGFR and overexpression of the mutant EGFRvIII in culture (Fig. 2; refs. 21 and 35). FISH for EGFR on metaphase spreads of BS-153 suggests that the amplification is extra-chromosomally organized on double minutes as displayed in Supplementary Fig. S1. BS-153 was subjected to gefitinib treatment in vitro and in vivo. In vitro experiments carried out over 24 hours showed as expected dephosphorylation of the EGFRvIII, and also reduced phosphorylation of key signal transducers, such as AKT that is involved in cell survival signaling, and p90RSK a regulator of cell growth and differentiation (Fig. 6). Treatment of mice with established subcutaneous BS-153 xenografts comparable to the human gefitinib dosing schedule also resulted in efficient dephosphorylation of the EGFRvIII. However, in contrast to the in vitro experiments phosphorylation of downstream signal transducers were not modulated. Hence, in the in vivo setting the results are similar to those obtained from the human glioblastoma samples. Interestingly, mTOR and elf4G that are involved in nutrition sensing and regulation of protein translation, respectively, were generally less activated in the BS-153 xenografts as compared to the cell lines in vitro. This may indicate differences of metabolism in the 2 model systems. Modulation of mTOR and elf4G phosphorylation was observed in vitro on stimulation with EGF or treatment with gefitinib (Fig. 6B).

**Discussion**

Small molecule inhibitors of the EGFR have shown little activity in glioblastoma despite the fact that this pathway is frequently affected through amplification.
and overexpression of the EGFR gene. The present phase II trial aimed at elucidating gefitinib-mediated modulation of known EGFR downstream signaling. Patients were moved onto non-EIAEDs before study entry, to exclude reduced drug exposure through induction of CYP3A4. Intratumoral gefitinib concentrations reached 22-fold higher concentrations in the resected tumor tissue than in coincident plasma samples, consistent with previous reports from lung and breast cancer (28, 29). Most importantly, gefitinib treatment

Figure 5. Modulation of the EGFR signaling pathway. A, the EGFR pathway interaction map is adapted from Bertotti and colleagues (41) and indicates the phospho-proteins measured by Bio-Plex analysis in pink, and proteins determined by immunohistochemistry on the tissue microarray or by Western blot in yellow. B, the heatmap clusters the samples and phospho-proteins by similarity. Tumors with EGFR amplification are marked in dark green (A1; no amplification; A0, light green). Tumors under gefitinib treatment are indicated in red (T1) and blue for the controls (T0). For 1 patient with an amplified EGFR, a sample at second relapse was obtained and showed high p-EGFR (ZH-06.1) in contrast to the tumor under gefitinib treatment (ZH-06). The pathway constituents PTEN, CycD, and mTOR were evaluated semiquantitatively by immunohistochemistry and have been added as labels (blue, no expression; grades of pink, increasing expression 1 to 3; white, no information; for mTOR, 0/vs, tumor negative/vessels positive, dark blue). The dendrogram of the phospho-proteins indicates that p-EGFR is very distant to the other pathway signaling transductors. C, principal component analysis of the phospho-proteins showed the first vectorial plan based on the correlation matrix. The first axis (x-axis) of the principal component analysis represented 60.6% of the variance (total inertia) of the table and organized the phospho-proteins in function of their total intensity. All phospho-proteins were oriented in the same sense, with the exception of p-EGFR and to a lesser extent p-PDGFR-B. This representation highlights the low association between p-EGFR and other phospho-proteins. The second axis was mainly built by the variable p-EGFR and explained 9.5% of the variance of all phospho-proteins. On the first vectorial plan of the principal component analysis, we observed that the expression of p-EGFR was not correlated with the other phospho-proteins, except for p-PDGFR-B ($\gamma = 0.523$). Pairwise correlations of all phospho-proteins are displayed in a matrix of scatter plots in Supplementary Fig. S5.
was associated with efficient dephosphorylation of the EGFR. This is in contrast to drug concentrations reported for erlotinib or its active metabolite (OSI-420) in glioblastoma that were low, and reaching only 6% to 50% of the respective plasma concentrations (36, 37). It may thus not be surprising that the authors reported inconsistent EGFR dephosphorylation in the respective tumor tissues (36, 37).

Despite the efficient EGFR dephosphorylation by gefitinib, a phospho-screen of signal transducers downstream of EGFR did not show a statistically significant modulatory effect on the pathway (Table S1). The overall inertness of the pathway signal transductors to EGFR dephosphorylation by gefitinib may not surprise given our observation that EGFR phosphorylation was not indicative of overall activation of the pathway regardless of the treatment (Fig. 5C). It has been proposed that the signaling network, constituted by the ERBB family of receptors of which EGFR is a member (ERBB1), and other mitogenic receptors involved in the malignant behavior of glioblastoma such as MET, or PDGFR, is very robust, because it shares modularity (parts of the pathway), and shows redundancy of regulatory circuits (38, 39). Interestingly, an in vivo model treating established human tumor xenografts with endogenous amplification and overexpression of the EGFRwt\&vIII, recapitulated the efficient EGFR dephosphorylation by gefitinib in glioblastoma, and reproduced lack of downstream signaling modulation. In contrast, treatment of the same cell line in vitro modulated phosphorylation of the signal transducers, thus failing to predict in vivo behavior. Reasons comprise the fact that in vitro experiments usually model acute exposure (here 24 hours), whereas treatment in glioblastoma and in vivo models may allow escape through adaptive changes utilizing the redundancy of the regulatory circuits. Moreover, respective analysis at resection likely shows a snapshot of a newly established steady state. In addition, in vitro systems lack stress signaling induced in vivo by metabolic stress, or hypoxia that share some of the downstream signal transductors.

In conclusion, this study suggests that the EGFR inhibitor gefitinib reaches the tumor in high concentrations, efficiently dephosphorylates the target, which, however, is not sufficient for the control of pathway activity. EGFR-phosphorylation independent regulatory circuits seem to dominate the pathway. To find therapeutic opportunities, the fragilities of the network need to be probed, to design promising combination therapies for patients with respective molecular characteristics (38, 40). We are aware of the limits of this study, due small sample size, and inherent issues on molecular integrity of human tumor samples.
Disclosure of Potential Conflicts of Interest

AstraZeneca supported the clinical trial and respective translational research, helped with protocol writing, and provided logistic support for data management of the trial, and study monitoring. The commercial funder had no role in the clinical and translational study design, data analysis and interpretation of the data, decision to publish, or preparation of the paper.

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