

## Differential Effects of Gefitinib and Cetuximab on Non-small-cell Lung Cancers Bearing Epidermal Growth Factor Receptor Mutations

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**Background:** Many patients with non-small-cell lung cancer (NSCLC) who achieve radiographic responses to treatment with the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors gefitinib and erlotinib have somatic mutations in the EGFR tyrosine kinase domain. However, little is known about the efficacy of cetuximab, an antibody against the EGFR extracellular domain, in EGFR mutant NSCLC. **Methods:** NSCLC cell lines carrying wild-type EGFR (A549, H441, and H1666) or mutant EGFR (H3255, DFCILU-011, PC-9, and HCC827) were treated with various dilutions of gefitinib or cetuximab relative to maximal achievable serum concentration. Cell growth was analyzed by the MTS assay, with differences between dose-response curves analyzed non-parametrically. Apoptosis was analyzed by propidium iodide staining and immunoblotting for PARP. Phosphorylation of EGFR and the downstream signaling components ERK1/2 and Akt were analyzed by immunoblotting. Statistical tests were two-sided. **Results:** Growth of NSCLC lines with wild-type EGFR was slightly (A549 and H441) or moderately (H1666) inhibited by gefitinib and cetuximab, and the effects of the two agents were similar. Both agents also induced no (H441) or moderate (H1666) apoptosis in NSCLC cells with wild-type EGFR. By contrast, gefitinib was statistically significantly more effective than cetuximab at inhibiting growth of EGFR mutant cells (H3255:  $P = .003$ , DFCILU-011:  $P = .011$ , and PC-9:  $P = .003$ ), and gefitinib-treated EGFR mutant cells had higher levels of apoptosis than cetuximab-treated cells (mean fold increase in apoptosis by 1  $\mu\text{M}$  of gefitinib and 10  $\mu\text{g}/\text{mL}$  of cetuximab relative to control, H3255: 8.3 [95% confidence interval {CI} = 4.8 to 11.8] and 2.1 [95% CI = 2.0 to 2.2], respectively,  $P = .025$ ; DFCILU-011: 5.7 [95% CI = 5.1 to 6.3] and 0.9 [95% CI = 0.3 to 1.5], respectively,  $P < .001$ ). Gefitinib treatment decreased EGFR, ERK1/2, and Akt phosphorylation in EGFR mutant cell lines whereas cetuximab had relatively little effect. Both gefitinib and cetuximab inhibited the growth of HCC827 cells, but gefitinib inhibited growth to a greater extent ( $P = .003$ ). **Conclusions:** EGFR mutations in NSCLC cells are associated with sensitivity to gefitinib but not to cetuximab. [J Natl Cancer Inst 2005;97:1185-94]

Epidermal growth factor receptor (EGFR), a member of the ErbB family of receptor tyrosine kinases, is frequently overex-

pressed in non-small-cell lung cancer (NSCLC) and has been implicated in the pathogenesis of the disease (1). EGFR inhibitors have been studied extensively as therapies for patients with relapsed NSCLC (2-4). Two main strategies for therapeutic targeting of EGFR have been developed: small-molecule inhibitors of the tyrosine kinase domain, such as gefitinib and erlotinib, and monoclonal antibodies, such as cetuximab, that are directed against the extracellular domain of EGFR and that inhibit phosphorylation and activation and stimulate internalization. Both gefitinib and erlotinib are approved by the U.S. Food and Drug Administration for the treatment of patients with relapsed NSCLC, and cetuximab has been approved for the treatment of metastatic colorectal carcinomas that express EGFR.

We and others have recently found that approximately 85% of NSCLC patients who show radiographic responses to treatment with gefitinib or erlotinib have somatic mutations in the EGFR gene (5-9). These mutations found to date are located in the first four exons that encode the kinase domain (i.e., exons 18-21) and include small overlapping deletions, insertions, and missense mutations. The most common mutations, which account for approximately 85% of the mutations described to date, include deletions in exon 19 and the L858R missense mutation in exon 21 (5-7,9-12). Autophosphorylation of both of these mutant EGFR proteins is inhibited at concentrations of gefitinib that are 10- to 100-fold lower than those that are necessary to inhibit

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wild-type EGFR (5,13). In addition, NSCLC cells with mutant but not wild-type EGFR undergo apoptosis following gefitinib treatment (5,13).

Little is known about the effects of cetuximab on NSCLC and the efficacy of cetuximab in EGFR mutant and wild-type NSCLC cell lines or tumors. To determine whether cetuximab, like gefitinib and erlotinib, is more effective in cells that harbor mutant EGFR than in cells with wild-type EGFR, we examined the effects of gefitinib and cetuximab in NSCLC cell lines with and without EGFR mutations, in NIH-3T3 cells stably expressing wild-type or mutant EGFR, and in NSCLC patients whose tumors carried EGFR mutations and who were treated with both agents in sequence.

## MATERIALS AND METHODS

### Cell Culture and Reagents

Seven NSCLC cell lines—one bronchioloalveolar cancer line (H1666) and six adenocarcinoma lines (H441, A549, H3255, DFCILU-011, PC-9, and HCC827)—were used in this study. H441, H1666, and A549, which all contain wild-type EGFR, were purchased from the American Type Culture Collection (Manassas, VA). H3255 was provided by the National Cancer Institute and has been previously characterized (5,13,14); it is heterozygous for the L858R missense mutation in exon 21 of the EGFR gene. HCC827 was kindly provided by Dr. Adi Gazdar and has been previously characterized (15); it is heterozygous for the E746\_A750 mutation, which is a deletion in exon 19 of the EGFR gene. PC-9 cells were kindly provided by Dr. Kazuto Nishio and have been previously characterized (16,17). These cells are heterozygous for the E746\_A750 mutation (K. Nishio, personal communication). DFCILU-011 cells were isolated from a male nonsmoking NSCLC patient at Dana-Farber Cancer Institute who responded clinically to gefitinib; these cells are heterozygous for the EGFR exon 19 deletion L747\_E749 (5). DFCILU-011 cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gemini-Bio-Products, Inc., Woodland, CA), 100 U/mL penicillin, 100 U/mL streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate. H1666 and H3255 cells were maintained in ACL-4 media (Life Technologies, Inc., Rockville, MD) supplemented with 5% FBS, 100 U/mL penicillin, 100 U/mL streptomycin, and 2 mM glutamine. H441, A549, HCC827 and PC-9 cells were maintained in RPMI 1640 medium (Cellgro; Mediatech, Inc., Herndon, VA) supplemented with 10% FBS, 100 U/mL penicillin, 100 U/mL streptomycin, and 2 mM glutamine. All cells were grown at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub> and were in the logarithmic growth phase at the initiation of the experiments.

Gefitinib was a gift from AstraZeneca. Stock solutions of 10 mM were prepared in dimethyl sulfoxide and stored at -20 °C. Cetuximab (2 mg/mL; ImClone Systems Inc., Branchburg, NJ) was purchased from the pharmacy at Dana-Farber Cancer Institute and stored at 4 °C. Cetuximab was diluted in 8.48 mg/mL sodium chloride, 1.88 mg/mL sodium phosphate dibasic heptahydrate, and 0.42 mg/mL sodium phosphate monobasic monohydrate. The drugs were diluted in fresh DMEM containing 0.1% FBS before each experiment, and the final dimethyl sulfoxide concentration in all experiments was less than 0.1%.

Antibodies to EGFR (SC-03) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies directed against phosphorylated EGFR (pY1068), total ERK1/2, and phosphorylated ERK1/2 (pT185/pY187) were purchased from Biosource International, Inc. (Camarillo, CA). Antibodies directed against phosphorylated Akt (pS473) and total Akt were purchased from Cell Signaling Technology (Beverly, MA). The  $\beta$ -actin antibody was purchased from Sigma-Aldrich (St. Louis, MO).

### Growth Inhibition Assay

Growth inhibition was assessed by using the MTS assay (Promega, Madison, WI). This assay, a colorimetric method for determining the number of viable cells, is based on the bioreduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) by cells to a formazan product that is soluble in tissue culture medium and can be detected spectrophotometrically. Cells diluted in 180  $\mu$ L/well of maintenance cell culture media (*see* "Cell Culture and Reagents") were plated in 96-well flat-bottom plates (Corning, Inc., Corning, NY). The number of cells for each cell line required to obtain an optical density (OD) of 1.3–2.2 at a wavelength of 490 nm, the linear range of the assay, after 72 hours of growth was determined empirically. The number of cells per well used in these experiments were as follows: H1666, 4000 cells; H441, 6000 cells; A549, 2500 cells; H3255, 8000 cells; DFCILU-011, 10000 cells; HCC827, 8000 cells; PC-9, 6000 cells. Twenty-four hours after plating, cell culture media were replaced with DMEM containing 0.1% FBS with and without gefitinib or cetuximab. Gefitinib was used at concentrations ranging from 3.3 nM to 10 mM and cetuximab at concentrations ranging from 33 ng/mL to 100 mg/mL, similar to amounts used in prior reports (18–20). The cells were incubated for another 72 hours. All experimental points were set up in six to 12 wells, and all experiments were repeated at least three times. The data are expressed as percentage of growth relative to that of untreated control cells. Each data point represents the mean value (percentage) and 95% confidence interval (CI). To make it possible to compare cells treated with gefitinib and cetuximab, the concentrations were expressed relative to the maximum achievable serum concentration (i.e., 1 mM for gefitinib and 100  $\mu$ g/mL for cetuximab) to obtain standardized relative doses and allow for graphical comparisons of the effects of the two drugs. The data were displayed graphically using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). The curves were fitted using a nonlinear regression model with a sigmoidal dose response.

### Western Blotting

Cells were seeded on 3-cm<sup>2</sup> cell culture plates at a density of  $0.6 \times 10^6$  to  $0.9 \times 10^6$  cells/plate and allowed to grow overnight in appropriate maintenance cell culture media for each cell line (*see* "Cell Culture and Reagents") containing 5%–10% FBS. The media were then replaced with DMEM containing 0.1% FBS with or without increasing concentrations of gefitinib (10 nM–10  $\mu$ M) or cetuximab (100 ng/mL–100  $\mu$ g/mL). The cells were incubated for another 24 hours (NSCLC cell lines) or 8 hours (NIH3T3 cells). Cells were washed with ice-cold phosphate-buffered saline (PBS) and scraped immediately after adding 30–75  $\mu$ L of lysis buffer (*see* below). The protein lysates were

collected in 1.5-mL microcentrifuge tubes. For evaluation of levels of phosphorylated EGFR, the lysates were immediately boiled at 100 °C for 7 minutes (this treatment quickly denatures cetuximab and was done whether or not samples were from cells treated with cetuximab). All lysates were centrifuged at 14 000 relative centrifugal force (rcf) for 10 minutes, and supernatants (protein extracts) were collected. The protein extracts were separated by electrophoresis on 7.5% polyacrylamide–sodium dodecyl sulfate (SDS) gels and transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) by electroblotting. The membranes were incubated with 5% nonfat skin milk diluted in TBS-T (10 mM Tris [pH 7.4], 150 mM NaCl, 0.05% Tween 20) for 1 hour to block nonspecific binding and were then incubated with appropriate primary antibodies under the conditions recommended by the manufacturers. The blots were then washed with TBS-T for 30 minutes and incubated with horseradish peroxidase–conjugated secondary antibody (Amersham Biosciences, Buckinghamshire, UK) for 1 hour. Antibody binding was detected using an enhanced chemiluminescence system (New England Nuclear Life Science Products, Inc., Boston, MA).

Previous studies have suggested that cetuximab can induce EGFR autophosphorylation in cells that are lysed with buffers that contain only mild detergent (18,19). This artificial autophosphorylation can be eliminated by using lysis buffers that contain more than 0.1% SDS (18). Therefore, to avoid this artifact, for studies that evaluated the effects of gefitinib or cetuximab on EGFR phosphorylation we used lysis buffer containing 1% SDS (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1% SDS, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride [PMSF], leupeptin at 25 µg/mL, and aprotinin at 25 µg/mL). In parallel, for immunoblot analysis of other proteins, we used NP-40 lysis buffer (20 mM Tris–HCl [pH 8.0], 150 mM NaCl, 10% glycerol, 1% NP-40, 0.42% NaF, 1 mM PMSF, 1 mM sodium orthovanadate, aprotinin at 2 mg/mL, and leupeptin at 5 mg/mL), as used in our previous studies (5,13). Gefitinib inhibited EGFR phosphorylation at similar concentrations, regardless of whether 1% SDS or NP-40 lysis buffer was used (data not shown).

### Apoptosis Analysis

We assessed levels of apoptosis induced by gefitinib or cetuximab using fluorescence-activated cell sorter (FACS) analysis and immunoblotting for PARP. Cells were seeded at densities of  $0.7\text{--}1.0 \times 10^5$  cells/plate in 10-cm<sup>2</sup> plates (Becton Dickinson, Franklin Lakes, NJ) for FACS analysis and  $0.35\text{--}0.5 \times 10^6$  cells/plate in 3-cm<sup>2</sup> plates (Becton Dickinson) for analysis of PARP cleavage. Twenty-four hours after plating, cell culture media were replaced by 15 mL (FACS analysis) or 5 mL (PARP analysis) of DMEM that contained 0.1% FBS with and without gefitinib (1 µM) or cetuximab (10 µg/mL), and the cells were incubated for another 72 hours. The cells were treated with trypsin, harvested, and collected by centrifuging at 14 000 rcf for 5 min. For FACS analysis, the collected cells were fixed overnight in 1 mL 40% ethanol at 4 °C. Fixed cells were then treated with 500 µg/mL RNase A (Sigma) and resuspended in 500 µL of 69 µM propidium iodide (Sigma). FACScan and Cell Quest software (Becton Dickinson) were used to analyze the proportions of subdiploid (i.e., apoptotic) cells (13). For PARP analysis, the collected cells were washed twice with ice-cold PBS and lysed in NP-40 lysis buffer. Subsequent immunoblotting procedures were

performed as described above, and PARP was detected with a PARP antibody (Cell Signaling Technology).

### EGFR Mutant Constructs and Retroviral Infection

The human EGFR gene was cloned into pDNR-Dual (BD Biosciences, San Jose, CA). Two mutants were constructed using the Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The L858R mutation was constructed using the following oligonucleotides: sense 5'-CACAGATTTTGGGCGGGCCAAACTGCTGGG-3' and antisense 5'-CCCAGCAGTTTGGCCCGCCCAAAATCTGTG-3'. The deletion mutation L747\_S752del, P753S was constructed using the following oligonucleotides: sense 5'-CCGTCGCTATCAAGGAATCGAAAGCCAACAAGGAAA-3' and antisense 5'-TTCCTTGTTGGCTTTCGATTCCTTGATAGCGACGG-3'. Both mutations were confirmed by DNA sequencing. As a control for transfection, we used a retroviral construct expressing green fluorescent protein (GFP) that has been previously described (21). All constructs (including the GFP construct) were shuttled into the retroviral vector JP1520 using the BD Creator System (BD Biosciences). NIH-3T3 cells were infected with retrovirus according to standard protocols, as described previously (21,22).

### Patients and EGFR Sequencing

We searched for patients with advanced NSCLC who had been treated with both gefitinib and cetuximab and whose tumors were known to contain an EGFR mutation from the clinical practices of the Dana-Farber Cancer Institute, the Beth Israel Deaconess Medical Center, and the Indiana University Medical Center. We identified four patients who were treated sequentially with both cetuximab and gefitinib, three of whom were treated with cetuximab in a phase II clinical trial (23). In all cases, tumor specimens had been obtained prior to any gefitinib or cetuximab therapy. Tumor tissue was separated from normal tissue by dissection prior to DNA preparation to yield tumor cell populations that were at least 70% pure. DNA was extracted by standard techniques, and exons 18–24 of the EGFR gene were sequenced as previously described (5). The EGFR primers and sequencing techniques have been previously published (5). All patients provided written informed consent for tumor DNA sequencing and were part of an IRB-approved study at the Dana-Farber Cancer Institute.

### Statistical Analysis

Statistical analyses were performed using StatView version 5.01 (SAS Institute, Cary, NC). Paired Student's *t* test was used to determine whether the percentage of apoptosis differed between gefitinib and cetuximab treatments. A *P* value of less than .05 was considered to be statistically significant. All statistical tests were two-sided.

The overall differences between the dose–response curves of cetuximab and gefitinib in each cell line were analyzed nonparametrically. Because of the different dosing units used for cetuximab (µg/mL) and gefitinib (µM), the original doses were expressed as a fraction of their respective maximum concentration in serum (i.e., 100 µg/mL for cetuximab and 1 µM for gefitinib) to obtain standardized relative doses for analysis. In particular, cetuximab doses were divided by 100 µg/mL, whereas gefitinib doses remained the same as the maximum serum

concentration, i.e., 1  $\mu\text{M}$ . At each standardized relative dose, the mean OD of cells treated with each drug was estimated from replicate experiments, typically six, except that 12 replicates were used to determine the mean OD in the absence of drug. The difference between the mean ODs of cetuximab- and gefitinib-treated cells at a given standardized relative dose was analyzed by the Jonckheere–Terpstra test to determine whether the trend has a natural ordering associated with the increasing range of standardized relative doses. Because OD and percent control are linearly related, the analysis is invariant using either outcome. Two-sided  $P$  values were computed by an exact algorithm using StatXact (Cambridge, MA).

## RESULTS

### Effects of Gefitinib and Cetuximab on Growth and Apoptosis of NSCLC Cell Lines

We used the MTS assay to examine the effects of gefitinib and cetuximab on the growth of six NSCLC cell lines in vitro. Three cell lines (A549, H441, and H1666) have wild-type EGFR and have previously been found to be resistant (A549 and H441) or moderately sensitive (H1666) to gefitinib in vitro (5,13). The other three cell lines contain mutant EGFR (L858R in H3255 cells, L747\_E749del in DFCILU-011 cells, and E746\_A750del in PC-9 cells). H3255 and PC-9 cells have previously been shown to be sensitive to gefitinib in vitro (5,13,16). We used a wide range of concentrations of gefitinib (3.3 nM–10  $\mu\text{M}$ ) and cetuximab (100 ng/mL–100  $\mu\text{g/mL}$ ). According to pharmacokinetic data obtained in phase I clinical studies, the mean steady-state plasma concentrations of gefitinib at the FDA-approved dosing (250 mg/day) ranges from 0.4  $\mu\text{M}$  to 1.4  $\mu\text{M}$  (24). On the basis of these findings we chose 1  $\mu\text{M}$  as the definition of the maximum achievable plasma concentration for gefitinib. According to the manufacturer, at the FDA-approved dosing level of cetuximab, the mean steady-state peak and trough concentrations across various studies range from 168 to 235  $\mu\text{g/mL}$  and from 41 to 85  $\mu\text{g/mL}$ , respectively. We thus chose 100  $\mu\text{g/mL}$  as the maximum achievable serum concentration to reflect an intermediate point of these values.

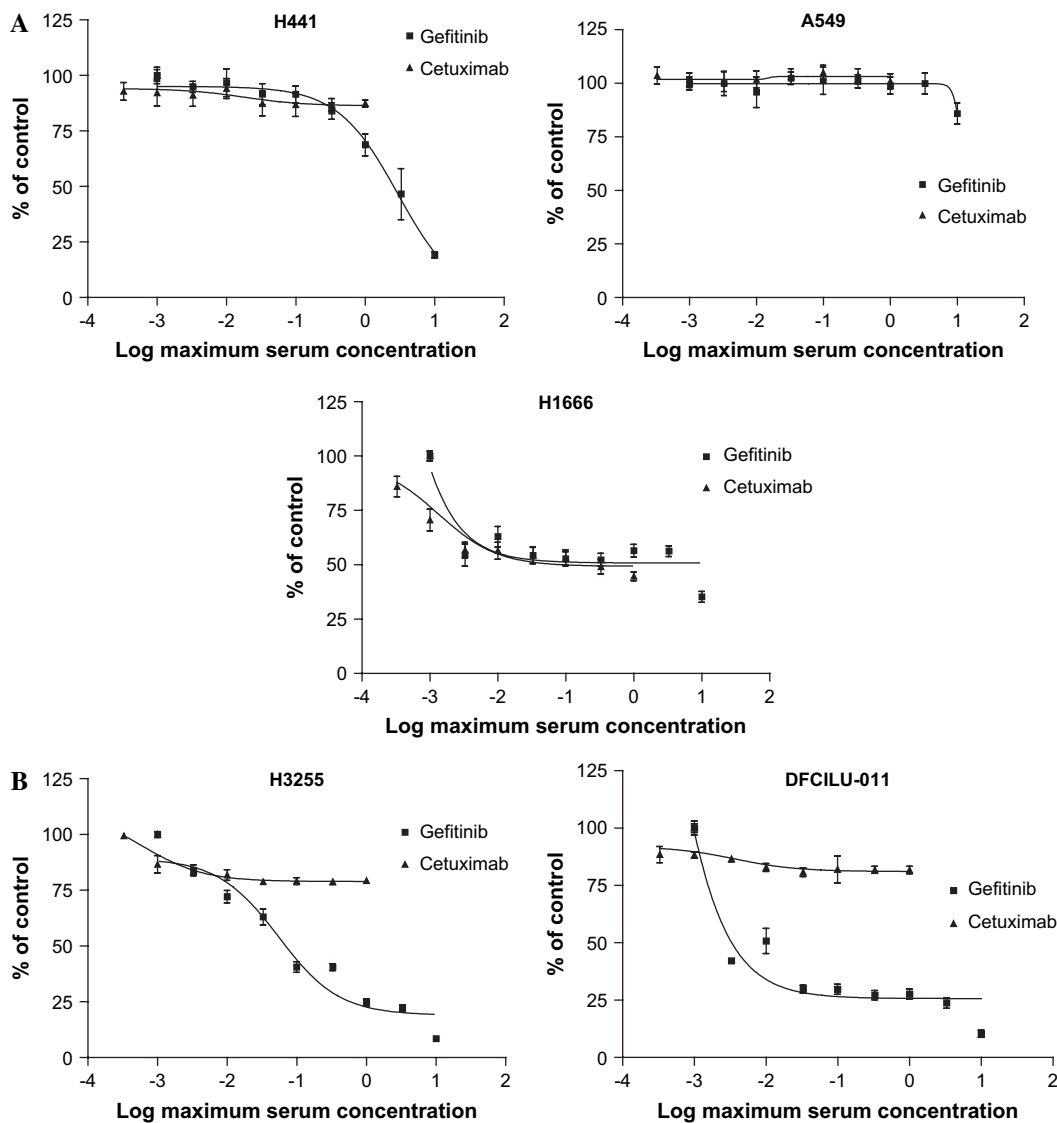
Growth was assayed 72 hours after the cells were exposed to each drug or to medium alone (control). In cell lines with wild-type EGFR (A549, H441, and H1666), the agents had similar effects on cell growth (A549:  $P = .38$ ; H441:  $P = .77$ , H1666:  $P = .56$ ) (Fig. 1, A). In H441 and A549 cells, both gefitinib and cetuximab showed little effect on cell growth except gefitinib at concentrations of at least 1  $\mu\text{M}$  (i.e., 0 on  $x$  axis in Fig. 1), which is equivalent to the maximum serum concentration at the FDA-approved dosing. Thus, we considered H441 and A549 cells to be resistant to both gefitinib and cetuximab. On the other hand, H1666 cells were more sensitive to both gefitinib and cetuximab than H441 or A549 cells. However, there was no statistically significant difference between the effects of gefitinib and cetuximab in H1666 cells ( $P = .56$ ). Although  $\text{IC}_{50}\text{s}$  (i.e., the drug concentrations required for 50% inhibition of growth) for H1666 were 4  $\mu\text{M}$  for gefitinib and 33  $\mu\text{g/mL}$  for cetuximab, we observed substantial growth inhibition at much lower concentrations, and a plateau was reached (Fig. 1, A).

By contrast to the cell lines with wild-type EGFR, each of which displayed similar sensitivity to gefitinib and cetuximab, the cell lines with EGFR mutations were effectively growth

inhibited by gefitinib but not by cetuximab. Gefitinib caused statistically significantly greater growth inhibition than cetuximab in all three cell lines (H3255:  $P = .003$ , DFCILU-011:  $P = .011$ , PC-9:  $P = .003$ ).  $\text{IC}_{50}\text{s}$  for gefitinib in H3255, DFCILU-011 and PC-9 were 63 nM, 10 nM, and 20 nM (Fig. 1, B and data not shown), respectively, and much lower than those in H1666. The  $\text{IC}_{50}\text{s}$  for cetuximab were not reached in the three EGFR mutant cell lines. The  $\text{IC}_{50}\text{s}$  of H3255 for gefitinib and PC-9 are consistent with previous findings (5,13,25). We tested even higher concentration of cetuximab than the limit of 100  $\mu\text{g/mL}$  shown in Fig. 1. Even at concentrations up to 1000  $\mu\text{g/mL}$ , which is approximately 10 times higher than the maximum serum concentration of cetuximab (26), no additional growth inhibition of H3255 cells was observed (data not shown). We chose H441 and H1666 as representative wild type cell lines and H3255 (with the L858R mutation) and DFCILU-011 (with the L747\_E749 mutation) as representative EGFR mutant cell lines for further studies.

To further examine the mechanism by which gefitinib and cetuximab inhibit growth, we treated NSCLC cell lines with 1  $\mu\text{M}$  gefitinib, 10  $\mu\text{g/mL}$  cetuximab, or neither drug and determined the percentage of apoptotic cells by propidium iodide staining. We used these particular concentrations of each drug because they can be achieved in patient serum, because they have been used in earlier studies by us and others, and because we did not observe any additional growth inhibition for cetuximab at higher concentrations in any of the cell lines examined (Fig. 1) (13,26–29). In H441 cells, neither drug had an effect on apoptosis compared with untreated control cells (Fig. 2, A; mean fold increase in apoptosis by 1  $\mu\text{M}$  of gefitinib or 10  $\mu\text{g/mL}$  of cetuximab relative to control = 1.1 [95% CI = 0.8 to 1.4] and 1.0 [95% CI = 0.7 to 1.3], respectively). Both drugs caused similar and statistically significant increases in apoptosis in H1666 cells compared with control treatment (Fig. 2, A; mean fold increase in apoptosis by 1  $\mu\text{M}$  of gefitinib and 10  $\mu\text{g/mL}$  of cetuximab relative to control = 10.4 [95% CI = 8.5 to 12.3] and 12.3 [95% CI = 10.8 to 13.8], respectively; control versus gefitinib,  $P = .002$ ; control versus cetuximab,  $P < .001$ ; gefitinib versus cetuximab,  $P = .196$ ), consistent with their similar degrees of growth inhibition (Fig. 1, A).

In contrast to their similar effects on cells with wild-type EGFR, gefitinib and cetuximab had statistically significantly different effects on apoptosis in EGFR mutant cell lines. For H3255 cells, treatment with gefitinib led to substantially higher levels of apoptosis than treatment with cetuximab, although cetuximab also increased apoptosis relative to control treatment (Fig. 2, A; mean fold increase in apoptosis by 1  $\mu\text{M}$  of gefitinib and 10  $\mu\text{g/mL}$  of cetuximab relative to control = 8.3 [95% CI = 4.8 to 11.8] and 2.1 [95% CI = 2.0 to 2.2], respectively; control versus gefitinib,  $P = .014$ ; control versus cetuximab,  $P = .002$ ; gefitinib versus cetuximab,  $P = .025$ ). For DFCILU-011 cells, treatment with gefitinib also resulted in a statistically significant increase in apoptosis relative to cetuximab treatment, but cetuximab did not increase apoptosis relative to control treatment (Fig. 2, A; mean fold increase in apoptosis by 1  $\mu\text{M}$  of gefitinib and 10  $\mu\text{g/mL}$  of cetuximab relative to control was 5.7 [95% CI = 5.1 to 6.3] and 0.9 [95% CI = 0.3 to 1.5], respectively; control versus gefitinib,  $P < .001$ ; gefitinib versus cetuximab,  $P < .001$ ). Again, these findings are consistent with the differential effects of these drugs on growth of H3255 and DFCILU-011 cells (Fig. 1, A).



**Fig. 1.** Effects of gefitinib and cetuximab on growth of non-small-cell lung cancer cell lines. Cells were treated with gefitinib (3.3 nM–10  $\mu$ M) or cetuximab (33 ng/mL–100  $\mu$ g/mL) for 72 hours, and cell viability was then measured by the MTS assay. Percentage of viable cells is shown relative to that of untreated control. Results are shown as mean value with 95% confidence intervals calculated based on results

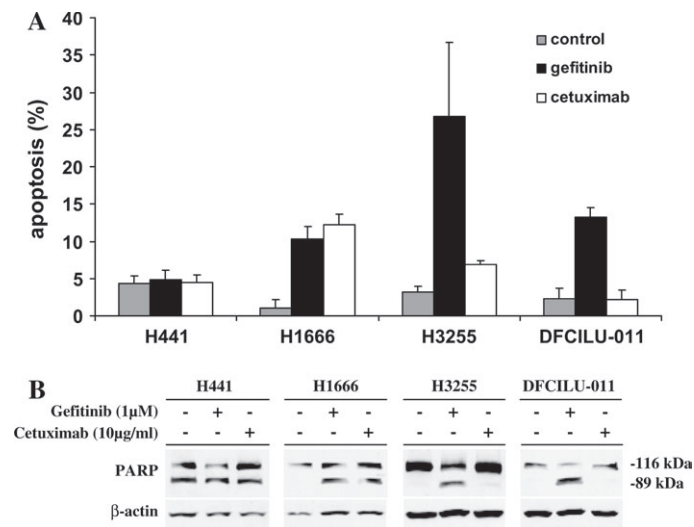
from six to 12 replicate wells. The concentrations are represented as the logarithm of the maximum achievable serum concentration for gefitinib (1  $\mu$ M) and cetuximab (100  $\mu$ g/mL). All experiments were repeated at least three times. **A)** EGFR wild-type cell lines A549, H441, and H1666. **B)** EGFR mutant cell lines H3255 (with the L858R mutation) and DFCILU-011 (with the L747\_E749del mutation).

We also assessed apoptosis by analyzing PARP cleavage, which is an indicator of caspase-mediated apoptosis, by immunoblotting (Fig. 2, B). Again, we treated cells with 1  $\mu$ M gefitinib, 10  $\mu$ g/mL cetuximab, or neither drug. We obtained results that were qualitatively consistent with those of the propidium iodide analyses. That is, gefitinib treatment led to substantial increases in levels of the cleaved fragment in H3255 and DFCILU-011 cells relative to levels in cells treated with neither drug. In H1666 cells, treatment with either drug led to increases in the level of cleaved fragment relative to that in untreated cells, whereas in H441 cells there was some baseline PARP cleavage that was not altered by either treatment.

#### Differential Effects of Gefitinib and Cetuximab on Cell Signaling in NSCLC Cell Lines

To explore the molecular mechanisms underlying the difference in cellular responses to treatment with gefitinib and

cetuximab, we compared the effects of gefitinib and cetuximab on autophosphorylation of EGFR and of the downstream signaling intermediates Akt and ERK1/2. In H441 cells, which are resistant to both gefitinib and cetuximab, treatment with high concentrations of either drug led to a decrease in EGFR phosphorylation but did not alter phosphorylation of ERK1/2 or Akt (Fig. 3, A). By contrast, both gefitinib and cetuximab inhibited phosphorylation of both EGFR and ERK1/2 in H1666 cells, consistent with the moderate effects of these drugs on growth and apoptosis. Phosphorylation of Akt was also reduced in these cells but to a lesser extent than that of ERK1/2, as seen in our earlier study (13). Gefitinib treatment of the EGFR mutant cell lines H3255 and DFCILU-011 led to complete inhibition of EGFR phosphorylation and concomitant inhibition of ERK1/2 and Akt phosphorylation (Fig. 3, B). By contrast, cetuximab caused only partial inhibition of EGFR, ERK1/2, and Akt phosphorylation in H3255 cells and had no effect on phosphorylation of these signaling molecules in DFCILU-011 cells (Fig. 3, B).



**Fig. 2.** Effects of gefitinib and cetuximab on apoptosis of non-small-cell lung cancer cell lines. Cells were grown in the presence of no drug (control; **hatched bar**), 1  $\mu\text{M}$  gefitinib (**solid bar**), or 10  $\mu\text{g}/\text{mL}$  cetuximab (**open bar**) for 72 hours. Apoptosis was analyzed by propidium iodide staining (**A**) or immunoblot analysis of the formation of the 89-kD PARP cleavage product (**B**). In panel A, the percentage of apoptosis is given relative to the total cell population analyzed. Each experiment was repeated three times, and mean values with 95% confidence intervals are shown. In panel B, blots were stripped and probed for  $\beta$ -actin as a loading and transfer control. The experiments were repeated at least twice.

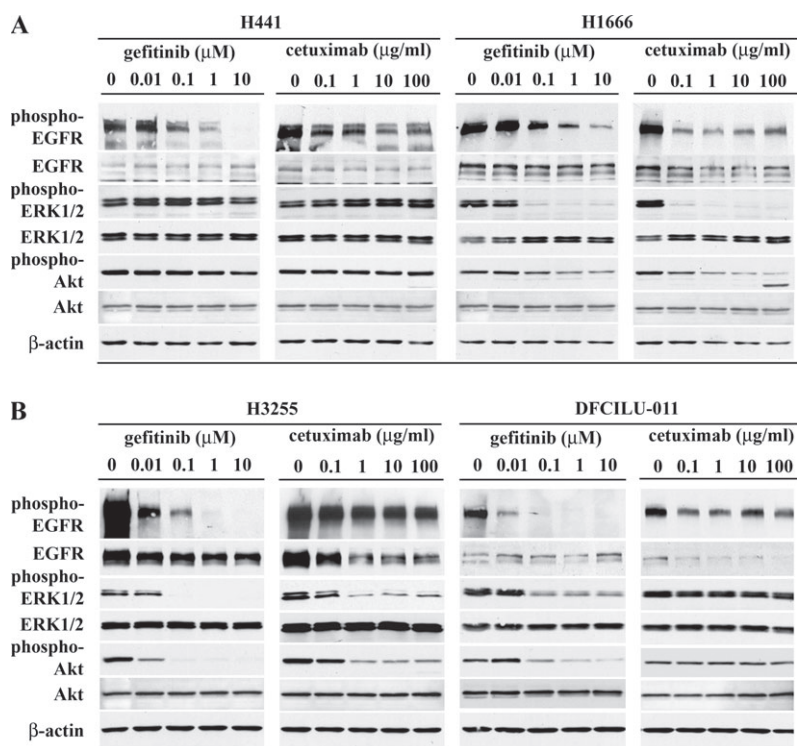
### Effects of Gefitinib and Cetuximab on Wild-type and Mutant EGFR Receptors

To more directly compare the inhibitor sensitivity conferred by wild-type and mutant EGFR, we stably transfected NIH-3T3 cells with different EGFR sequences. This approach eliminates differences in cellular context that could influence drug sensitivity. We chose NIH-3T3 cells because they have low levels

of endogenous EGFR. We used wild-type EGFR and two different EGFR mutations, L858R and L747\_S752del, P753S; the latter was chosen because it has been observed in patients responding to gefitinib and was used in previous studies (6,7). For controls, we transfected cells with a vector that contains the gene for green fluorescent protein (GFP). We treated the transfected NIH-3T3 cells with a range of concentrations of gefitinib and cetuximab. Both drugs decreased wild-type EGFR autophosphorylation only modestly and only at high concentrations (10  $\mu\text{M}$  and 100  $\mu\text{g}/\text{mL}$ , respectively) (Fig. 4). By contrast, much lower concentrations of gefitinib (0.1  $\mu\text{M}$  and above) completely inhibited phosphorylation of both EGFR mutants. Cetuximab had no substantial effects on phosphorylation of either mutant.

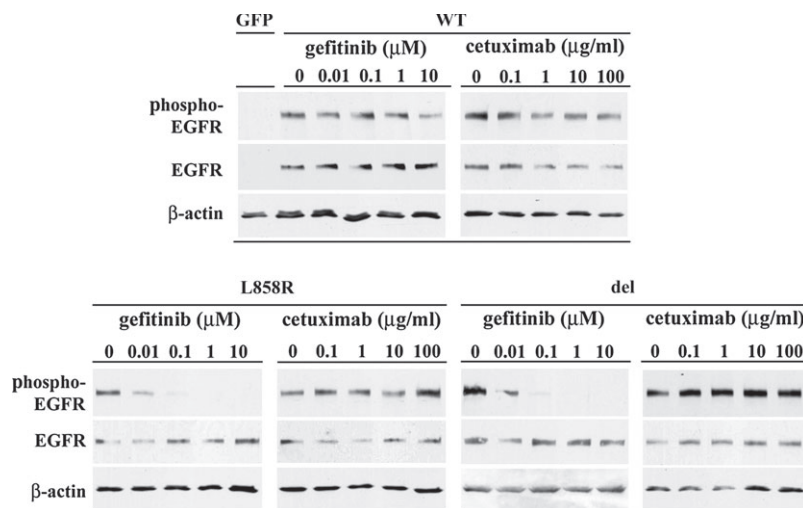
### An EGFR Mutant NSCLC Cell Line Growth Inhibited by Both Gefitinib and Cetuximab

While this article was in review, Amann et al. (15) published a study demonstrating that both gefitinib and cetuximab effectively inhibit the growth of the NSCLC cell line HCC827, which contains the E746\_A750del mutation of EGFR. We thus examined the effects of both gefitinib and cetuximab on the growth of HCC827 cells under our study conditions. Both drugs inhibited the growth of HCC827 cells, with  $\text{IC}_{50}$ s of 6.6 nM and 277 ng/mL, respectively (Fig. 5, A). Although both agents caused substantial growth inhibition in this cell line, gefitinib was still statistically significantly more effective than cetuximab ( $P = .0028$ ). However, whereas gefitinib effectively inhibited EGFR phosphorylation at concentrations of 0.1  $\mu\text{M}$  or higher, cetuximab treatment did not affect EGFR phosphorylation at any concentration tested (Fig. 5, B). These findings are similar to those of Amann et al. (15) and are consistent with our results on cell lines carrying other EGFR mutations. Thus, HCC827 cells behaved differently from the other cell lines containing mutant EGFR in that cetuximab inhibited growth of HCC827 cells. However, as



**Fig. 3.** Effects of gefitinib and cetuximab on phosphorylation of EGFR, Akt, and ERK1/2 in non-small-cell lung cancer cell lines. **A**) EGFR wild-type cell lines H441 and H1666. **B**) EGFR mutant cell lines H3255 (with the L858R mutation) and DFCILU-011 (with the L747\_E749del mutation). Cells were treated with gefitinib or cetuximab at the indicated concentrations for 24 hours. Immunoblots of cellular protein were analyzed for phosphorylated and total EGFR, ERK1/2, and AKT. Blots were stripped and re-probed for  $\beta$ -actin as a loading and transfer control. The experiments were repeated at least twice.

**Fig. 4.** Effects of gefitinib and cetuximab on levels of phospho-EGFR in NIH-3T3 cells stably transfected with wild-type EGFR (WT), mutant EGFR (L858R or L747\_S752del, P753S [del]), or green fluorescent protein (GFP; control). Transfected cells were treated with gefitinib or cetuximab at the indicated concentrations for 8 hours. Cell lysates were analyzed for phospho- and total EGFR by immunoblotting. The blot was stripped and reprobed for  $\beta$ -actin as a loading and transfer control. The experiments were repeated at least twice.



in the other EGFR mutant cell lines, cetuximab had no effect on EGFR phosphorylation in HCC827 cells.

#### Outcome of Patients With EGFR Mutations Treated Sequentially With Cetuximab and Gefitinib

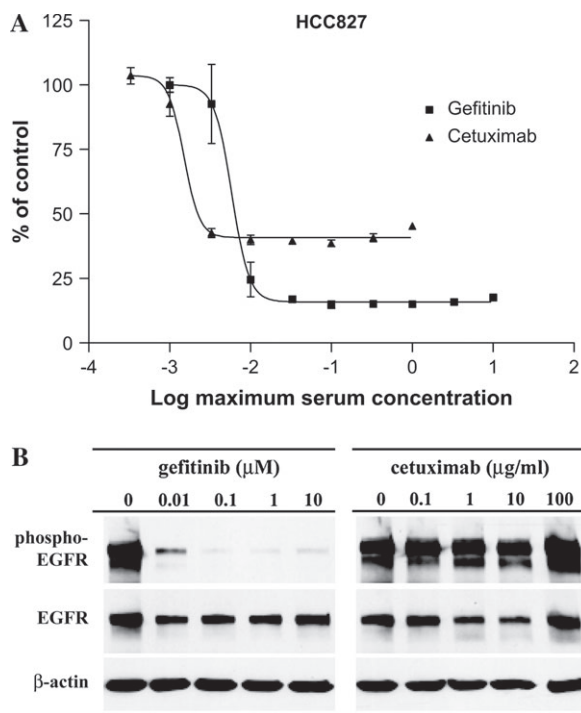
We identified four patients with advanced NSCLC with EGFR mutations in their tumors who were treated with both gefitinib

and cetuximab for relapsed disease (Table 1). Three patients were treated with cetuximab first and achieved stable disease as their best response. On disease progression, these three patients were switched to gefitinib and subsequently achieved a partial response. A fourth patient was initially treated with gefitinib and achieved a partial response that lasted 24 months. On disease progression, he was treated with single-agent cetuximab, but his disease progressed after 8 weeks of treatment and the treatment was discontinued. Thus, the results with these patients are consistent with the observation made in NSCLC cell lines, that mutant EGFRs are more sensitive to gefitinib than to cetuximab.

#### DISCUSSION

EGFR is one of the most widely studied molecular targets for cancer therapy. We compared the effects of two different EGFR inhibitors—the small-molecule tyrosine kinase inhibitor gefitinib, which is approved for use in patients with previously treated, advanced NSCLC, and the monoclonal antibody cetuximab, which is approved for treatment of previously treated metastatic colorectal cancer—in NSCLC cell lines carrying wild-type and mutant EGFR. The growth of NSCLC cell lines with the most common EGFR mutations (L858R and a deletion in exon 19) was inhibited by gefitinib but not by cetuximab; the growth inhibition caused by gefitinib appeared to result from increased apoptosis. Phosphorylation of mutant EGFRs and of the downstream signaling intermediates Akt and ERK1/2 in EGFR mutant cells was inhibited strongly by gefitinib but only minimally by cetuximab. In cells containing wild-type EGFR, by contrast, the two inhibitors had largely similar effects. Treatment of H1666 cells with either drug caused modest growth inhibition, increased apoptosis, and decreased EGFR, Akt, and ERK1/2 phosphorylation. However, neither drug had an effect on A549 or H441 cells. Taken together, our findings suggest that, although the *in vitro* effects of cetuximab and gefitinib are similar in EGFR wild-type cell lines, they are different in EGFR mutant cell lines. Our results further demonstrate that these differences are likely due to the inability of cetuximab to inhibit the phosphorylation of mutant EGFR.

Despite frequent overexpression of EGFR in NSCLC, gefitinib and erlotinib (another small-molecule inhibitor of the EGFR tyrosine kinase) cause partial radiographic regressions in a small percentage of patients (2–4,30,31). Recent studies have demonstrated that somatic mutations in the EGFR tyrosine kinase



**Fig. 5.** Comparison of gefitinib and cetuximab in HCC827 non-small-cell lung cancer cells. **A**) Cells were treated with gefitinib (3.3 nM–10  $\mu$ M) or cetuximab (33 ng/mL–100  $\mu$ g/mL) for 72 hours, and cell viability was measured by the MTS assay. Percentage of cell growth is shown relative to untreated control. Results are shown as means with 95% confidence intervals. The concentrations are represented as the logarithm of the maximum achievable serum concentration for gefitinib (1  $\mu$ M) and cetuximab (100  $\mu$ g/mL). The experiments were repeated twice. **B**) Effects of gefitinib and cetuximab on EGFR phosphorylation. Cells were treated with gefitinib or cetuximab at the indicated concentrations for 24 hours, and phospho- and total EGFR were analyzed by immunoblotting. The blot was stripped and reprobed for  $\beta$ -actin as a loading and transfer control. The experiments were repeated twice.

**Table 1.** Characteristics of advanced non–small-cell lung cancer patients with EGFR mutations treated with both gefitinib and cetuximab

| Patient No. | Sex | Age, y | Smoking history, pack-years* | Histology† | First treatment | Response‡ | Second treatment | Response‡ | EGFR mutation |
|-------------|-----|--------|------------------------------|------------|-----------------|-----------|------------------|-----------|---------------|
| 1           | F   | 53     | <10                          | NSCLC      | Cetuximab       | SD (8.0)  | Gefitinib        | PR (4.0)  | E746_A750del  |
| 2           | F   | 65     | <10                          | Adeno      | Cetuximab       | SD (2.0)  | Gefitinib        | PR (6.0+) | E746_A750del  |
| 3           | F   | 68     | <10                          | Adeno      | Cetuximab       | SD (5.0)  | Gefitinib        | PR (8.0+) | E746_A750del  |
| 4           | M   | 71     | 40                           | Adeno      | Gefitinib       | PR (24.0) | Cetuximab        | PD (N/A)  | L747_S752del  |

\*Pack-years are defined as number of packs of cigarettes smoked per day times number of years smoked.

†NSCLC; non–small-cell lung cancer not otherwise specified, adeno; adenocarcinoma.

‡SD, stable disease; PR, partial response; PD, progressive disease; N/A, not applicable. Number in parentheses represents duration of response in months.

domain are found in approximately 85% of patients who develop radiographic regressions following treatment with gefitinib or erlotinib (5–7). These observations suggest that gefitinib and erlotinib are more effective against mutant EGFR than they are against wild-type EGFR. Cetuximab has a response rate of 9% in previously treated colorectal cancer patients (32). Only a few EGFR mutations have been detected in colorectal cancer to date, and these mutations are not associated with clinical responses to cetuximab (33–35). According to a preliminary report of a phase II clinical trial, the response rate to single-agent cetuximab in NSCLC patients previously treated with chemotherapy is 6% (23). However, only limited examinations of EGFR mutation status were performed on patients treated in this study.

The finding that H1666 cells responded differently to the EGFR inhibitors than A549 and H441 cells, even though all three cell lines contain wild-type EGFR, is interesting and remains to be explained. One possible explanation is differences in the mutation status of K-ras. Both A549 and H441 cells have K-ras mutations, whereas H1666 have wild-type K-ras (13). K-ras mutations have been associated with resistance to gefitinib in vitro and in patients treated with either gefitinib or erlotinib (36,37), although such K-ras mutations have not previously been associated with resistance to cetuximab. A second possible explanation lies in the fact that EGFR undergoes autocrine activation in H1666 cells but not in the other cell lines (13). Among the NSCLC cell lines examined in this study, H1666 and H441 secrete transforming growth factor  $\alpha$  (TGF- $\alpha$ ), which suggests the presence of a TGF- $\alpha$ /EGFR autocrine loop (13). These findings raise the possibility that expression of wild-type K-ras and interruption of the autocrine pathway may account for growth inhibition in H1666 cells treated with cetuximab. In this context, it is interesting to note that two other cancer cell lines that are sensitive to cetuximab, A431 (epidermoid carcinoma) and DiFi (colon cancer), are also both wild type for K-ras and secrete TGF- $\alpha$  (38–40). Similarly, Raben et al. (41) recently found that three NSCLC cell lines (H332, H292 and Calu 3) are sensitive to cetuximab in vitro. All three sensitive cell lines have previously been reported to be wild type for K-ras (21,41,42). Additional studies of K-ras in NSCLC and colorectal cancer patients treated with cetuximab may help to clarify whether mutational status of K-ras is a critical determinant of sensitivity to cetuximab.

Our studies of three different EGFR mutant NSCLC cell lines (H3255, PC-9, and DFCILU-011) demonstrated that cetuximab does not inhibit their growth in vitro, probably because it does not inhibit EGFR phosphorylation. In contrast to our findings, Amann et al. (15) recently demonstrated that the growth of HCC827, an EGFR mutant (E746\_A750 del) NSCLC cell line, was inhibited by both gefitinib and cetuximab, and we made the same observation (Fig. 5). Nevertheless, like Amann

et al., we also found that only gefitinib was able to inhibit EGFR phosphorylation. This disconnect between inhibition of growth and lack of inhibition of EGFR phosphorylation, which we observed in the HCC827 cell line only, raises questions as to the mechanism by which cetuximab inhibits growth of this cell line in vitro.

Our finding that phosphorylation of mutant EGFRs (those in NSCLC cell lines as well as those in stably transfected NIH-3T3 cells) was completely inhibited by gefitinib at concentrations as low as 100 nM, which is substantially below the mean plasma steady state concentration of gefitinib (0.4–1.4  $\mu$ M) but was only minimally affected by even the maximum serum achievable concentration of cetuximab (i.e., 100  $\mu$ g/mL), indicates that cetuximab, which binds to the extracellular portion of EGFR, is unable to inhibit phosphorylation of the mutant EGFRs. By contrast, both drugs had minimal effects on EGFR phosphorylation in cells with wild-type EGFR. Although these findings are similar to those of Lynch et al. (6,15) and Amman et al. (6,15), they partly differ from those of Pao et al. (7), who found that EGFR phosphorylation in cells transiently transfected with L858R but not with L747\_S752del was inhibited by 10-fold lower concentrations of gefitinib or erlotinib than were necessary to inhibit wild-type EGFR. The differences between our findings and those of Pao et al. may reflect differences in the cell lines (NIH-3T3 versus 293T cells) used and/or the amount of EGFR expression in the transfectants because we generated stable cell lines with retroviral infection, whereas Pao et al. assessed transiently transfected cells.

A potential limitation of our study is that the experiments were performed in vitro; therefore, we could not evaluate the antibody-dependent cellular cytotoxicity effects that have been observed with anti-EGFR monoclonal antibodies (43). Because of these effects, responses to cetuximab in vivo and in vitro could be different. Indeed, Huang et al. (29) found that, in vitro, gefitinib caused a greater degree of growth inhibition of the NSCLC line H226 than cetuximab, whereas cetuximab was more effective at inducing tumor regression in a xenograft model using these same cells. Another potential limitation lies in the fact that we examined only four EGFR mutant NSCLC cell lines with two different types of mutations (L858R and exon 19 deletion), and these may not be representative of all of the different EGFR mutations found in patients with NSCLC. However, L858R and exon 19 deletions are the most common EGFR mutations, and no NSCLC cell lines harboring mutations other than these have, to our knowledge, been described. Our study also examined more EGFR mutant NSCLC cell lines than any other previously published study.

To gain insight into the effects of cetuximab in EGFR mutant tumors in vivo, we examined the outcome of four NSCLC patients



with EGFR mutations in their tumors who were treated sequentially with cetuximab and gefitinib. In this small patient series, we found that, although patients who received cetuximab treatment had stable disease, none of the patients had shrinkage of their tumors while on cetuximab. By contrast, all patients treated with gefitinib had partial responses. These clinical observations are consistent with our *in vitro* findings—that is, mutant EGFR appears to be more sensitive to gefitinib than cetuximab. One patient (Table 1; patient 4) with an EGFR mutation, who achieved an initial partial response to gefitinib, developed a secondary mutation in EGFR (T790M) while on gefitinib and progressed (44). Subsequent treatment of this patient with cetuximab was ineffective. Recent studies have revealed that this secondary mutation (T790M) confers *in vitro* resistance to gefitinib (44,45). To further evaluate cetuximab's efficacy against an EGFR protein harboring the secondary mutation, we examined the effect of cetuximab on EGFR phosphorylation in Cos-7 cells expressing EGFR del 747–752 or in conjunction with the secondary mutation (EGFR del747–752/T790M). Cetuximab did not substantially inhibit EGFR phosphorylation of either EGFR construct (data not shown). This finding is consistent with the patient's lack of response to cetuximab, and it suggests that this agent may not be effective in patients who develop this secondary mutation in EGFR.

Overall, our results raise the possibility that NSCLC patients with EGFR mutations may derive the greatest benefit, in terms of tumor regression, if treated with gefitinib rather than with cetuximab. This hypothesis is supported by results of multiple studies using gefitinib, in which the response rates to gefitinib are greater in patients with EGFR mutations than in those without such mutations (5–7). Only one study of single-agent cetuximab in NSCLC has been conducted, and the findings of this study, although limited, are also consistent with our hypothesis (23). In a preliminary analysis from this phase II trial of cetuximab in previously treated patients with NSCLC, the response rate was 6% (23). Fourteen patients with available tumor tissue had undergone EGFR sequencing. One patient who had a partial response to cetuximab treatment had no detectable EGFR mutation. On the other hand, EGFR mutations were detected in one patient with stable disease and one patient with disease progression. The former patient was subsequently treated with gefitinib (Table 1, patient 3). Another patient's tumor contained an L861Q mutation in EGFR, which has previously been found in patients who responded to gefitinib (6), and that patient developed disease progression with cetuximab treatment and was never treated with gefitinib (23). Thus, the growth of at least some NSCLC tumors and cell lines (i.e., H1666) with wild-type EGFR can be inhibited by cetuximab. Future studies that examine the association between EGFR mutation status and type with the efficacy of cetuximab and other EGFR inhibitors will help guide future EGFR-directed therapies to those patients who are most likely to benefit from such treatments.

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## NOTES

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