

Resistance to Tyrosine Kinase Inhibition by Mutant Epidermal Growth Factor Receptor Variant III Contributes to the Neoplastic Phenotype of Glioblastoma Multiforme

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ABSTRACT

Purpose: We have reported previously that tumors expressing wild-type epidermal growth factor receptor (EGFR) in a murine model are sensitive to the EGFR tyrosine kinase inhibitor gefitinib, whereas tumors expressing mutant EGFR variant III (EGFRvIII) are resistant. Determination of how this differential inhibition occurs may be important to patient selection and treatment criteria, as well as the design of future therapeutics for glioblastoma multiforme.

Experimental Design: We have determined and quantified how treatment with gefitinib at commonly used, nontoxic doses affects neoplastic functions ascribed to EGFRvIII, including downstream signaling by Akt, DNA synthesis, and cellular invasion. In doing so, we have tested and compared a series of wild-type and mutant EGFRvIII-expressing fibroblast and glioblastoma cell lines *in vitro* after treatment with gefitinib.

Results: The results of these experiments demonstrate that short-term treatment with gefitinib (~24 h) does not reduce phosphorylation of EGFRvIII, whereas EGFR phosphorylation is inhibited in a dose-dependent manner. However, after daily treatment with gefitinib, phosphorylation declines for EGFRvIII by day 3 and later. Nevertheless, after 7 days of daily treatment, cells that express and are dependent on EGFRvIII for tumorigenic growth are not effectively growth inhibited. This may be due in part to

phosphorylation of Akt, which is inhibited in EGFR-expressing cells after treatment with gefitinib, but is unaffected in cells expressing EGFRvIII. Cell cycle analysis shows that nascent DNA synthesis in EGFR-expressing cells is inhibited in a dose-dependent manner by gefitinib, yet is unaffected in EGFRvIII-expressing cells with increasing dosage. Furthermore, cells expressing EGFRvIII demonstrate greater invasive capability with increasing gefitinib concentration when compared with cells expressing EGFR after treatment.

Conclusions: We conclude that the neoplastic phenotype of EGFRvIII is relatively resistant to gefitinib and requires higher doses, repeated dosing, and longer exposure to decrease receptor phosphorylation. However, this decrease does not effectively inhibit the biologically relevant processes of DNA synthesis, cellular growth, and invasion in cells expressing EGFRvIII.

INTRODUCTION

The *epidermal growth factor receptor (EGFR)* gene is often amplified and mutated in human neoplasms including a high percentage of malignant gliomas (1–3), as well as breast carcinomas, non-small cell lung carcinomas, and ovarian tumors (2, 4). High expression of EGFR is believed to contribute to the malignant progression of these cancers by promoting DNA synthesis via its tyrosine kinase (TK; Refs. 5–7). The most frequent mutation of this gene, *EGFR variant III (EGFRvIII)*, is characterized by a consistent and tumor-specific in-frame deletion of 801 bp from the extracellular domain that splits a codon and produces a novel glycine at the fusion junction (8, 9). This mutation encodes a protein with a constitutively active TK (10) that greatly enhances the tumorigenicity of the cells containing this mutation (11, 12). In addition, this mutated protein sequence is clonally expressed on the cell surface of ~40% of glioblastomas (13, 14) but is not present in any normal adult tissues making it an ideal target for tumor-specific immunotherapy of malignant gliomas (4, 8, 15, 16). Furthermore, mutant EGFRvIII, which is expressed only in transformed cells, has been clinically correlated with enhanced tumor cell growth, metastasis, and invasion (17, 18). Its constitutive kinase activity is thought to dysregulate signal transduction and cell cycle arrest (18–21).

Gefitinib (Iressa, ZD1839), an orally administered EGFR TK inhibitor, blocks signaling pathways implicated in the proliferation, survival, and other host-dependent processes promoting tumor growth (22–25). We have shown previously that whereas growth of tumors highly expressing EGFR were inhibited by gefitinib, tumors expressing the EGFRvIII mutation were resistant, despite a 70% reduction in receptor phosphorylation *in vivo* (26). However, whereas the success achieved clinically with gefitinib treatment of EGFR-expressing tumors is

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encouraging (25, 27–30), little is known as to whether or not it is an effective therapy for the treatment of tumors expressing mutant EGFRvIII. Additionally complicating our understanding of mutant EGFRvIII pathogenesis are the mechanisms behind its unattenuated signal transduction and cellular growth, which are not fully understood. Previous studies from our laboratory suggest that wild-type EGFR and mutant EGFRvIII use some common signaling pathways, whereas EGFRvIII may also signal by mechanisms not used by EGFR (26). Yet, it remains unclear what molecular events and which signaling pathways are required for transformation by EGFRvIII (19, 21, 31–33).

From our previous work, we found that therapeutic resistance of mutant EGFRvIII tumors to gefitinib *in vivo* (26) resulted from only partially decreased phosphorylation of EGFRvIII at the maximally tolerated dose, whereas up-regulating overall receptor expression. These findings suggest that the mutant receptor may not be susceptible to the same molecular mechanisms of TK inhibition as EGFR. The purpose of this study was to explain how this therapeutic resistance may occur, and to do so we examined the effect of gefitinib on wild-type EGFR and mutant EGFRvIII receptor phosphorylation, cell-cycle progression, and invasive potential *in vitro*. For our investigations, we have used cell lines that express only wild-type EGFR (A431 and NR6W) or mutant EGFRvIII (NR6M), as well as a malignant glioblastoma multiforme cell line (U87MG), overexpressing only wild-type EGFR (U87MG.wtEGFR) or both types of receptor (U87MG.ΔEGFR). The studies described herein demonstrate that whereas mutant EGFRvIII may require higher doses, repeated dosing, and longer exposure to gefitinib to decrease receptor phosphorylation, the overall neoplastic phenotype contributed by EGFRvIII is not effectively inhibited.

MATERIALS AND METHODS

Cell Lines, Tissue Culture, and Inhibitor Studies. All of the tissue culture was performed in antibiotic-free Zinc Option medium (Life Technologies, Inc., Grand Island, NY) containing 10% heat-inactivated FCS, and all of the cell lines were shown to be free of *Mycoplasma* contamination as described previously (3). For the purposes of these investigations, the following cell lines were used as described previously, A431, human epidermoid carcinoma cells which express high levels of wild-type EGFR (26), and NR6, Swiss 3T3 murine fibroblasts mock-transfected (NR6 Null, shown previously to be devoid of either wild-type EGFR or mutant EGFRvIII expression), or transfected with either wild-type EGFR (NR6W) or mutant EGFRvIII (NR6M) cDNA (12). NR6M is fully tumorigenic *in vivo* and is dependent on mutant EGFRvIII expression for its neoplastic phenotype (12). The U87MG.wtEGFR and U87MG.ΔEGFR malignant glioma cell lines, derived from transfection of the parental U87 malignant glioma (U87MG) cell line stably transfected with and expressing human wild-type EGFR or mutant EGFRvIII (19), respectively, were provided by Dr. Webster Cavenee (Ludwig Institute, University of California San Diego, San Diego, CA). Stock solutions of gefitinib (courtesy of Dr. Alan Wakeling, AstraZeneca, Cheshire, United Kingdom) were prepared in DMSO. Cell lines were cultured in humidified incubators at 37°C/5%CO₂ and treated with 0.1, 1, or 10 μM gefitinib unless otherwise indicated, for the reported

Table 1 Wild-type EGFR^a and mutant EGFRvIII surface expression for the cell lines described in these experiments

Cell line	No. of wild-type EGFR receptors/cell	No. of mutant EGFRvIII receptors/cell
A431	2.0–3.8 × 10 ⁶	0
NR6 null	0	0
NR6W	1.1–2.8 × 10 ⁶	0
NR6M	0	7.4–10 × 10 ⁵
U87MG	1 × 10 ⁵	0
U87MG.wtEGFR	1 × 10 ⁶	0
U87MG.ΔEGFR	1 × 10 ⁵	2 × 10 ⁶

^a EGFR, epidermal growth factor receptor.

times. Control samples were mock-treated with an equal amount of DMSO. Growth inhibition and viability were assessed by trypan blue dye exclusion using light microscopy and a standard hemocytometer for cell counting.

Determination of Receptor Density by Quantitative Fluorescence Activated Cell Sorting Analysis. As described previously by Wikstrand *et al.* (4), we have standardized the Quantitative Fluorescence Activated Cell Sorting methodology as marketed by Banks Laboratory (Fisher, IN) for the determination of wild-type EGFR and mutant EGFRvIII receptor density by comparing to standard Scatchard analysis with iodinated epidermal growth factor and iodinated monoclonal antibodies. Results presented in Table 1 for A431, NR6 Null, NR6W, and NR6M cells were provided from that analysis.

Western Blot Analysis. All cell lines were mock-treated or treated with gefitinib and were cultured for up to 7 days, as indicated in the figure legends. Briefly, at the time of harvest, cells were washed once in 1× Dulbecco's PBS, centrifuged, lysed, equivalent amounts of protein were separated by SDS-PAGE (4–20% acrylamide), and were transferred to polyvinylidene difluoride (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) for enhanced chemiluminescence detection, as described previously (26). The polyvinylidene difluoride membranes were blocked for 1 h with 5% nonfat milk in 1× Tris-buffered saline/0.1% Tween 20 and were then probed with rabbit polyclonal IgG phospho-Tyr-1173 EGFR (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal IgG pan EGFR (Cell Signaling Technology, Beverly, MA), rabbit polyclonal IgG phospho-Ser-473 Akt (Cell Signaling Technology); rabbit polyclonal IgG pan Akt (Cell Signaling Technology); and monoclonal IgG1 anti-β-actin clone AC-15 (Sigma-Aldrich, Inc., St. Louis, MO). The membranes were washed three times in 1× Tris-buffered saline/0.1% Tween 20 and incubated for 2 h with antirabbit IgG (Cell Signaling) or antimouse IgG (Cell Signaling) conjugated to horseradish peroxidase. The membranes were washed six times in 1× Tris-buffered saline/0.1% Tween 20, and immunoblotted proteins were visualized using the Phototope-horseradish peroxidase Western Blot Detection System (Cell Signaling), according to the manufacturer's instructions. For Western analysis, a mean constitutive activity or fold induction was determined for each experiment, with expression levels normalized to β-actin for all of the blots. To compare results between lanes, densitometry data were normalized to the 45.5 kDa β-actin band, which was constitutively expressed to similar levels in all of the lanes.

Values were calculated using scanning densitometry software (Kodak Digital Science 1D Image Analytical Software, Rochester, NY).

Flow Cytometric Cell Cycle Analysis. *In vitro* mock-treated and gefitinib-treated cells were harvested with 0.125% trypsin, washed twice in $1 \times$ Dulbecco's PBS with 0.1% glucose, counted, and fixed overnight with 70% (vol/vol) ethanol. Cells were then centrifuged at $1333 \times g$ for 10 min, resuspended at a concentration of 1×10^6 cells/ml in $1 \times$ Dulbecco's PBS with 0.1% glucose, 50 $\mu\text{g/ml}$ propidium iodide (Sigma), and 100 units/ml of RNase A (Sigma), and analyzed by flow cytometry.

In Vitro Invasion Assay. The BD Biocoat Matrigel Invasion Chamber assay was performed according to manufacturer's instructions (Becton Dickinson Labware, Bedford, MA). Briefly, 1×10^6 cells were mock-treated or treated with gefitinib (1 or 10 μM) in equivalent amounts of DMSO for 24 h, at which time 5×10^4 cells were added to control or invasion-chamber wells for an additional 24 h with fresh gefitinib. Twenty-four h later, the cells on the upper side of the chamber were scraped, and the ones on the lower side of the chamber were stained with Diff Quik (Dade Behring, Newark, DE) and counted using light microscopy with a standardized grid. All of the experimental treatment and control samples were performed in triplicate.

Statistical Analysis. The Student's *t* test was used to determine significant changes in activities and assess statistical significance for cell cycle analysis. Data are presented as the mean \pm SE, and were analyzed using Microsoft Excel XP software (Microsoft, Seattle, WA).

RESULTS

Inhibition of Mutant EGFRvIII Receptor Phosphorylation Requires Higher Concentration and Longer Exposure to Gefitinib Than Wild-Type EGFR. To determine the effectiveness of inhibition of phosphorylation by gefitinib, we tested multiple cell lines expressing no or low levels of EGFR and high levels of wild-type EGFR or mutant EGFRvIII (Table 1). For no or low-level EGFR expression, we assayed NR6 Swiss murine fibroblasts expressing neither EGFR nor EGFRvIII (NR6 Null), as well as the human glioblastoma multiforme cell line U87 (U87MG), endogenously expressing low amounts of wild-type EGFR. A431 human epidermoid carcinoma cells, NR6 cells expressing wild-type EGFR (NR6W), and U87 cells expressing wild-type EGFR (U87MG.wtEGFR) were tested because of their high level of stably expressed wild-type EGFR. Similarly, NR6 cells expressing mutant EGFRvIII (NR6M), as well as U87 cells (U87MG. Δ EGFR), which express the mutant receptor, we assayed because of their stable expression of EGFRvIII. All of the cells were mock-treated (DMSO only) or treated daily for 7 days with fresh medium and gefitinib at a final concentration of 0.1, 1, or 10 μM . Cells were harvested, lysed, and total cell lysates were subjected to SDS-PAGE for Western blot analysis.

After *in vitro* daily treatment with gefitinib at various concentrations (0.1, 1, or 10 μM), phosphorylation of EGFR tyrosine 1173, a key regulatory tyrosine residue involved in EGFR (34, 35), as well as EGFRvIII signal transduction (18,

19), was reduced significantly in high EGFR-expressing A431, NR6W, and U87MG.wtEGFR cells, but was not as decreased in mutant EGFRvIII-expressing cells NR6M and U87MG. Δ EGFR at 24 h (Fig. 1, A and B). However, after 3 days, phosphorylated EGFR expression in mutant EGFR-expressing cell lines was reduced with higher concentrations of gefitinib and was beginning to decline at lower treatment concentrations with prolonged exposure. Inasmuch, reductions in both wild-type and mutant receptor phosphorylation were sustained after day 3, continuing to decrease at days 5 and 7 for mutant EGFRvIII (Fig. 1, A and B). As expected, neither form of EGFR was detected in NR6 Null cells (Fig. 1A). Parental U87MG cells, which endogenously express low levels of wild-type EGFR (18), did not display receptor phosphorylation, regardless of concentration or time tested (Fig. 1A). Thus, mutant EGFRvIII does not appear to be as sensitive to TK inhibition as wild-type EGFR when treated with lower concentrations of gefitinib for shorter periods of time, but displays a dose-dependent inhibition with higher concentrations (1 and 10 μM) at day 5 and later (Fig. 1, A and B). From this, EGFRvIII-expressing tumors may require increased concentration and repeated dosing to inhibit mutant receptor phosphorylation.

Phosphorylation of Akt Is Decreased with Increasing Concentration of Gefitinib in Cells Expressing Wild-Type EGFR but Is Resistant to Inhibition in Mutant EGFRvIII-Expressing Cells. To determine what effect decreased phosphorylation of EGFR has on downstream signaling, we investigated the expression of the phosphatidylinositol-3'-kinase downstream effector Akt, considered to be a promitotic kinase central to EGFR-promoted growth, neoplasia, and invasion (21, 36, 37). Whereas the tumor suppressor PTEN, a lipid phosphatase that is a key negative regulator of Akt activity, is commonly mutated in glioblastoma multiforme tumors and cell lines such as U87MG (38, 39), it was not known if PTEN is functional in NR6 cells. Because mutant EGFRvIII expression and mutation of PTEN often occur concomitantly in glioblastoma multiformes, we were interested in the expression of Akt and PTEN in cells expressing wild-type EGFR or mutant EGFRvIII.

For the purposes of determining Akt expression in our studies, we investigated the phosphorylation of Akt serine 473, a residue that has been shown to be constitutively phosphorylated in some HER-2 overexpressing malignancies (40) and head and neck cancers (41). We found that phosphorylated Akt was down-regulated after treatment with gefitinib in a dose-dependent manner in cells expressing EGFR, including U87MG.wtEGFR (Fig. 2, A and B). However, phosphorylated Akt expression was unchanged in all of the mutant EGFRvIII-expressing cells assayed at all of the concentrations and times tested after treatment with gefitinib (Fig. 2, A and B). It is interesting to note that Akt phosphorylation was not reduced in U87MG. Δ EGFR, which expresses low levels of wild-type EGFR and high levels of mutant EGFRvIII. These data would suggest that cells expressing mutant EGFRvIII, which is significantly inhibited in phosphorylation with repeated dosing and prolonged exposure, still signal intracellularly via Akt after treatment with gefitinib. As expected, immunoblot detection of PTEN in U87MG.wtEGFR and U87MG. Δ EGFR was unsuccessful (data not shown), as *PTEN* is mutated in this malignant glioma line (42). However, PTEN was detected and constitu-

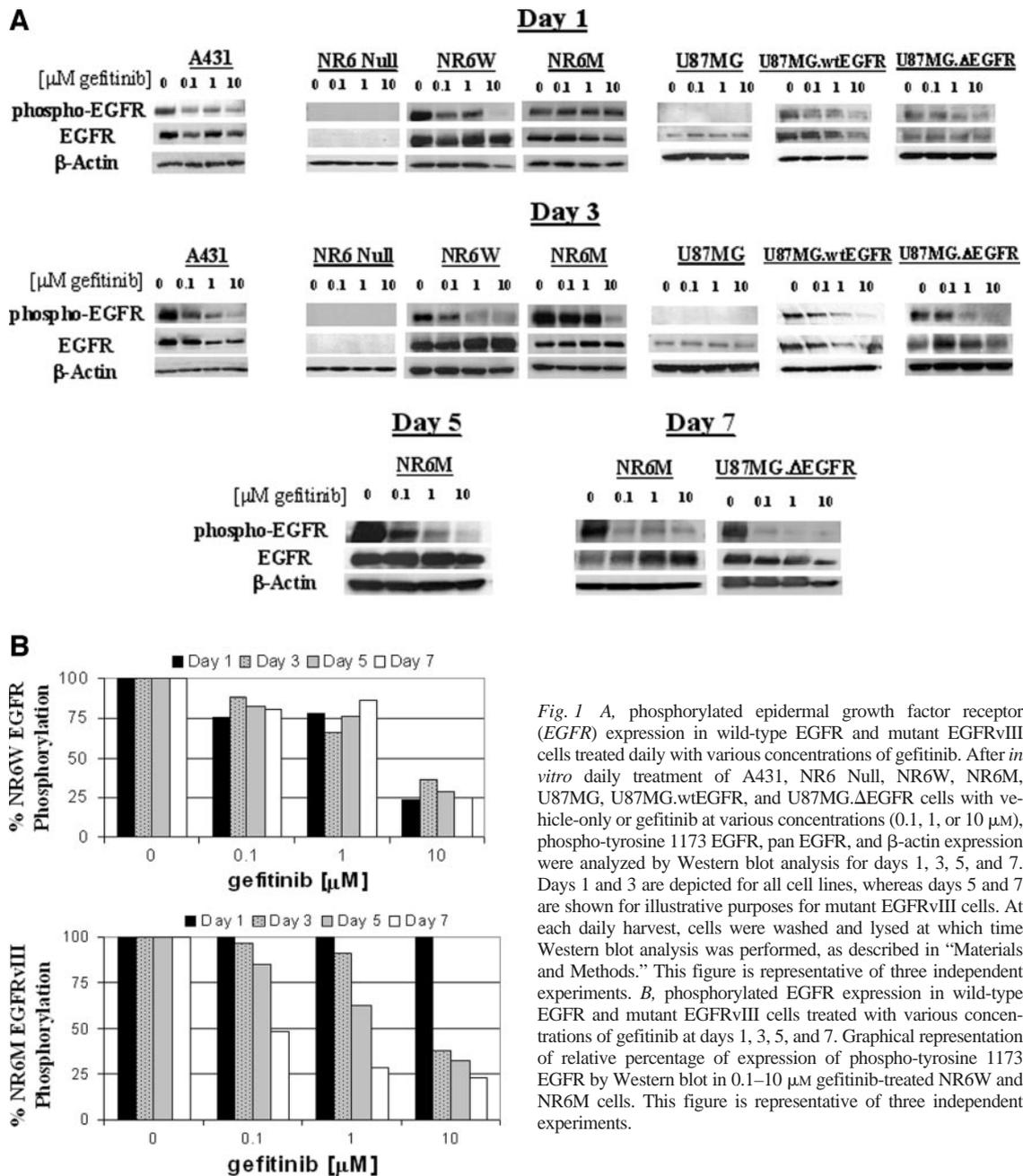


Fig. 1 A, phosphorylated epidermal growth factor receptor (EGFR) expression in wild-type EGFR and mutant EGFRvIII cells treated daily with various concentrations of gefitinib. After *in vitro* daily treatment of A431, NR6 Null, NR6W, NR6M, U87MG, U87MG.wtEGFR, and U87MG.ΔEGFR cells with vehicle-only or gefitinib at various concentrations (0.1, 1, or 10 μ M), phospho-tyrosine 1173 EGFR, pan EGFR, and β -actin expression were analyzed by Western blot analysis for days 1, 3, 5, and 7. Days 1 and 3 are depicted for all cell lines, whereas days 5 and 7 are shown for illustrative purposes for mutant EGFRvIII cells. At each daily harvest, cells were washed and lysed at which time Western blot analysis was performed, as described in "Materials and Methods." This figure is representative of three independent experiments. B, phosphorylated EGFR expression in wild-type EGFR and mutant EGFRvIII cells treated with various concentrations of gefitinib at days 1, 3, 5, and 7. Graphical representation of relative percentage of expression of phospho-tyrosine 1173 EGFR by Western blot in 0.1–10 μ M gefitinib-treated NR6W and NR6M cells. This figure is representative of three independent experiments.

tively phosphorylated in both NR6W and NR6M cells (data not shown).

Growth of Cells Expressing Wild-Type EGFR Are Inhibited in a Dose-Dependent Manner by Gefitinib, but Cells Expressing EGFRvIII Are Resistant to Inhibition. On the basis of the observation that phosphorylation of Akt was differentially affected in cells expressing wild-type EGFR *versus* mutant EGFRvIII after treatment with gefitinib, we investigated the growth of these cells under the same conditions. Growth of NR6 Null cells was unaffected at all of the concentrations of gefitinib tested (Fig. 3). NR6W cells demonstrated a dose-dependent reduction in growth (Fig. 3) and viability (data not

shown) after treatment with gefitinib. In contrast, NR6M cells displayed an enhanced relative growth rate (Fig. 3) and unaffected viability (data not shown) with increasing gefitinib concentration and length of exposure when compared with cells expressing wild-type EGFR after treatment. These data were recapitulated in the U87MG glioblastoma cells, in which U87MG.ΔEGFR demonstrated a significantly greater growth rate than U87MG.wtEGFR, even at the highest concentrations of gefitinib tested (data not shown). We did not observe any nonspecific cytotoxicity for any of the cell lines tested at the concentration of 10 μ M. However, we have determined gefitinib to be nonspecifically toxic at concentrations of 20 μ M and

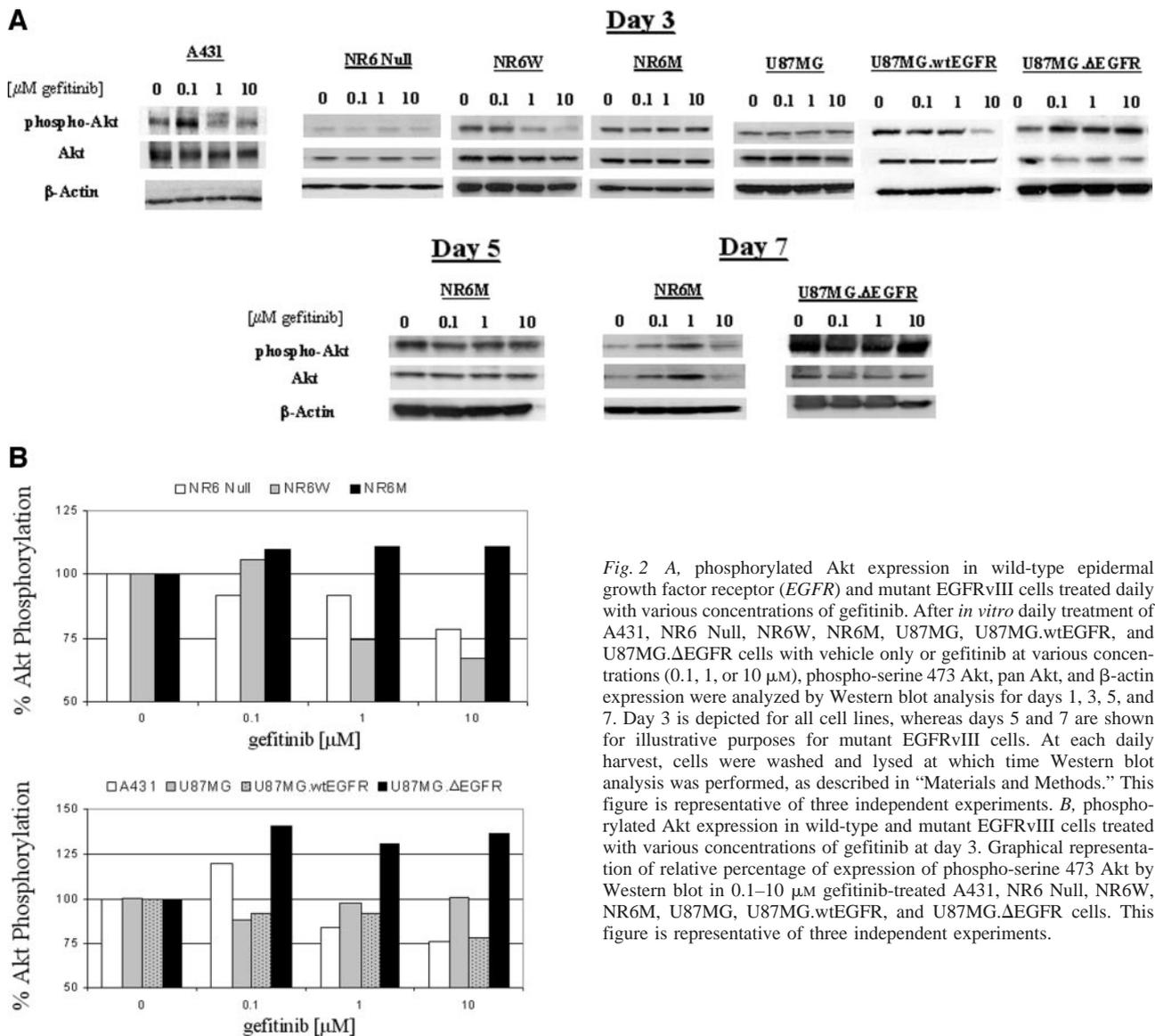


Fig. 2 A, phosphorylated Akt expression in wild-type epidermal growth factor receptor (EGFR) and mutant EGFRvIII cells treated daily with various concentrations of gefitinib. After *in vitro* daily treatment of A431, NR6 Null, NR6W, NR6M, U87MG, U87MG.wtEGFR, and U87MG. Δ EGFR cells with vehicle only or gefitinib at various concentrations (0.1, 1, or 10 μ M), phospho-serine 473 Akt, pan Akt, and β -actin expression were analyzed by Western blot analysis for days 1, 3, 5, and 7. Day 3 is depicted for all cell lines, whereas days 5 and 7 are shown for illustrative purposes for mutant EGFRvIII cells. At each daily harvest, cells were washed and lysed at which time Western blot analysis was performed, as described in “Materials and Methods.” This figure is representative of three independent experiments. B, phosphorylated Akt expression in wild-type and mutant EGFRvIII cells treated with various concentrations of gefitinib at day 3. Graphical representation of relative percentage of expression of phospho-serine 473 Akt by Western blot in 0.1–10 μ M gefitinib-treated A431, NR6 Null, NR6W, NR6M, U87MG, U87MG.wtEGFR, and U87MG. Δ EGFR cells. This figure is representative of three independent experiments.

higher *in vitro* for all of the cell lines tested here (data not shown).

To additionally investigate the effect that gefitinib has on cell cycle progression after treatment, propidium iodide staining and flow cytometric cell cycle analysis were performed. As described above, wild-type and mutant EGFRvIII-expressing cells were mock-treated or treated with 0.1, 1, or 10 μ M gefitinib for 3 days. At the end of 3 days, cells were harvested, treated with RNase A, stained with propidium iodide, and analyzed by flow cytometry. G₀/G₁ gates were set to characterize whole cells with normal DNA content, *i.e.*, indicative of cells in the resting/nondividing state, whereas S + G₂-M gates were set to characterize whole cells undergoing DNA synthesis or having twice the baseline DNA content, *i.e.*, indicative of cells in the mitotic state. Treatment with gefitinib had no effect on NR6 Null at any concentration tested (Fig. 4). However, gefitinib reduced DNA synthesis and cell cycle progression of wild-type EGFR-

expressing cells in a dose-dependent manner, such that there were statistically significant increases in the resting G₀/G₁ phases and decreases in the mitotic S + G₂-M phases of A431, U87MG.wtEGFR (data not shown), and NR6W cells (Fig. 4) treated with increasing concentrations of gefitinib. There were statistically significant increases in G₀/G₁ for 1 μ M and 10 μ M gefitinib-treated NR6W cells compared with mock-treated NR6W cells ($P = 0.036$ and $P = 0.032$, respectively), whereas there were statistically significant decreases in S + G₂-M for 1 μ M and 10 μ M gefitinib-treated NR6W cells compared with mock-treated NR6W cells ($P = 0.045$ and $P = 0.040$, respectively). Collectively, these data are consistent with the observation that a dose-dependent growth reduction was observed for wild-type EGFR-expressing cells, as determined by cell counting (Fig. 3).

In contrast, gefitinib was not effective in reducing DNA synthesis in mutant EGFRvIII-expressing NR6M (Fig. 4) and

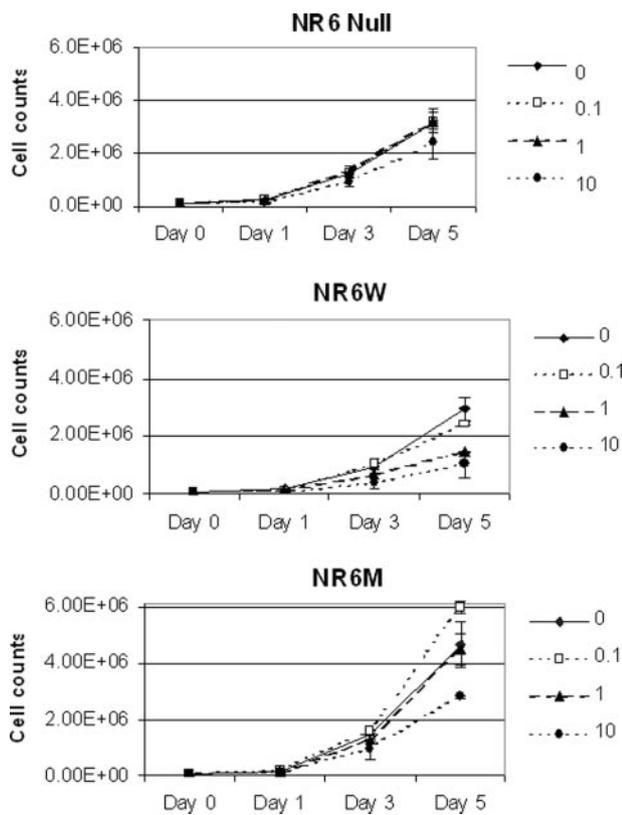


Fig. 3 Growth of cells expressing no epidermal growth factor receptor (EGFR), wild-type EGFR, or mutant EGFRvIII when treated daily with gefitinib at various concentrations. Graphical representation of cell counting as determined by hemacytometry for NR6 Null, NR6W, and NR6M when treated with 0.1–10 μM gefitinib for 5 days. This figure is representative of three independent experiments; bars, \pm SE.

U87MG. Δ EGFR cells (data not shown). Decreases in DNA synthesis for gefitinib-treated NR6M cells compared with mock-treated cells did not reach statistical significance ($P > 0.05$) at any concentration tested. These findings support the observation that mutant EGFRvIII-expressing cells survived treatment with gefitinib better (*i.e.*, higher cell counts and higher viability) than cells expressing wild-type EGFR (Fig. 3).

Invasion by Wild-Type EGFR-Expressing Cells Is Inhibited by Gefitinib, Whereas Invasion by Mutant EGFRvIII-Expressing Cells Is Resistant to Inhibition. We have shown previously that expression of mutant EGFRvIII is correlated with the up-regulation of genes and molecular effectors intrinsically involved in tumor invasion (43), as determined by quantitation of specific mRNA transcripts. In addition, we have demonstrated that mutant EGFRvIII confers an invasive phenotype when expressed in different cellular backgrounds (3, 12). To assess if the invasive effects of mutant EGFRvIII were inhibited by gefitinib, we determined how treatment with gefitinib affected invasion of wild-type EGFR and mutant EGFRvIII cells *in vitro*. NR6 Null, NR6W, and NR6M cells were mock-treated (DMSO only) or treated with gefitinib at 1 or 10 μM (in equivalent volumes of DMSO) for an initial 24 h and were then placed into control (migration) or Matrigel invasion

assay chambers with fresh medium and drug for an additional 24 h, for a total culture time of 48 h. In comparison to mock-treated cells, invasion of NR6W cells was reduced by 48% and 69% after treatment with 1 μM and 10 μM gefitinib, respectively (Fig. 5). In contrast, invasion by NR6M was reduced by 33% and 40% after treatment with 1 μM and 10 μM gefitinib, respectively (Fig. 5). Thus, it appears that unlike wild-type EGFR, invasive characteristics conferred by mutant EGFRvIII are more resistant to inhibition by gefitinib, even with increasing drug concentration (Fig. 5). We have obtained similar results that demonstrate there is a dose-dependent inhibition of invasion by A431, U87MG, and U87MG.wtEGFR cells, whereas U87MG. Δ EGFR cells are resistant to this inhibitor (data not shown). These data are consistent with previous observations we have made, as well as those made by others that mutant EGFRvIII expression strongly correlates with enhanced growth rate and invasion (43, 44), which help contribute to its resistance to inhibition even at the maximally tolerated dose *in vitro* as well as *in vivo* (26).

DISCUSSION

The data presented here provide evidence that gefitinib, an EGFR TK inhibitor, effectively inhibits cell cycle progression, growth, and invasion of cells expressing wild-type EGFR but not mutant EGFRvIII *in vitro*. We have shown previously that whereas gefitinib is efficacious in the treatment of s.c. and intracranial xenograft tumors expressing wild-type EGFR *in vivo* in mice, it failed to inhibit the growth of xenograft tumors expressing mutant EGFRvIII (26). These new findings may in part provide a potential mechanism of therapeutic resistance for this promising drug, and for the other EGFR TK inhibitors currently in clinical development. By their nature, mutant receptors as a whole may be more resistant to inhibition with EGFR TK inhibitors, due to their altered structure and any corresponding modifications to the active site upon which the drug works.

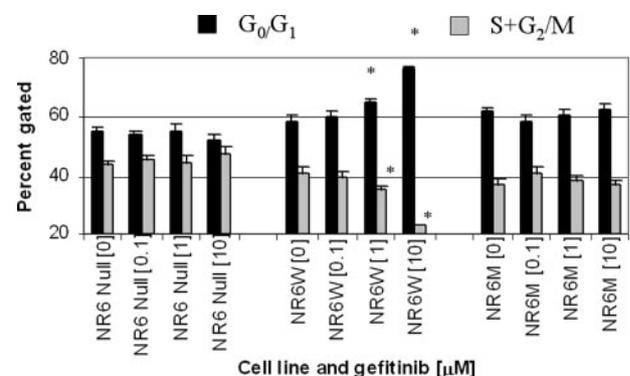


Fig. 4 A, cell cycle analysis of S + G₂/M phases for cells expressing no epidermal growth factor receptor (EGFR), wild-type EGFR, or mutant EGFRvIII when treated daily with gefitinib at various concentrations. Propidium iodide staining assay for *in vitro* mock-treated and 0.1, 1, and 10 μM gefitinib-treated NR6 Null, NR6W, and NR6M cells, as described in “Materials and Methods.” $P = 0.045$ for NR6W (0) versus NR6W (1); $P = 0.04$ for NR6W (0) versus NR6W (10); $P = 0.076$ for NR6M (0) versus NR6M (10). This figure is representative of three independent experiments; bars, \pm SE.

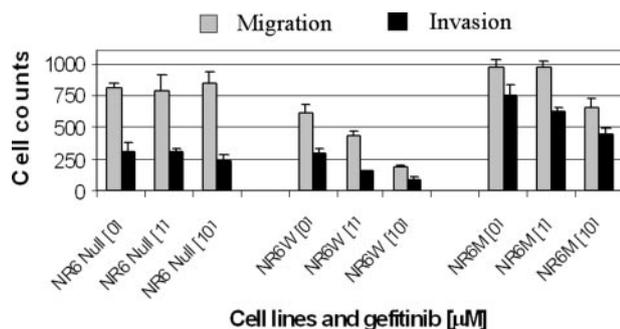


Fig. 5 Cell counts for migration and invasion of cells expressing no epidermal growth factor receptor (EGFR), wild-type EGFR, or mutant EGFRvIII when treated daily with gefitinib at various concentrations. Graphical representation of raw cell counts for NR6 Null, NR6W, and NR6M cells treated with 0.1–10 μM gefitinib for 48 h. This figure is representative of three independent experiments; bars, \pm SE.

The *in vitro* experiments described herein demonstrate that treatment with gefitinib at commonly used, noncytotoxic doses was not effective in inhibiting neoplastic functions ascribed to the EGFRvIII mutation, including downstream signaling by Akt, DNA synthesis, and cellular invasion. We have shown that short-term treatment with gefitinib did not significantly reduce autophosphorylation of a key EGFR regulatory residue, tyrosine 1173 (18, 19), involved in signal transduction in mutant EGFRvIII-expressing cells, whereas phosphorylation of this same residue in wild-type EGFR-expressing cells was substantially decreased in a dose-dependent manner. Yet, whereas gefitinib potentially inhibited autophosphorylation in wild-type EGFR and mutant EGFRvIII-expressing cells with daily treatment for ≥ 3 days, it still failed to inhibit the neoplastic phenotype conferred with mutant EGFRvIII expression. These data are consistent with the idea that despite significantly reduced or ablated receptor autophosphorylation, the neoplastic phenotype of EGFRvIII does not appear as sensitive to gefitinib as EGFR in the short-term, and may require repeated dosing and longer exposure to completely inhibit receptor phosphorylation. However, continued and significant inhibition of EGFR phosphorylation after prolonged exposure to gefitinib does not correlate with meaningful reductions in DNA synthesis, growth, or invasion promoted by mutant EGFRvIII. Nevertheless, it should be noted that given the present levels of understanding of the complexities of signaling mechanisms, the relevance of these studies to the clinical efficacy of the drug has not yet been determined.

Whereas phosphorylation of EGFRvIII can be significantly inhibited through increased drug concentration and repeated dosing, nascent DNA synthesis in mutant EGFRvIII-expressing cells does not appear to be affected. Furthermore, Cavenee *et al.* have reported that phosphorylation of EGFR tyrosine 1173 in mutant EGFRvIII requires as little as 10% the level of phosphorylation required to promote the same cellular processes and effects that occur in fully phosphorylated wild-type EGFR receptors (18, 19). On the basis of this, as well as some of our previous studies (26), we believe that mutant EGFRvIII receptors are more resistant to EGFR TK inhibitor therapy, due to their requirement for higher concentrations of inhibitor (often

beyond the maximally tolerated dose) and repeated dosing, to decrease or shut off promitotic *cis*-signaling from the receptor.

Our data demonstrate that inhibition of receptor phosphorylation of mutant EGFRvIII may not be the only issue influencing mutant EGFRvIII-promoted growth and resistance. We have shown that despite significantly decreased or ablated phosphorylation of EGFRvIII, there remains undiminished signaling through Akt and possibly other promitotic signaling moieties. Phosphorylation of Akt, a promitotic kinase involved in signal transduction through the phosphatidylinositol-3'kinase signaling pathway (21, 36, 40) as well as increased cellular invasion (37), was down-regulated in a dose-dependent manner in cells expressing wild-type EGFR, including U87MG.wtEGFR. We find this observation of particular interest because the U87MG parental cell line is known to have mutated and dysfunctional PTEN (45), suggesting that gefitinib may inhibit wild-type EGFR-induced signaling through Akt via PTEN-independent mechanisms. Nevertheless, phosphorylated Akt expression was unaffected always and concentrations of gefitinib tested in cells expressing mutant EGFRvIII. Consistent with the observations that gefitinib did not decrease phosphorylation of Akt in cells expressing mutant EGFRvIII, daily treatment with gefitinib did not inhibit DNA synthesis or growth of mutant EGFRvIII-expressing cells. We find these results particularly noteworthy because we have used a 'pure' model system with NR6, in which NR6W only expresses wild-type EGFR and the fully tumorigenic NR6M only expresses mutant EGFRvIII, which is required for its neoplastic phenotype (12). Thus, the only difference between cell types should be the receptor and its corresponding influence on intracellular signaling, *i.e.*, phosphorylated Akt in these studies. Further to this point, Rosen *et al.* have demonstrated in a panel of human breast cancer and other epithelial tumor cell lines that growth inhibition was associated with dephosphorylation of EGFR and down-regulation of Akt activity (40). In consideration of this, our findings would lend support to the idea that a fundamental difference between wild-type EGFR sensitivity to gefitinib and mutant EGFRvIII resistance is due to the differential regulation and activity of Akt, and possibly other functionally redundant promitotic signaling pathways, in cells expressing mutant EGFRvIII. Alternatively, mutant EGFRvIII-mediated resistance could be due to a potential *trans*-phosphorylation or *trans*-signaling event by other signaling pathways involving Akt. To this end, others have reported that the Akt-dependent antiapoptotic pathway and the mitogen-activated kinase (MAPK) cascade are alternatively activated in human glioblastoma multiforme in response to different stimuli (36, 46).

There are several potential arguments to explain why EGFRvIII responds differently to gefitinib, despite having the same nucleotide and amino acid sequence as EGFR in the COOH-terminal intracellular domain. The simplest explanation rests in the possibility that the deleted extracellular domain of EGFRvIII sterically affects the transmembrane and intracellular domain, thereby structurally altering its intracellular configuration. Precedence for this argument comes from Huang *et al.* (18) and others (47–50) who demonstrated that EGFRvIII does not traffic intracellularly as well as EGFR. They argued that altered intracellular conformations of EGFRvIII did not result in the exposure of receptor sequence motifs required for endocytosis

and lysosomal sorting. Furthermore, Fernandes *et al.* (51) reported that EGFRvIII receptor dimerization is highly dependent on a conformational change induced by *N*-linked glycosylation. Presumably, these structural differences would allow for the binding of different adaptor proteins and the subsequent different pathways to be activated (20, 21, 31, 52, 53). Another possible related explanation is that EGFR and EGFRvIII have different binding affinities for gefitinib secondary to intracellular conformational differences. As a result, gefitinib easily and specifically binds into the ATP binding site to prevent EGFR autophosphorylation. However, because of a decreased affinity for gefitinib, EGFRvIII autophosphorylation proceeds at a low level and only becomes significantly dephosphorylated with increased concentration and prolonged exposure. Finally, it is possible that once constitutive mitogenic EGFRvIII signaling pathways are established in a cell, rather than continuously divided at a pace that results in the elimination of nutrients, the cell adapts to constitutive growth signals. That is, the cell no longer responds to the constant EGFRvIII autophosphorylation. In doing so, mutant kinase activity becomes no longer essential or necessary for tumorigenicity. Using serial analysis of gene expression, we have demonstrated previously that a number of genes are induced simply by the expression of EGFRvIII (43). Specifically, they provide evidence that EGFRvIII up-regulates the molecular effectors of tumor invasion. It is plausible that other neoplastic genes are also up-regulated and function without the need for EGFRvIII phosphorylation.

The most significant finding in our study is that despite significantly inhibited or ablated phosphorylation, the neoplastic phenotype conferred by mutant EGFRvIII is more resistant to treatment with gefitinib than wild-type EGFR. This is consistent with the idea that whereas *cis*-phosphorylation of EGFRvIII is inhibited significantly or ablated by repeated dosing of gefitinib *in vitro*, a *trans*-signaling event in conjunction with Akt may help promote cell cycle progression and the neoplastic phenotype. We conclude that transactivation of certain oncogenes and signaling redundancy may in part contribute to EGFRvIII-induced tumorigenesis, such that therapeutic resistance may not be specific to mutant EGFRvIII alone.

REFERENCES

- Jungbluth AA, Stockert E, Huang HJ, et al. A monoclonal antibody recognizing human cancers with amplification/overexpression of the human epidermal growth factor receptor. *Proc Natl Acad Sci USA* 2003;100:639–44.
- Wikstrand CJ, McLendon RE, Friedman AH, Bigner DD. Cell surface localization and density of the tumor-associated variant of the epidermal growth factor receptor, EGFRvIII. *Cancer Res* 1997;57:4130–40.
- Sampson JH, Crotty LE, Lee S, et al. Unarmed, tumor-specific monoclonal antibody effectively treats brain tumors. *Proc Natl Acad Sci USA* 2000;97:7503–8.
- Wikstrand CJ, Hale LP, Batra SK, et al. Monoclonal antibodies against EGFRvIII are tumor specific and react with breast and lung carcinomas and malignant gliomas. *Cancer Res* 1995;55:3140–8.
- Schlessinger J. Mutational analysis of the epidermal growth factor-receptor kinase. *Biochem Soc Symp* 1990;56:13–9.
- Wong AJ, Croce CM. *Oncogenes and signal transduction*. Hospital Practice. Office Edition. New York: HP Pub. Co. 1993;28:128–32, 36, 41.
- Mendelsohn J, Baselga J. The EGF receptor family as targets for cancer therapy. *Oncogene* 2000;19:6550–65.
- Bigner SH, Humphrey PA, Wong AJ, et al. Characterization of the epidermal growth factor receptor in human glioma cell lines and xenografts. *Cancer Res* 1990;50:8017–22.
- Sugawa N, Ekstrand AJ, James CD, Collins VP. Identical splicing of aberrant epidermal growth factor receptor transcripts from amplified rearranged genes in human glioblastomas. *Proc Natl Acad Sci USA* 1990;87:8602–6.
- Chu CT, Everiss KD, Wikstrand CJ, et al. Receptor dimerization is not a factor in the signalling activity of a transforming variant epidermal growth factor receptor (EGFRvIII). *Biochem J* 1997;324:855–61.
- Nishikawa R, Ji XD, Harmon RC, et al. A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. *Proc Natl Acad Sci USA* 1994;91:7727–31.
- Batra SK, Castelino-Prabhu S, Wikstrand CJ, et al. Epidermal growth factor ligand-independent, unregulated, cell-transforming potential of a naturally occurring human mutant EGFRvIII gene. *Cell Growth Differ* 1995;6:1251–9.
- Wikstrand C, Stenzel TT, McLendon RE, Bigner DD. Correlation of epidermal growth factor receptor wild type (EGFRWT) and variant (EGFRvIII) RNA transcript levels and glioma biopsy protein expression by immunohistochemistry (IHX). *Neuro-oncol* 2002;4:S102.
- Frederick L, Wang X-Y, Eley G, James CD. Diversity and frequency of epidermal growth factor receptor mutations in human glioblastomas. *Cancer Res* 2000;60:1883–7.
- Moscatello DK, Holgado-Madruga M, Godwin AK, et al. Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. *Cancer Res* 1995;55:5536–9.
- Garcia de Palazzo IE, Adams GP, Sundareshan P, et al. Expression of mutated epidermal growth factor receptor by non-small cell lung carcinomas. *Cancer Res* 1993;53:3217–20.
- Reist CJ, Archer GE, Kurpad SN, et al. Tumor-specific anti-epidermal growth factor receptor variant III monoclonal antibodies: use of the tyramine-cellobiose radioiodination method enhances cellular retention and uptake in tumor xenografts. *Cancer Res* 1995;55:4375–82.
- Huang HS, Nagane M, Klingbeil CK, et al. The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. *J Biol Chem* 1997;272:2927–35.
- Prigent SA, Nagane M, Lin H, et al. Enhanced tumorigenic behavior of glioblastoma cells expressing a truncated epidermal growth factor receptor is mediated through the Ras-Shc-Grb2 pathway. *J Biol Chem* 1996;271:25639–45.
- Moscatello DK, Montgomery RB, Sundareshan P, et al. Transformational and altered signal transduction by a naturally occurring mutant EGF receptor. *Oncogene* 1996;13:85–96.
- Moscatello DK, Holgado-Madruga M, Emler DR, Montgomery RB, Wong AJ. Constitutive activation of phosphatidylinositol 3-kinase by a naturally occurring mutant epidermal growth factor receptor. *J Biol Chem* 1998;273:200–6.
- Sirotnak FM, Zakowski MF, Miller VA, Scher HI, Kris MG. Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), and inhibitor of EGFR tyrosine kinase. *Clin Cancer Res* 2000;6:4885–92.
- Ciardello F, Caputo R, Bianco R, et al. Tortora inhibition of growth factor production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. *Clin Cancer Res* 2001;7:1459–65.
- Lichtner RB, Menrad A, Sommer A, Klar U, Schneider MR. Signaling-inactive epidermal growth factor receptor/ligand complexes in intact carcinoma cells by quinazoline tyrosine kinase inhibitors. *Clin Cancer Res* 2001;6:5790–5.
- Baselga J, Averbuch SD. ZD1839 (“Iressa”) as an anticancer agent. *Drugs* 2000;60:33–40.

26. Heimberger AB, Learn CA, Archer GE, et al. Brain tumors in mice are susceptible to blockade of epidermal growth factor receptor (EGFR) with the oral, specific, EGFR-tyrosine kinase inhibitor ZD1839 (Iressa). *Clin Cancer Res* 2002;8:3496–502.
27. Villano JL, Mauer AM, Vokes EE. A case study documenting the anticancer activity of ZD1839 (Iressa) in the brain. *Ann Oncol* 2003;14:656–8.
28. Culy CR, Faulds D. Gefitinib. *Drugs* 2002;62:2237–48.
29. Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized Phase II trial of gefitinib for previously treated patients with advanced non-small cell lung cancer. *J Clin Oncol* 2003;21:2237–46.
30. Cappuzzo F, Gregorc V, Rossi E, et al. Gefitinib in pretreated non-small cell lung cancer (NSCLC): analysis of efficacy and correlation with HER2 and epidermal growth factor receptor expression in locally advanced or metastatic NSCLC. *J Clin Oncol* 2003;21:2658–63.
31. Narita Y, Nagane M, Mishima K, et al. Mutant epidermal growth factor receptor signaling down-regulates p27 through activation of the phosphatidylinositol 3-kinase/Akt pathway in glioblastomas. *Cancer Res* 2002;62:6764–9.
32. Clarke K, Smith S, Gullick WJ, Harris AL. Mutant epidermal growth factor receptor enhances induction of vascular endothelial growth factor by hypoxia and insulin-like growth factor-1 via a PI3 kinase dependent pathway. *Br J Cancer* 2001;84:1322–9.
33. Boerner JL, Danielsen A, McManus MJ, Mairle NJ. Activation of Rho is required for ligand-independent oncogenic signaling by a mutant epidermal growth factor receptor. *J Biol Chem* 2001;276:3691–5.
34. Keilhack H, Tenev T, Nyakatura E, et al. Phosphotyrosine 1173 mediates binding of the protein-tyrosine phosphatase SHP-1 to the epidermal growth factor receptor and attenuation of receptor signaling. *J Biol Chem* 1998;273:24839–46.
35. Shah BH, Soh J-W, Catt KJ. Dependence of gonadotropin-releasing hormone-induced neuronal MAPK signaling on epidermal growth factor receptor transactivation. *J Biol Chem* 2003;278:2866–75.
36. Schlegel J, Piontek G, Budde B, Neff F, Kraus A. The Akt/protein kinase B-dependent anti-apoptotic pathway and the mitogen-activated protein kinase cascade are alternatively activated in human glioblastoma multiforme. *Cancer Lett* 2000;158:103–8.
37. Arboleda MJ, Lyons JF, Kabbinavar FF, et al. Overexpression of AKT2/protein kinase B β leads to up-regulation of β 1 integrins, increased invasion, and metastasis of human breast and ovarian cancer cells. *Cancer Res* 2003;63:196–206.
38. Li J, Yen C, Liaw D, et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science (Wash DC)* 1997;275:1943–7.
39. Steck PA, Pershouse MA, Jasser SA, et al. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet* 1997;15:356–62.
40. Moasser M, Basso A, Averbuch SD, Rosen N. The tyrosine kinase inhibitor ZD1839 (“Iressa”) inhibits HER-2 driven signaling and suppresses the growth of HER-2 overexpressing tumor cells. *Cancer Res* 2001;61:7184–8.
41. Magne N, Fischel J, Tiffon C, et al. Molecular mechanisms underlying the interaction between ZD1839 (“Iressa”) and cisplatin/5-fluorouracil. *Br J Cancer* 2003;89:585–92.
42. Ishii N, Maier D, Merlo A, et al. Frequent co-alterations of TP53, p16/CDK2NA, p14ARF, PTEN tumor suppressor genes in human glioma cell lines. *Brain Pathol* 1999;9:469–79.
43. Lal A, Glazer CA, Martinson HM, et al. Mutant epidermal growth factor receptor up-regulates molecular effectors of tumor invasion. *Cancer Res* 2002;62:3335–9.
44. Feldkamp MM, Lala P, Lau N, Roncari L, Guha A. Expression of activated epidermal growth factor receptors, Ras-guanosine triphosphate, and mitogen-activated protein kinase in human glioblastoma multiforme specimens. *Neurosurgery (Baltim)* 1999;45:1442–53.
45. Furnari FB, Lin H, Huang HS, Cavenee WK. Growth suppression of glioma cells by PTEN requires a functional phosphatase catalytic domain. *Proc Natl Acad Sci USA* 1997;94:12479–84.
46. Jo M, Thomas KS, O'Donnell DM, Gonias SL. Epidermal growth factor receptor-dependent and -independent cell-signaling pathways originating from the urokinase receptor. *J Biol Chem* 2003;278:1642–6.
47. Schlessinger J. Ligand-induced, receptor-mediated dimerization and activation of EGF receptor. *Cell* 2002;110:669–72.
48. Waterman H, Katz M, Rubin C, et al. A mutant EGF-receptor defective in ubiquitylation and endocytosis unveils a role for Grb-2 in negative signaling. *EMBO J* 2002;21:303–13.
49. Stang E, Johannessen LE, Knardal SL, Madhus IH. Polyubiquitination of the epidermal growth factor receptor occurs at the plasma membrane upon ligand-induced activation. *J Biol Chem* 2000;275:13940–7.
50. Haugh JM, Huang AC, Wiley HS, Wells A, Lauffenburger DA. Internalized epidermal growth factor receptors participate in the activation of p21 (ras) in fibroblasts. *J Biol Chem* 1999;274:34350–60.
51. Fernandes H, Cohen S, Bishayee S. Glycosylation-induced conformational modification positively regulates receptor-receptor association: a study with an aberrant epidermal growth factor receptor (EGFRvIII/ δ EGFR) expressed in cancer cells. *J Biol Chem* 2001;276:5375–83.
52. Antonyak MA, Kenyon LC, Godwin AK, et al. Elevated JNK activation contributes to the pathogenesis of human brain tumors. *Oncogene* 2002;21:5038–46.
53. Antonyak MA, Moscatello DK, Wong AJ. Constitutive activation of c-Jun N-terminal kinase by a mutant epidermal growth factor receptor. *J Biol Chem* 1998;273:2817–22.

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