

ORIGINAL ARTICLE

Molecular Determinants of the Response of Glioblastomas to EGFR Kinase Inhibitors

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ABSTRACT

BACKGROUND

The epidermal growth factor receptor (EGFR) is frequently amplified, overexpressed, or mutated in glioblastomas, but only 10 to 20 percent of patients have a response to EGFR kinase inhibitors. The mechanism of responsiveness of glioblastomas to these inhibitors is unknown.

METHODS

We sequenced kinase domains in the *EGFR* and human EGFR type 2 (*Her2/neu*) genes and analyzed the expression of EGFR, EGFR deletion mutant variant III (EGFRvIII), and the tumor-suppressor protein PTEN in recurrent malignant gliomas from patients who had received EGFR kinase inhibitors. We determined the molecular correlates of clinical response, validated them in an independent data set, and identified effects of the molecular abnormalities in vitro.

RESULTS

Of 49 patients with recurrent malignant glioma who were treated with EGFR kinase inhibitors, 9 had tumor shrinkage of at least 25 percent. Pretreatment tissue was available for molecular analysis from 26 patients, 7 of whom had had a response and 19 of whom had rapid progression during therapy. No mutations in *EGFR* or *Her2/neu* kinase domains were detected in the tumors. Coexpression of EGFRvIII and PTEN was significantly associated with a clinical response ($P < 0.001$; odds ratio, 51; 95 percent confidence interval, 4 to 669). These findings were validated in 33 patients who received similar treatment for glioblastoma at a different institution ($P = 0.001$; odds ratio, 40; 95 percent confidence interval, 3 to 468). In vitro, coexpression of EGFRvIII and PTEN sensitized glioblastoma cells to erlotinib.

CONCLUSIONS

Coexpression of EGFRvIII and PTEN by glioblastoma cells is associated with responsiveness to EGFR kinase inhibitors.

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TYROSINE KINASES ARE KEY REGULATORS of intracellular signaling.^{1,2} Overexpressed or mutated tyrosine kinases occur in many types of cancer and contribute to the development and progression of tumors.³⁻⁵ The dependence of tumor cells on persistently activated tyrosine kinases may render tumors susceptible to inhibitors of these kinases.³⁻⁷ The epidermal growth factor receptor (EGFR), a receptor tyrosine kinase, is a target for such inhibitors because it is amplified, mutated, or both in a number of neoplasms.⁸ A small subgroup of patients with lung cancer have a response to EGFR inhibitors,⁹⁻¹¹ and mutations in the EGFR kinase domain have been associated with responsiveness.¹²⁻¹⁴ It is not known, however, whether such mutations affect the responsiveness of other types of cancer to EGFR kinase inhibitors.

Among patients with glioblastoma, the most common primary malignant brain tumor of adults, a small subgroup seems to benefit from the EGFR kinase inhibitors erlotinib and gefitinib.^{15,16} However, the infrequency of mutations in the EGFR kinase domain in glioblastomas^{17,18} suggests that such *EGFR* mutations cannot account for responsiveness to EGFR kinase inhibitors.¹⁹ The *EGFR* gene is commonly amplified in glioblastoma,²⁰ but this abnormality also does not correlate with responsiveness to EGFR kinase inhibitors.¹⁶ Glioblastomas often express EGFRvIII, a constitutively active genomic deletion variant of EGFR.²¹⁻²⁵ This variant of EGFR strongly and persistently activates the phosphatidylinositol 3' kinase (PI3K) signaling pathway, which provides critical information for cell survival, proliferation, and motility.²⁶⁻³⁰ Persistent PI3K signaling — as would be instigated by EGFRvIII — is believed to cause “pathway addiction”³¹; addicted tumor cells die if the pathway is disrupted by tyrosine kinase inhibitors. By promoting chronic dependence on PI3K signaling, EGFRvIII may sensitize glioblastoma cells to EGFR kinase inhibitors.

The PTEN (phosphatase and tensin homologue deleted in chromosome 10) tumor-suppressor protein, an inhibitor of the PI3K signaling pathway, is commonly lost in glioblastoma.^{20,27,32} This loss may promote cellular resistance to EGFR kinase-inhibitor therapy by dissociating EGFR inhibition from downstream PI3K pathway inhibition (Fig. 1).³³ We hypothesized that EGFRvIII would sensitize tumors to EGFR kinase inhibitors, whereas PTEN loss would impair the response to such inhibitors.³³ To test this hypothesis, we analyzed EGFRvIII and PTEN at the gene and protein levels in glioblastomas from patients before treatment

with EGFR kinase inhibitors. We also searched for mutations in *EGFR* and in its heterodimerization partner *Her2/neu*, which has been reported to be mutated in glioblastoma and could also affect the response to EGFR kinase inhibitors.³⁴ We found a strong association between the coexpression of EGFRvIII and PTEN in glioblastoma cells and responsiveness to EGFR kinase inhibitors.

METHODS

TEST SET

Since 2001, 49 patients with recurrent glioblastoma have been treated at the University of California, Los Angeles (UCLA) — 37 with gefitinib and 12 with erlotinib — as part of three multi-institutional clinical trials approved by the institutional review board. The clinical studies were performed through the Cancer Therapy Evaluation Program of the National Institutes of Health or Genentech. The correlative study described here was conducted independently and without industry support. Diagnoses were established by two neuropathologists and confirmed by a third pathologist who was unaware of the results of molecular analyses. Tumor specimens were obtained according to a protocol approved by the institutional review board of UCLA. All patients had measurable disease on magnetic resonance imaging (MRI) and had stopped receiving any previous cancer treatment at least four weeks before the start of monotherapy with an EGFR inhibitor.

MRI and clinical assessment were performed at baseline, every two months thereafter, and at the time of progression by a neuroradiologist and a neuro-oncologist who were unaware of the results of the molecular analyses. A response was defined as a decrease of at least 25 percent in the bidirectional area of the contrast-enhancing tumor on MRI in the absence of an increased dose of corticosteroids. Progression was defined as an increase of at least 25 percent in the bidirectional tumor area. Thirty-seven patients, 26 with clear-cut evidence of a response or progression, had sufficient tissue for molecular analysis (Tables 1 and 2). Ten patients fell between these extremes of response to treatment, with tumor growth or shrinkage of 25 percent or less (details are provided in Table 1 of the Supplementary Appendix, available with the full text of this article at www.nejm.org). One patient was excluded because a response occurred coincidentally with an increase in the dose of decadron. The statistical methods used are described in the Supplementary Appendix.

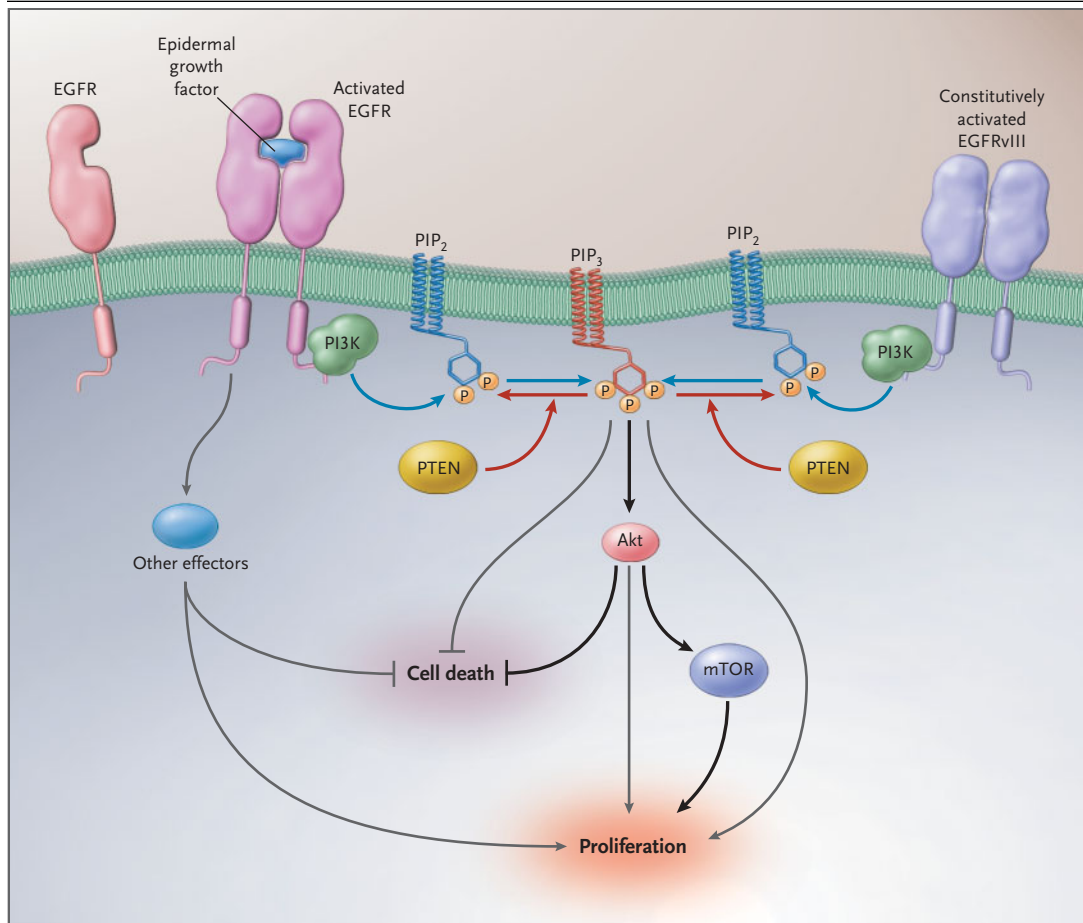


Figure 1. The PI3K–Akt Signaling Pathway.

The EGFR becomes activated on binding to epidermal growth factor and recruits PI3K to the cell membrane. PI3K converts phosphatidylinositol-4,5-bisphosphate (PIP₂) to the second-messenger molecule PIP₃ (blue arrows). This second messenger then activates downstream effector molecules, such as Akt and the mammalian target of rapamycin (mTOR), which help induce cellular proliferation and block apoptosis. PTEN terminates the PIP₃ signal (red arrows). The mutant receptor EGFRvIII is persistently activated in the absence of ligand, owing to an in-frame deletion within the extracellular ligand-binding domain.

VALIDATION SET

We obtained paraffin-embedded slides from biopsy specimens of malignant glioma from 33 patients who received erlotinib at the University of California, San Francisco (UCSF). Frozen tissue was not available from these patients. Immunohistochemical evaluation of EGFRvIII and PTEN was scored semiquantitatively by two UCLA pathologists who were unaware of the clinical results.

MOLECULAR STUDIES

Sequencing of Genomic Tumor DNA

Exons and flanking intronic sequences for *EGFR* (kinase domain), *HER2/neu* (kinase domain), and

PTEN (all exons) were amplified with the use of specific primers in a 384-well format involving a nested polymerase chain reaction (PCR), as performed by Agencourt Bioscience. Details are provided in the Supplementary Appendix.

Fluorescence in Situ Hybridization

Dual-probe fluorescence in situ hybridization (FISH) was performed on paraffin-embedded sections with locus-specific probes for *EGFR* and the centromere of chromosome 7 (*CEP7*) (Vysis). Standard FISH protocols for pretreatment, hybridization, and analyses were followed according to the manufacturer's instructions.²⁰

Table 1. Characteristics of the Patients.*

| Patient No. | Sex | Age yr | Diagnosis | Prior Therapy† | EGFR Inhibitor and Dose | EIAED | Change in Bidirectional Tumor Volume on MRI % | Time to Progression mo | Survival (Time from Treatment) |
|--------------------|-----|-----------|-----------|----------------|---------------------------|-------|--|---------------------------|--------------------------------|
| Response | | | | | | | | | |
| 1 | M | 47 | GBM | F | Erlotinib, 300–500 mg | Yes | –82 | 9.7 | >19.5 |
| 2 | M | 60 | GBM | T, A | Erlotinib, 150–200 mg | No | –57 | 10.1 | >19.4 |
| 3 | F | 19 | GBM | T, A | Gefitinib, 1500, 1000 mg‡ | Yes | –44 | 6.5 | 18.7 |
| 4§ | M | 27 | AO | P | Gefitinib, 500 mg | No | –57 | >40 | >40 |
| 5 | F | 41 | GBM | T, A | Gefitinib, 500, 250 mg‡ | No | –87 | 15.2 | 24.7 |
| 6 | F | 60 | GBM | T, A | Gefitinib, 500–750 mg | No | –50 | 5.6 | 7.8 |
| 7¶ | M | 65 | GBM | T, A | Erlotinib, 150–200 mg | No | –35 | NA | 2.7 |
| No response | | | | | | | | | |
| 8 | M | 50 | GBM | T, A | Gefitinib, 150 mg | No | 35 | 0.9 | 6.1 |
| 9 | F | 39 | GBM | T | Erlotinib, 150–200 mg | No | 100 | 1.8 | 3.4 |
| 10 | M | 64 | GBM | T | Erlotinib, 300–450 mg | Yes | 106 | 1.8 | 3.7 |
| 11 | F | 56 | GBM | T | Gefitinib, 500–750 mg | No | 121 | 1.6 | 5.2 |
| 12 | M | 38 | GBM | T, A | Gefitinib, 500 mg | No | 697 | 0.9 | 6.1 |
| 13 | M | 46 | GBM | T, A | Erlotinib, 300–350 mg | Yes | 38 | 0.8 | 0.8 |
| 14 | M | 40 | GBM | T, A | Erlotinib, 150 mg | No | 453 | 1.8 | 10.6 |
| 15 | M | 60 | GBM | T, A | Gefitinib, 500–1000 mg | No | 628 | 1.9 | 4.8 |
| 16 | M | 58 | GBM | T, A | Erlotinib, 150 mg | No | 105 | 1.7 | 5.8 |
| 17 | M | 57 | GBM | T, I | Gefitinib, 500 mg | No | 319 | 1.3 | 5.6 |
| 18 | F | 42 | GBM | T, A | Gefitinib, 500 mg | No | 450 | 1.8 | 10.6 |
| 19 | M | 55 | GBM | T, A | Gefitinib, 150 mg | No | 150 | 1.1 | 5.8 |
| 20 | F | 52 | GBM | T, A | Gefitinib, 150–200 mg | No | 200 | 1.8 | 6.1 |
| 21 | M | 39 | GBM | T, A | Gefitinib, 150 mg | Yes | 87 | 0.7 | 6.7 |
| 22§ | F | 31 | AO | P | Gefitinib, 500 mg | No | 418 | 1.8 | 3.5 |
| 23¶ | F | 32 | GBM | T | Gefitinib, 500 mg | No | 182 | 1.4 | 6.3 |
| 24¶ | F | 41 | GBM | T, A | Gefitinib, 500 mg | No | 332 | 0.9 | 6.6 |
| 25 | F | 58 | GBM | C, T, A | Erlotinib, 150–200 mg | No | 34 | 1.8 | 6.6 |
| 26¶ | M | 26 | GBM | P | Gefitinib, 500 mg | No | 350 | 1.8 | 2.2 |

* EIAED denotes enzyme-inducing antiepileptic drugs, GBM glioblastoma, AO anaplastic oligodendroglioma, and NA not applicable.

† All patients underwent surgery and received radiation as first-line therapy. Additional treatments before therapy with an EGFR kinase inhibitor included farnesyl transferase inhibitor (tipifarnib [F]), temozolomide (T), *cis*-retinoic acid (A), procarbazine, lomustine, and vincristine (P), irinotecan (I), and carmustine (C).

‡ Dose was reduced.

§ This patient was excluded from the analysis of the time to progression and survival.

¶ This patient died of an unrelated cardiac arrhythmia. Minimal residual tumor was found at autopsy.

|| Glioblastoma developed in this patient from lower-grade glioma. Therapy with an EGFR kinase inhibitor was given after the tumor had progressed to glioblastoma. All other patients with glioblastoma had primary glioblastoma.

Reverse Transcriptase–PCR

For the reverse-transcriptase–PCR (RT-PCR) assay, high-quality total RNA was extracted from 13 fresh-frozen tumor samples (4 from patients with a response and 9 from patients without a response). Complementary DNA (cDNA) was synthesized and amplified with the use of primers designed

specifically to amplify *EGFR* (1044-bp product) and *EGFRvIII* (243-bp product). Details are provided in the Supplementary Appendix.

Real-Time PCR

Genomic DNA was extracted from samples from 15 patients (7 with a response and 8 without a re-

Table 2. EGFR and PTEN Status in Tumor Tissue.*

| Patient No. | EGFR | | EGFRvIII | | | PTEN IHC |
|--------------------|---------------------------|---------------|----------|--------|----------------|----------|
| | Analysis for KD Mutations | FISH | IHC | RT-PCR | Immunoblotting | |
| Response | | | | | | |
| 1 | – | Amplified | + | ND | + | No loss |
| 2 | – | Not amplified | – | – | – | No loss |
| 3 | – | Polysomy | + | NA | NA | No loss |
| 4 | – | Polysomy | + | + | + | No loss |
| 5 | – | Polysomy | + | + | NA | No loss |
| 6 | – | Amplified | +† | NA | NA | No loss |
| 7 | – | Amplified | + | + | + | No loss |
| No response | | | | | | |
| 8 | – | Not amplified | – | – | – | No loss |
| 9 | NA | Polysomy | – | NA | NA | Loss |
| 10 | NA | Amplified | + | NA | NA | No loss |
| 11 | NA | Amplified | – | NA | NA | Loss |
| 12 | NA | Amplified | – | NA | NA | Loss |
| 13 | NA | Amplified | – | NA | NA | Loss |
| 14 | – | Polysomy | – | – | – | No loss |
| 15 | NA | Amplified | – | NA | NA | Loss |
| 16 | – | Not amplified | – | – | – | Loss |
| 17 | – | Amplified | NT | + | + | No loss |
| 18 | NA | Polysomy | + | NA | NA | Loss |
| 19 | NA | Amplified | + | + | NA | Loss |
| 20 | – | Amplified | + | + | + | Loss |
| 21 | – | Polysomy | – | – | – | Loss |
| 22 | – | Not amplified | – | – | – | Loss |
| 23 | – | Not amplified | – | – | – | No loss |
| 24 | NA | Not amplified | – | NA | NA | No loss |
| 25 | NA | Amplified | + | NA | NA | Loss |
| 26 | NA | NT | – | NA | NA | Loss |

* KD denotes kinase domain, FISH fluorescence in situ hybridization, IHC immunohistochemical analysis, ND RNA degraded, NA no frozen tissue available, NT no tissue slide available, plus sign positive results, and minus sign negative results.

† The results were confirmed by a ratio of EGFR exon 9 DNA to exon 4 DNA of 4.79.

sponse) and subjected to real-time PCR with the use of the iCycler thermocycler (Bio-Rad Laboratories). All measurements were made in triplicate and confirmed by independent experiments. The Supplementary Appendix lists the primer sequences used and provides complete details.

Immunohistochemistry and Immunoblotting

Paraffin-embedded tissue sections underwent immunohistochemical analysis in which the results

were scored independently by two pathologists who were unaware of the findings of the molecular analyses. Scores of 0 and 1 were considered to indicate PTEN loss.²⁷ If staining for PTEN was heterogeneous, tumors with diminished or absent staining in at least 25 percent of the section were considered PTEN-deficient. Tumors demonstrating focal, moderate-to-strong immunostaining for EGFRvIII were considered positive.²⁷ Quantitative image analysis to confirm the pathologists' scor-

Table 3. Biomarkers of a Response to EGFR Kinase Inhibitors.*

| Group | Response | No Response | P Value | Odds Ratio (95% CI) |
|---|----------------|-------------|---------|---------------------|
| | <i>no. (%)</i> | | | |
| UCLA patients | | | | |
| Clinical variables | | | | |
| Total no. | 7 | 19 | | |
| Median age — yr | 47 | 46 | 0.70 | NC |
| Sex — no. | | | | |
| Male | 4 | 11 | 1.0 | NC |
| Female | 3 | 8 | | NC |
| Mean Karnofsky performance-status score | 88.6 | 86.3 | 0.52 | NC |
| Gross total surgical resection — no. (%) | 3 (43) | 8 (42) | 1.0 | NC |
| EGFR inhibitor | | | 1.0 | NC |
| Erlotinib | 3 | 7 | | |
| Gefitinib | 4 | 12 | | |
| Enzyme-inducing antiepileptic drugs | | | | |
| No. | 2 | 3 | | |
| Mean dose of erlotinib — mg (no.) | 500 (1) | 317 (3) | 0.50 | NC |
| Mean dose of gefitinib — mg (no.) | 1500 (1) | 0 | NC | NC |
| No enzyme-inducing antiepileptic drugs | | | | |
| No. | 5 | 16 | | |
| Mean dose of erlotinib — mg (no.) | 200 (2) | 175 (4) | 0.80 | NC |
| Mean dose of gefitinib — mg (no.) | 583 (3) | 479 (12) | 0.63 | NC |
| Molecular biomarkers — no./total no. (%) | | | | |
| EGFR amplification or polysomy | 6/7 (86) | 13/18 (72) | 0.66 | NC |
| EGFRvIII expression | 6/7 (86) | 6/19 (32) | 0.03 | 13 (1–130) |
| PTEN expression | 7/7 (100) | 6/19 (32) | 0.005 | NC† |
| Coexpression of EGFRvIII and PTEN‡ | 6/7 (86) | 2/19 (11) | <0.001 | 51 (4–669) |
| UCSF patients | | | | |
| Clinical variables | | | | |
| Total no. | 8 | 25 | | |
| Median age — yr | 50 | 53 | 0.67 | NC |
| Sex — no. | | | 0.12 | NC |
| Male | 6 | 10 | | |
| Female | 2 | 15 | | |
| Concurrent temozolomide — no.§ | 2 | 4 | 0.61 | NC |
| Enzyme-inducing antiepileptic drugs | | | | |
| Mean dose of erlotinib — mg (no.) | 367 (6) | 267 (15) | 0.12 | NC |
| No enzyme-inducing antiepileptic drugs | | | | |
| Mean dose of erlotinib — mg (no.) | 175 (2) | 180 (10) | 1.0 | NC |
| Molecular biomarkers — no./total no. (%) | | | | |
| EGFRvIII expression | 7/8 (88) | 11/25 (44) | 0.05 | 9 (1–84) |
| PTEN expression | 5/8 (62) | 4/25 (16) | 0.02 | 9 (1.5–52) |
| Coexpression of EGFRvIII and PTEN¶ | 5/8 (62) | 1/25 (4) | 0.001 | 40 (3–468) |

* CI denotes confidence interval, and NC not calculated.

† An odds ratio could not be calculated because none of the UCLA patients with PTEN-deficient tumors had a response, but if 0.5 is added to each cell count, the odds ratio is 31 (95 percent confidence interval, 1.5 to 633.0).

‡ The test had a sensitivity of 86 percent and a specificity of 89 percent in the UCLA group and a positive predictive value of 75 percent.

§ A subgroup of patients in the UCSF study received concurrent temozolomide. All UCLA patients received monotherapy with an EGFR kinase inhibitor.

¶ The test had a sensitivity of 63 percent, a specificity of 96 percent, and a positive predictive value of 89 percent.

ing was also performed with the use of Soft Imaging System software (complete details are provided in the Supplementary Appendix).

CELL-LINE MODELS

U87MG Model

EGFR and *EGFRvIII* cDNAs were introduced into U87MG cells and U87MG-PTEN cells (which overexpress PTEN) by means of a retroviral vector; 1000 cells per well were seeded into eight sets of 96-well plates. Twenty-four hours later, erlotinib was added at final concentrations ranging from 0 to 10 μ M. Plates were incubated for 7 to 10 days, fixed, stained with 0.25 percent crystal violet in methanol, and quantified. The results were confirmed in several independent clones.

Other Cell-Line Models

Mouse-embryonic fibroblasts with deletion of the *PTEN* locus were kindly provided by Dr. Hong Wu (UCLA). A431 cell lines stably expressing vector RNA interference (RNAi), short hairpin RNA (shRNA) to human *PTEN* (5'ATAGCTACCTGTTAAAGAA3'), or pWZL-MyrAkt Δ 4-129 (kindly provided by Dr. Russell Pieper, UCSF) were derived by retroviral infection and selection. *PTEN* shRNAi and vector shRNA were also stably expressed in amphotropic 293T cells (Orbigen). PKI-166 was provided by Novartis. Cell viability was determined in triplicate by means of trypan blue exclusion, and the results were confirmed in three independent experiments. Cells were plated at a density of 50,000 cells per well in six-well plates, recounted after the cells had adhered to the plate, and then treated with vehicle or an EGFR kinase inhibitor. After four to five days of incubation, the supernatant (containing floating cells) and trypsin-treated adherent cells were pooled, spun at 1500 rpm for 90 seconds, and resuspended in trypan blue for cell counting. The occurrence of apoptosis was confirmed in independent experiments by immunoblotting for caspase-cleaved poly(adenosine diphosphate ribose) polymerase (number 9546, Cell Signaling Technology) and by flow cytometry.

RESULTS

PATIENTS

For the purposes of this study, we selected 7 patients with unequivocal evidence of a response and 19 who had no response (as reflected by evidence of tumor progression on MRI within eight weeks

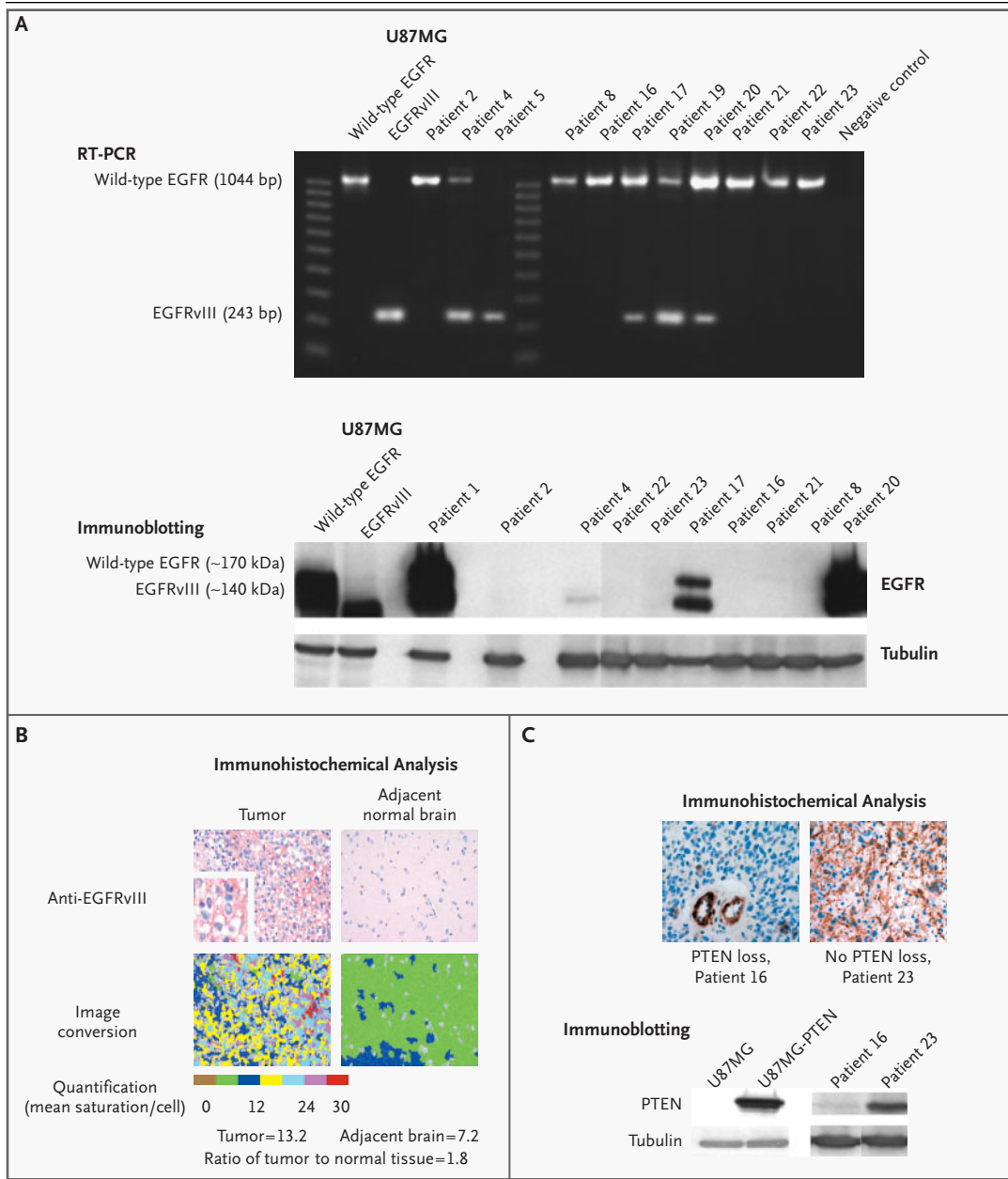
Figure 2 (facing page). EGFRvIII and PTEN in Glioblastomas.

Panel A shows the detection of EGFRvIII in fresh-frozen tumor specimens from patients with glioblastomas by RT-PCR and immunoblotting. In the upper part of the panel, primers flanking the deleted portion (exons 2 through 7) of EGFRvIII amplified cDNA fragments from both full-length EGFR (1044 bp) and the truncated EGFRvIII (243 bp) on RT-PCR. Plasmid cDNAs for wild-type EGFR (lane 1) and EGFRvIII (lane 2) in U87MG cells were included as size controls. In the lower portion of the panel, immunoblotting of tumor lysates with an antibody against EGFR detected full-length EGFR (approximately 170 kDa) and the truncated EGFRvIII (approximately 140 kDa). Whole-cell lysates of wild-type EGFR (lane 1) and EGFRvIII (lane 2) in U87MG cell lines were included as controls. Tubulin was used as a control for gel loading. Additional details are provided in Figure 3 of the Supplementary Appendix. Panel B shows immunohistochemical detection and quantification of EGFRvIII in paraffin-embedded tumor samples (mean saturation per cell in arbitrary units). Tumor samples and adjacent normal brain tissue were stained with antibody against EGFRvIII (L8A4) (explained in detail in Fig. 3 of the Supplementary Appendix at www.nejm.org). The inset shows a higher magnification. Representative "false-color" images were generated with the use of the Soft Imaging Systems image-analysis software. Panel C shows the detection of PTEN in glioblastomas. Representative immunohistochemical staining (upper portion of the panel) and immunoblotting (lower portion of the panel) from a PTEN-deficient tumor (from Patient 16) and a PTEN-positive tumor (from Patient 23) are shown. In the specimen from Patient 16, PTEN staining is absent in tumor cells, but not in vascular endothelial cells. PTEN immunoblotting of whole-cell lysates from isogenic U87MG cells that overexpressed PTEN (U87MG-PTEN) and cells that did not express PTEN demonstrates the specificity of the antibody.

after initiating therapy) (Fig. 1A and 1B of the Supplementary Appendix). Median survival after the initiation of treatment with an EGFR kinase inhibitor was significantly longer among patients with a response than among those without a response (21.7 vs. 5.8 months, $P=0.01$), as was the median time to progression (9.7 vs. 1.7 months, $P<0.001$) (Table 1, and Fig. 1C of the Supplementary Appendix). There were no significant correlations between response and age, sex, the extent of surgical resection, Karnofsky performance status, or the dose of EGFR inhibitor (Table 3).

MUTATIONS IN THE EGFR KINASE DOMAIN

We sequenced the kinase domain of *EGFR* in the glioblastomas from the 26 patients. The quality of



the DNA was sufficient to allow us to sequence the entire kinase domain of *EGFR* (exons 18 through 24) in six of seven patients with a response and exon 21 alone in Patient 6. No mutations were detected in the seven patients with a response or in eight of the patients with no response for whom DNA was available for sequencing (Table 2). Thus, mutations in the *EGFR* kinase domain are unlikely to determine the sensitivity of glioblastomas to EGFR kinase inhibitors. We also sequenced the kinase domain of *Her2/neu* and found no mutations.

EGFR GENE AMPLIFICATION

FISH showed — and real-time PCR confirmed — that the *EGFR* gene was amplified in 12 of 25 glioblastomas (48 percent); 7 of these 12 demonstrated polysomy. No association between amplification of *EGFR* and a response to EGFR inhibitors was found (Table 3).¹⁶

EXPRESSION OF EGFRvIII AND PTEN PROTEIN

We used immunohistochemical analysis to screen for EGFRvIII and validated the results with the use

of nucleic acid–based assays (RT-PCR and the ratio of EGFR exon 9 DNA to exon 4 DNA) and immunoblotting in the 15 available samples of frozen tumor (Fig. 2A and 2B and Table 2). We found complete agreement between the nucleic acid–based assays and immunohistochemical analysis ($\kappa=1.0$, $P<0.001$) and between immunoblotting and immunohistochemical analysis ($\kappa=1.0$, $P<0.001$) (Fig. 3 of the Supplementary Appendix). These results are consistent with recent findings by another group²¹ and provide support for the use of immunohistochemical analysis to determine the EGFRvIII status in the remaining 11 patients for whom no frozen tissue was available.

EGFRvIII was detected in 12 of 26 malignant gliomas (46 percent) (Table 2), similar to the previously reported frequency in glioblastomas.^{21,27} It was found only in tumors with EGFR amplification or a gain of chromosome 7. Of 12 patients whose tumors expressed EGFRvIII, 6 had a response to EGFR kinase inhibitors, whereas 1 of 14 patients whose tumors did not express EGFRvIII had a response to EGFR kinase inhibitors ($P=0.03$). These data suggested that the expression of EGFRvIII sensitizes gliomas to EGFR kinase inhibitors. However, the lack of response in 50 percent of patients with EGFRvIII-expressing tumors indicates that other factors influence the outcome of treatment.

Since PTEN may be required for a response to the EGFR family of kinase inhibitors,³³ we studied this protein in glioblastomas using immunohistochemical analysis and immunoblotting (Fig. 2C). The results of both assays were concordant ($\kappa=0.8$, $P=0.005$). None of 13 patients whose tumors lacked PTEN had a response to EGFR inhibitors, whereas 7 of 13 patients with PTEN-positive tumors had a response ($P=0.005$) (Table 3). To rule out the possibility that the immunohistochemical assay detected a mutant protein, we sequenced PTEN; no mutations were detected in the responsive tumors. The greatest likelihood of a clinical response to EGFR kinase inhibitors was associated with coexpression of EGFRvIII and PTEN (odds ratio, 51; 95 percent confidence interval, 4 to 669; $P<0.001$) (Table 3).

Ten patients who had neither tumor regression nor substantial tumor growth while receiving an EGFR kinase inhibitor (Table 1 of the Supplementary Appendix) were excluded from the original analysis because they did not fit the extremes of a clear response or treatment failure. None of these patients, who had a median time to progression of

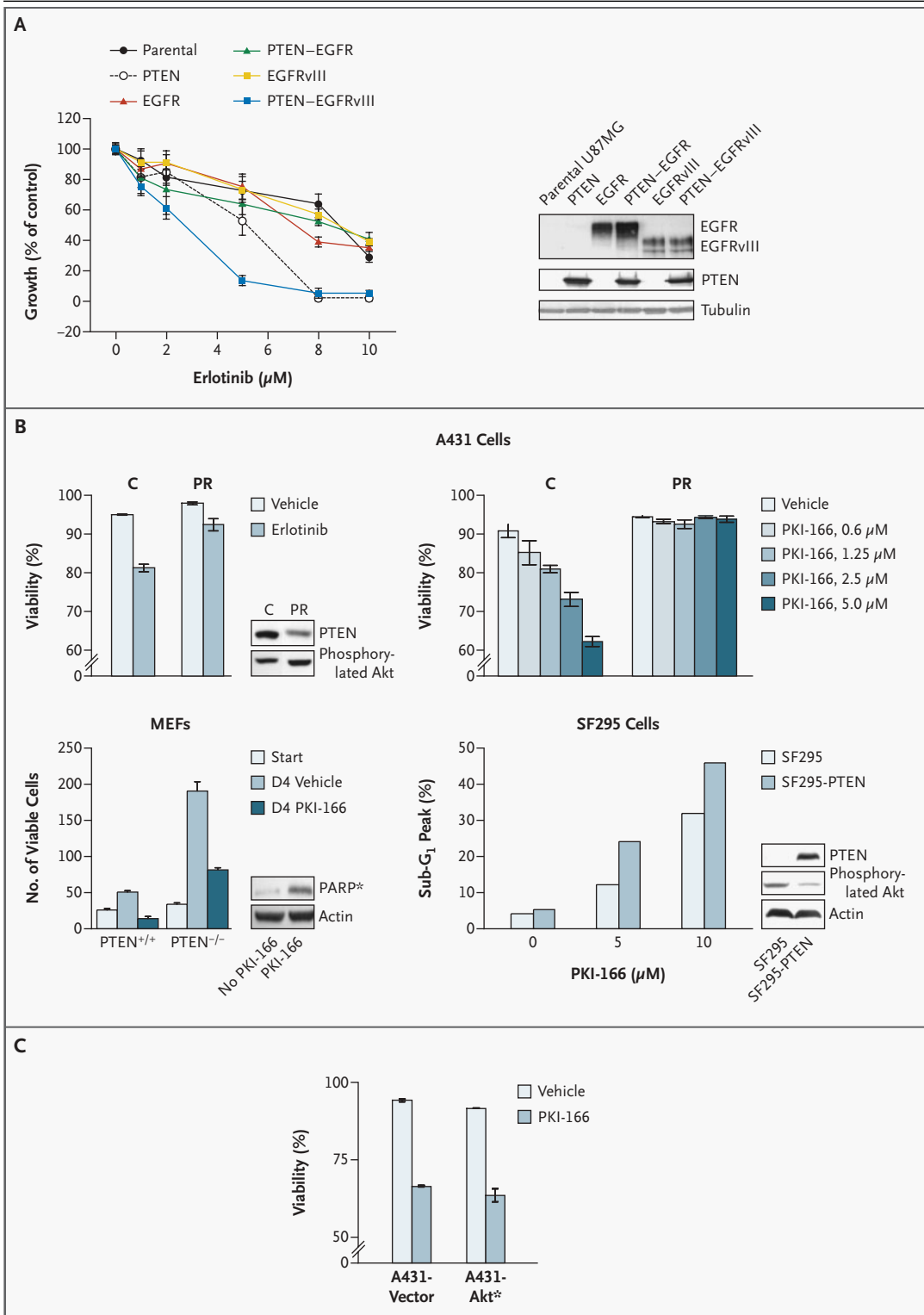
Figure 3 (facing page). Loss of PTEN and Resistance to EGFR Kinase Inhibitors in EGFR-Sensitized Cells.

In Panel A, the coexpression of EGFRvIII and PTEN sensitizes U87MG glioblastoma cells to the antiproliferative effects of erlotinib. The graph shows the effect of erlotinib on the mean (\pm SD) growth of the parental cell line and U87MG sublines. The immunoblot of the parental cell line and U87MG sublines shows EGFR (170 kD) and EGFRvIII (140 kD) in cells with PTEN and without PTEN. In Panel B, the loss of PTEN nullifies the induction of apoptosis by two EGFR kinase inhibitors, erlotinib and PKI-166. PKI-166 inhibits both EGFR and Her2, but the 50 percent inhibitory concentration is less by a factor of 10 for EGFR than for Her2.⁴¹ Cell death was detected at concentrations of PKI-166 that primarily or exclusively inhibit EGFR.⁴²⁻⁴⁴ The upper part of Panel B shows the viability of A431 cells stably expressing PTEN shRNA (A431-PR [PR]) or vector control (A431-vector [C]) five days after the addition of 3 μ M of erlotinib or PKI-166. The inset shows immunoblots of PTEN and phosphorylated Akt from A431-vector and A431-PR stable cell lines. The graph on the bottom left of Panel B shows that PKI-166 induces cell death in PTEN^{+/+}, but not PTEN^{-/-} mouse-embryonic fibroblasts (MEFs). The number of viable cells was determined by means of trypan blue exclusion immediately before (start) and four days after (D4) the addition of 5 μ M of PKI-166. Immunoblots of cleaved poly(adenosine diphosphate ribose) polymerase (PARP) were obtained eight hours after the addition of 5 μ M of PKI-166. The graph on the bottom right of Panel B shows that stable overexpression of PTEN (SF295-PTEN) in PTEN-null SF295 glioblastoma cells enhances the apoptotic response to PKI-166. Apoptosis was determined with the use of flow cytometry (the sub-G₁ fraction was counted) 24 hours after the addition of PKI-166. The inset shows immunoblots for PTEN, phosphorylated Akt, and actin in the isogenic SF295 pair. Panel C shows that stable overexpression of an activated Akt allele (A431-Akt) does not block cell death induced by five days of incubation with 5 μ M of PKI-166. Figure 5 of the Supplementary Appendix (www.nejm.org) shows the corresponding immunoblots. In each panel, plus–minus values are means \pm SD. The asterisk denotes the cleaved product.

3.7 months, had tumors that coexpressed EGFRvIII and PTEN (Table 1 of the Supplementary Appendix).

VALIDATION SET

We analyzed the expression of EGFRvIII and PTEN in 33 gliomas from patients who were treated with erlotinib at a different institution, UCSF (Table 2 of the Supplementary Appendix). Of these 33 patients, 8 had a clinical response. A clinical response in this group was also significantly associated with the coexpression of EGFRvIII and PTEN (odds ratio, 40; 95 percent confidence interval, 3 to 468; $P=0.001$) (Table 3).



IN VITRO STUDIES OF THE COEXPRESSION OF EGFRvIII AND PTEN

We induced the expression of relevant combinations of PTEN, EGFR, and EGFRvIII in U87MG glioblastoma cells. U87MG cells are deficient in PTEN,³⁵⁻³⁸ express low levels of wild-type EGFR, and lack EGFRvIII.^{39,40} Coexpression of EGFRvIII and PTEN in these glioblastoma cells rendered them highly susceptible to arrest of growth by erlotinib, as compared with control cells and U87MG cells that were transfected with other components of the PTEN-EGFR system (Fig. 3A). In these experiments, erlotinib caused the arrest of growth but not apoptosis in U87MG cells. Because U87MG cells do not depend on EGFR signaling for survival, we examined the effect of a lack of PTEN in three cell lines that undergo apoptosis in the presence of EGFR kinase inhibitors: SF295 human glioblastoma cells, mouse embryonic fibroblasts from mice in which the *PTEN* gene has been conditionally inactivated,⁴⁵ and A431 cells with stable PTEN RNAi (Fig. 3B). These experiments were performed with two EGFR kinase inhibitors, erlotinib and PKI-166.⁴¹ In all three systems, the lack of PTEN abrogated or markedly reduced the extent of apoptosis induced by the two inhibitors of EGFR kinase (Fig. 3B).

We examined the contribution of Akt to the resistance to EGFR kinase inhibitors conferred by the loss of PTEN, because many of the effects that follow the loss of PTEN occur through the activation of the downstream kinase Akt.⁴⁶ Erlotinib-induced inhibition of the phosphorylation of Akt correlated with growth inhibition in U87MG cells (Fig. 4 of the Supplementary Appendix), but not in all cell lines. For example, in A431 cells expressing PTEN shRNA, phosphorylation of Akt was blocked by EGFR kinase inhibitors (Fig. 5 of the Supplementary Appendix, lanes 3 and 4), but the cells remained viable (Fig. 3B). Furthermore, overexpression of a membrane-targeted and persistently activated allele of Akt⁴⁷ (Fig. 5 of the Supplementary Appendix, lanes 5 and 6) did not prevent A431 cells from undergoing apoptosis mediated by EGFR kinase inhibitors (Fig. 3C). These findings suggest that Akt-independent branches of the PTEN pathway may contribute to the effects of PTEN on the sensitivity of tumors to EGFR kinase inhibitors. We also noted that the levels of EGFR and EGFRvIII phosphorylation were consistently higher in PTEN-deficient cells than in their PTEN-expressing counterparts, suggesting that PTEN affects the receptor protein itself

(Fig. 6 of the Supplementary Appendix). Taken together, these results suggest the existence of multiple mechanisms by which the loss of PTEN promotes resistance to EGFR kinase inhibitors in glioblastoma cells.

DISCUSSION

In this study of glioblastomas from patients who were treated with gefitinib or erlotinib, we found that responsiveness to EGFR kinase inhibitors was strongly associated with coexpression by the tumor of EGFRvIII and PTEN. EGFRs with mutations in the tyrosine kinase domain selectively activate anti-apoptotic signals through the PI3K-Akt signaling pathway (Fig. 1).²⁶ Akt, a kinase involved in cellular proliferation and apoptosis, is activated by signals generated by PI3K. Inhibition of this antiapoptotic signal by gefitinib appears to be critical to the efficacy of the drug.²⁶ Like the EGFR kinase domain mutants in lung cancer, EGFRvIII, a constitutively active mutant variant of EGFR, preferentially activates PI3K-Akt signaling and can sensitize glioblastoma cells to EGFR kinase inhibitors.²⁶⁻³⁰ Loss of PTEN, a tumor-suppressor protein that inhibits the PI3K signaling pathway, may promote resistance to EGFR kinase inhibitors.³³ We found that the lack of PTEN in gliomas is associated with resistance to EGFR kinase inhibitors. Moreover, in four isogenic cell lines, loss of PTEN markedly diminished responsiveness to EGFR kinase inhibitors. Our evidence suggests that both Akt-dependent and Akt-independent mechanisms underlie the resistance caused by a loss of PTEN.

These results suggest that screening of tumors for PTEN protein may be warranted in patients with cancers with EGFR kinase mutations that do not respond to gefitinib, erlotinib, or related EGFR kinase inhibitors. Our data also suggest that downstream inhibition of the PI3K pathway, perhaps at the level of the mammalian target of rapamycin (a kinase related to PI3K) (Fig. 1), could be combined with EGFR kinase inhibitors in patients with PTEN-deficient tumors to promote responsiveness.⁴⁸ These studies also raise the possibility that more complete inhibition of EGFR phosphorylation may overcome the resistance to kinase inhibitors caused by a deficiency of PTEN.

In summary, we have implicated EGFRvIII and PTEN as molecular determinants of the sensitivity of glioblastomas to EGFR kinase inhibitors. Pro-

spective validation of EGFRvIII and PTEN as predictors of the clinical response to EGFR kinase inhibitors in independent data sets is warranted.

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A patent application entitled "Molecular Determinants of EGFR Kinase Inhibitor Response in Glioblastoma" has been filed by the University of California. The patent includes the names of Drs. Mischel, Mellinghoff, Wang, Sawyers, and Cloughesy.

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CORRECTION

Molecular Determinants of the Response of Glioblastomas to EGFR Kinase Inhibitors

Molecular Determinants of the Response of Glioblastomas to EGFR Kinase Inhibitors . On page 2017, the last footnote in Table 3 should have stated that the test had a positive predictive value of 83 percent, rather than 89 percent, as printed.